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Cellular mechanisms of insulin resistance in skeletal muscle and the liver: the role of mitochondrial function and ectopic lipid deposition

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Frequently used abbreviations

^{13}C MRS	Carbon-13 magnetic resonance spectroscopy
^{1}H MRS	Proton magnetic resonance spectroscopy
^{31}P MRS	Phosphorus magnetic resonance spectroscopy
MRI	Magnetic resonance imaging
AMPK	AMP-activated protein kinase
ATP	Adenosine triphosphate, Adenosintriphosphat
BMI	Body mass index
CHREBP-1	Carbohydrate regulatory element binding protein-1
SREBP-1	Sterol regulatory element binding protein-1
CI, II, III	Complex I, II, III of the respiratory chain
CoA	Conezyme A
CPT1	Carnitine palmitoyltransferase-1
CS	Citrate synthase
DAG	Diacylglycerols
EGP	Endogenous glucose production
fATP	ATP synthase flux rate
FCCP	Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone
FFA	Free fatty acids
FFS	Freie Fettsäuren
FoxO1	Transcription factor forkhead box O1
G-6-P	Glucose-6-phosphate
GIR	Glucose infusion rate
GLUT2, GLUT4	Glucose transporter 2, 4
GNG	Gluconeogenesis
HbA_{1c}	Hemoglobin A_{1c}
HCL	Hepatocellular lipids
HLVF	Hepatic lipid volume fraction
IMCL	Intramyocellular lipids
IRS1, IRS2	Insulin receptor substrate proteins 1 and 2
LCFA-CoA	Long-chain fatty acyl-CoA
LDL-C	Low-density lipoprotein cholesterol levels
MELAS	Myopathy, encephalopathy, lactate acidosis, stroke-like episodes
MRS	Magnetic resonance spectroscopy
mtDNA	Mitochondrial DNA
NAFL	Non-alcoholic fatty liver
NAFLD	Non-alcoholic fatty liver diseases
NASH	Non-alcoholic steatohepatitis
NFκB	Nuclear factor κ-light-chain-enhancer of activated B cells
OXPHOS	Oxidative phosphorylation
PCr	Phosphocreatine
PDE	Phosphodiesters
PGC-1α, -1β	Peroxisome proliferator-activated receptor γ coactivator-1α, -1β
Pi	Inorganic phosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate

PKCθ, ε	Protein kinase C**θ, ε**
PKB/Akt	Protein kinase B/Akt
ROS	Reactive oxygen species
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TCA	Tricarboxylic acid cycle
TG	Triglycerides
UCP-3	Uncoupling protein-3
VLDL	Very-low density lipoproteins
VO_2max	Maximal aerobic capacity

Original publications underlying this thesis

1. **Szendroedi, J.**, Frossard, M.*, Klein, N., Bieglmayer, C., Wagner, O., Pacini, G., Decker, J., Nowotny, P., Muller, M., and Roden, M. (2012). Lipid-induced insulin resistance is not mediated by impaired transcapillary transport of insulin and glucose in humans. Diabetes *61*, 3176-3180. *both authors contributed equally. Impact factor 2015: 8.474

2. **Szendroedi, J.**, Anderwald, C., Krssak, M., Bayerle-Eder, M., Esterbauer, H., Pfeiler, G., Brehm, A., Nowotny, P., Hofer, A., Waldhausl, W., Roden, M. (2009a). Effects of high-dose simvastatin therapy on glucose metabolism and ectopic lipid deposition in nonobese type 2 diabetic patients. Diabetes Care *32*, 209-214. Impact factor 2015: 8.570

3. **Szendroedi, J.**, Yoshimura, T., Phielix, E., Koliaki, C., Marcucci, M., Zhang, D., Jelenik, T., Muller, J., Herder, C., Nowotny, P., Shulman, G., Roden, M. (2014b). Role of diacylglycerol activation of PKCθ in lipid-induced muscle insulin resistance in humans. Proc Natl Acad of Sci U S A *111*, 9597-9602. Impact factor 2015: 9.809

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8. **Szendroedi, J.**, Zwettler, E., Schmid, A.I., Chmelik, M., Pacini, G., Kacerovsky, G., Smekal, G., Nowotny, P., Wagner, O., Schnack, C., *et al.* (2008). Reduced basal ATP synthetic flux of skeletal muscle in patients with previous acromegaly. PloS One *3*, e3958. Impact factor 2015: 3.534

9. **Szendroedi, J.**, Chmelik, M., Schmid, A.I., Nowotny, P., Brehm, A., Krssak, M., Moser, E., and Roden, M. (2009b). Abnormal hepatic energy homeostasis in type 2 diabetes. Hepatology *50*, 1079-1086. Impact factor 2015: 10.401

10. Schmid, A.I., **Szendroedi, J.**,* Chmelik, M., Krssak, M., Moser, E., and Roden, M. (2011). Liver ATP synthesis is lower and relates to insulin sensitivity in patients with type 2 diabetes. Diabetes Care *34*, 448-453. *both authors contributed equally. Impact factor 2015: 8.570

Deutsche Zusammenfassung

Hintergrund: Muskuläre Insulinresistenz und Fettleber gehen der Manifestation des Typ 2 Diabetes (T2DM) um viele Jahre voraus und sind wichtige Risikofaktoren für Herzinfarkt und kardiovaskuläre Mortalität. Erhöhte Lipidverfügbarkeit könnte durch Anhäufung von Triglyzeriden (IMCL) oder Lipidmetaboliten wie Diazylglyzerolen (DAG) oder Ceramiden oder durch Abnahme der lokalen Durchblutung muskuläre Insulinresistenz auslösen. Andererseits wird auch die Störung der mitochondrialen Aktivität als Auslöser diskutiert. Funktionsstörungen der Mitochondrien könnten an der Entstehung der hepatischen Insulinresistenz und der Fettleber beteiligt sein. Folgende Hypothesen wurden daher geprüft: (i) Die Erhöhung zirkulierender Lipide hemmt über DAG die Insulinsignalübertragung und so die muskuläre Glukoseaufnahme, während Effekte auf andere Lipidmetaboliten oder auf die regionale Durchblutung keine Rolle spielen. (ii) Die Reduktion der muskulären mitochondrialen Aktivität ist eine Folge der Insulinresistenz, kann diese aber verstärken. (iii) Die Reduktion der mitochondrialen Aktivität in der Leber ist mit dem Leberfettgehalt und mit hepatischer Insulinresistenz assoziiert.

Methoden: In klinisch-experimentellen Studien wurden mittels hyperinsulinämisch-euglykämischen Clamps und stabil-markierter Glukose die Insulinsensitivität von Muskel und Leber, mittels Mikrodialyse und Muskelbiopsien Substratkonzentrationen, myozelluläre Lipide und Insulinsignalübertragung, und mittels Magnetresonanzbildgebung und -spektroskopie die Körperfettverteilung sowie die Aktivität der Mitochondrien in Muskel und Leber bestimmt. Dazu wurden neue Methoden zur nicht-invasiven Messung der Konzentrationen von ATP und der ATP-Synthese in der Leber entwickelt.

Resultate: Bei Menschen sind myozelluläre DAG, welche die Fettsäurereste C18:0, C18:1, C:18:2 und C20:4 enthalten, für die durch Erhöhung von freien Fettsäuren induzierte Insulinresistenz verantwortlich. Die DAG-vermittelte Aktivierung der Proteinkinase C θ hemmt in der Folge durch Serinphosphorylierung des Insulinrezeptor-Substrates 1 die Glukoseaufnahme. Diese Veränderungen finden sich auch in der Skelettmuskulatur von Patienten mit Adipositas oder manifestem T2DM. Lipid-vermittelte Effekte auf die regionale Durchblutung, interstitielle Konzentrationen von Glukose, Lipiden und Insulin und myozelluläre Ceramide spielen dabei keine Rolle. Interessanterweise korrelieren die Serumkonzentrationen freier Fettsäuren aber negativ mit der mitochondrialen Aktivität im Skelettmuskel, diese korrelieren wiederum negativ mit Insulinsensitivität, Markern muskulärer Membranschäden und dem Leberfettgehalt. Die verminderte muskuläre mitochondriale Aktivität könnte demnach eine Folge von lipotoxischer Schädigung der Mitochondrien sein. Dies begünstigt die Akkumulation von Lipidmetaboliten und Insulinresistenz, fördert letztlich den Substratfluß zur Leber und leitet so die Entwicklung einer

Fettleber ein. Eine Reduktion der basalen mitochondrialen Aktivität kann bei Insulinresistenz unterschiedlicher Ursachen beobachtet werden. Für Patienten mit manifestem T2DM ist die Reduktion der Stimulierbarkeit der muskulären Mitochondrienaktivität durch Insulin (Plastizität) typisch und tritt unabhängig von der Glukoseaufnahme auf. Patienten mit T2DM weisen aber auch eine verminderte mitochondriale Aktivität der Leber auf, die mit hepatischer Insulinresistenz und dem Leberfettgehalt korreliert.

Zusammenfassung: Die DAG-assoziierte Aktivierung der Proteinkinase C θ und die Störung der mitochondrialen Plastizität sind charakteristische Merkmale der Insulinresistenz und stellen einen neuen Ansatz nicht nur zur Therapie der Insulinresistenz, sondern auch von nicht-alkoholischen Fettleberkrankheiten dar.

Diese kumulative Habilitationsschrift beruht auf 10 begutachteten Originalarbeiten, die als Erstautorin publiziert wurden (Anlagen 1-10).

1. Introduction

Diabetes mellitus comprises metabolic diseases characterized by hyperglycemia resulting from impaired insulin action and/or inadequate insulin secretion. Diabetes mellitus is associated with chronic dysfunction and failure of various organs, especially the eyes, kidneys, nerves, heart and blood vessels [1].

1.1. Type 2 diabetes and the role of insulin resistance

Type 2 diabetes mellitus (T2DM) affects about 90% of all patients with diabetes. T2DM is characterized by a sustained prodromal stage of insulin resistance, which promotes hyperinsulinemia. With failing insulin secretion, glycemia increases until diagnostic thresholds for overt T2DM are reached [2]. In some individuals, β-cell impairment is the main defect [3], in most patients insulin resistance precedes defects in insulin secretion [4-6]. Genome-wide association studies suggest that gene variants affect T2DM susceptibility predominantly through β-cell failure [7]. These risk alleles are common in the general population, but their impact is small and not applicable for broad genetic screening [8]. Thus, modifiable factors underlying the development of insulin resistance are of high relevance for the prevention of T2DM and development of novel therapeutic targets.

T2DM tightly relates to microvascular complications (retino-, nephro- and neuropathy), cardiovascular diseases and increased risk of several malignancies [9]. Individuals with newly diagnosed T2DM already present with comorbidities presumably due to longer-term undetected insulin resistance and hyperglycemia [10]. The prognostic importance of the metabolic syndrome compared to insulin resistance has been challenged [11], pointing out that insulin resistance alone increases cardiac mortality independent of the onset of T2DM. The predictive value of whole body insulin resistance for the development of comorbidities and the effectiveness of individualized treatment strategies is far from being understood. One reason is that the majority of epidemiological studies have estimated insulin resistance from crude surrogate parameters [12-14]. The link between hyperglycemia, which is also a surrogate marker for insulin resistance, and comorbidities appears complex [15]. There is evidence for sustained protection by intensive glycemic control early after onset of T2DM, with reduction of mortality and micro- and macrovascular comorbidities decades thereafter, referred to as metabolic memory effect [15, 16]. Current concepts suggest that mitochondrial impairment by increased production of reactive oxygen species (ROS) and other processes referred to as glucolipotoxicity result in irreversible cell damage and induction of apoptosis [17]. Therefore,

novel therapy strategies presumably via amelioration of mitochondrial function or insulin sensitivity might be protective.

1.2. Insulin-mediated regulation of glucose metabolism

Insulin regulates metabolic processes maintaining glucose homeostasis throughout the fed and the fasted state. The pancreatic β-cells secrete insulin upon increased plasma glucose values but also when plasma concentrations of FFA, branched-chain amino acids or incretine hormones are increased. After meal ingestion, insulin acts as an anabolic hormone by stimulating glucose, lipid and protein storage, while during fasting, insulin inhibits FFA release from adipose tissue and hepatic glucose release. Insulin exerts its effects by binding to the insulin receptor and thereby stimulating its autophosphorylation and internalization on target tissues. Consequently, insulin receptor substrate proteins 1 and 2 (IRS1, IRS2) activate phosphatidylinositol 3 kinase, which converts phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol (3,4,5)-trisphosphate (PIP3). Binding to PIP3 phosphorylates and activates the serine/threonine kinase Akt (also known as protein kinase B), the key signal transducer for insulin-mediated glucose transport through recruitment of glucose transporter 4 (GLUT4) into the muscle cell membrane [18]. In skeletal muscle and adipose tissue, insulin-mediated Akt activation promotes the translocation of GLUT4 to the membrane to facilitate glucose uptake [19].

In adipose tissue, insulin stimulates glucose entry via GLUT4, inhibits lipolysis and promotes lipogenesis. In the liver, insulin binding to the insulin receptor leads to suppression of gluconeogenesis (GNG) by inactivation of the gluconeogenic gene transcription factor forkhead box O1 (FoxO1), by stimulation of fatty acid synthesis via activation of sterol and carbohydrate regulatory element binding protein-1 (SREBP-1, CHREBP-1) 1, transcription factors that foster expression of genes involved in fatty acid synthesis, and by stimulation of glycogen synthesis through inactivation of glycogen synthase kinase, the inhibitor of glycogen synthesis. Thus, in the liver, insulin decreases endogenous glucose production (EGP) by suppression of GNG and stimulation of glycogen synthesis. In insulin sensitive humans, the liver maintains glucose homeostasis after meal ingestion by insulin independent glucose uptake via GLUT2 and consequent glycogen synthesis and during fasting by releasing glucose from glycogenolysis under the tight control of insulin and glucagon. During prolonged fasting, the liver of healthy individuals gradually increases GNG from lactate, pyruvate, glycerol and branched-chain amino acids to maintain EGP [20, 21]. In the postprandial state, hyperglycemia stimulates insulin secretion, which lowers EGP by inhibiting

glycogenolysis, rather than GNG, and by stimulating glycogen synthesis to rapidly normalize blood glucose levels [22]. The regulation of these metabolic processes maintains glucose homeostasis within a narrow range of euglycemia.

1.3. Physiology of mitochondrial function

Mitochondria are dynamic intracellular organelles which are involved in a number of vital processes including substrate oxidative catabolism through tricarboxylic acid cycle (TCA), fatty acid β-oxidation, ketogenesis, adenosine triphosphate (ATP) synthesis through the mechanism of oxidative phosphorylation (OXPHOS) and formation of ROS (Murphy 2009; Rimessi *et al.* 2008; Starkov 2008). Mitochondria maintain vital processes such as substrate oxidation and energy conversion into ATP and play an essential role in cellular proliferation, apoptosis and signalling by modulating production of nitric oxide, ROS and intracellular calcium. Due to the complex diversity of mitochondrial features, the rating of possible impairments of mitochondrial function strongly depends on its definition and assessment (see also in Methods section **3.3.1.**).

In young, lean, glucose tolerant humans, mean unidirectional ATP synthase flux rates (fATP) measured by ^{31}P MRS range from ~8 [23, 24] up to 12 μmol (g muscle)$^{-1}$ min^{-1} [25]. Muscle fATP can be noticeably lower in glucose tolerant humans aged ~70 years (fATP of ~4±1 μmol g^{-1} min^{-1}) [23]. This is in line with the concept that the insulin resistance that emerges during ageing is correlated with, or even results from, reduced mitochondrial function. An age-related decline in mitochondrial function might be due to reduced overall and, in particular, insulin-stimulated protein synthesis [26-28], or be related to increasing body mass, insulin resistance and physical inactivity. Adjustment for physical activity might abolish differences in mitochondrial function between young and elderly humans [29, 30]. Accordingly, oxidative capacity can be maintained in humans aged up to 70 years even though mitochondrial DNA (mtDNA) accumulates mutations and deletions at high rates as a result of impaired DNA repair [31] and exposure to ROS [32-34]. However, it was yet unclear, if glucose tolerant humans matched for age, body mass and physical activity have higher or equal fATP compared to patients with T2DM.

Insulin stimulates fATP concomitantly with glucose uptake in skeletal muscle [35]. Transmembranous substrate gradients established during insulin stimulation change until a new equilibrium is reached [36, 37], and this immediate insulin effect enhances fATP for up to ~4 h [25, 38]. Following prolonged hyperinsulinemia, stimulation of electron chain enzyme activities (starting at 1–3 h) and augmentation of mitochondrial protein synthesis (after ~6 h) further

increase fATP [38]. Use of ^{31}P MRS during euglycemic–hyperinsulinemia clamps showed that skeletal muscle fATP increases by ~26–90% at 30–240 min in young, lean, glucose tolerant humans and by ~11% at 120–240 min in glucose tolerant elderly humans [25, 35].

1.4. General concepts of the pathophysiology of insulin resistance

Insulin resistance strongly relates to obesity both of which share characteristic risk factors such as high-caloric intake, subclinical inflammation and sedentary lifestyle (**Figure 1.**). Several concepts have been developed to that provide unifying cellular and molecular mechanisms contributing to the development of insulin resistance.

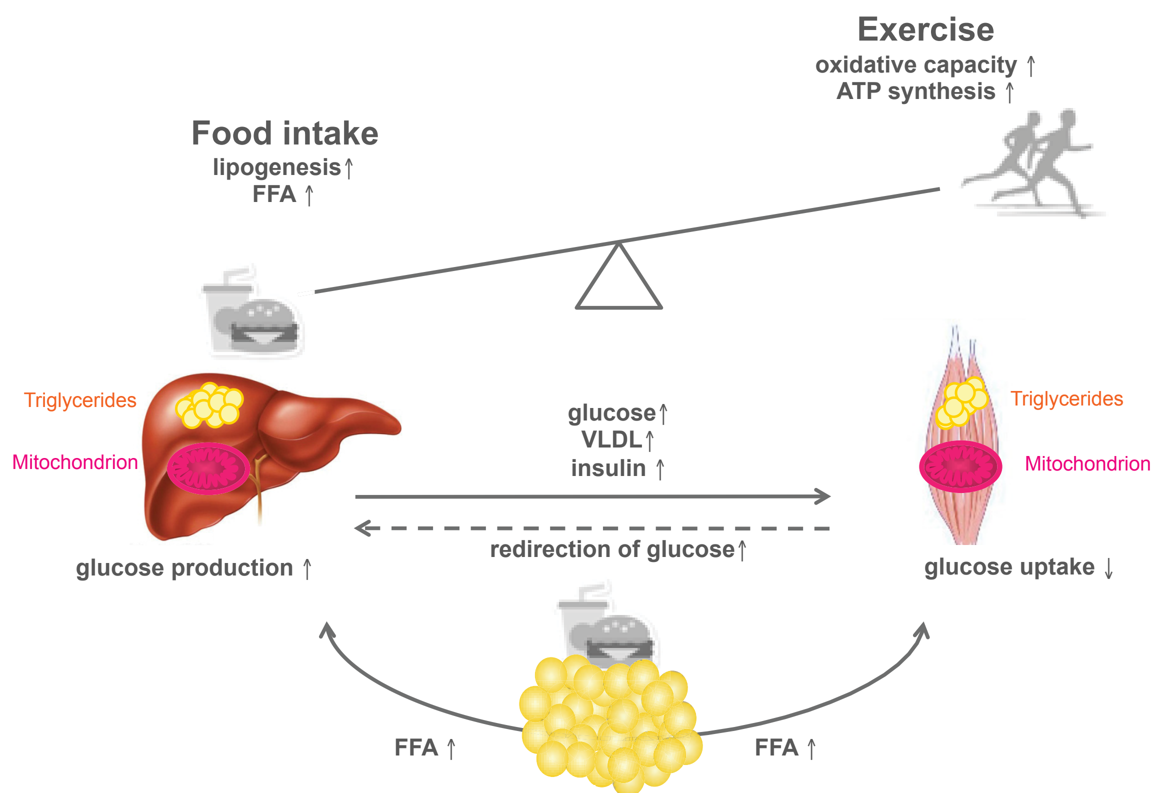

Figure 1.

Increased food intake and reduced physical activity augment substrate flux to liver and skeletal muscle. Lower oxidation in skeletal muscle directs higher amounts of glucose to the liver, while increased hepatic glucose and very-low density lipoproteins (VLDL) release and lipogenesis increases substrate flux to skeletal muscle. Lower storage of free fatty acids (FFA) in adipose tissue and higher lipolysis rates increase lipid flux to skeletal muscle and

liver. Ectopic lipid deposition in liver and skeletal muscle reflects substrate overload and insulin resistance.

1.4.1. The metabolic hypothesis

The lipid overflow hypotheses suggest that intracellular lipid metabolites and inflammatory pathways are involved in the development of insulin resistance in skeletal muscle and the liver. High lipid availability due to high caloric intake and/or increased release of free fatty acids (FFA) from adipose tissue, leads to lipid deposition in skeletal muscle and the liver and plays a central role in the development of insulin resistance [39]. Insulin resistance can result from (functional) lipodystrophy with increased storage of intracellular triacylglycerol (referred to as ectopic fat) in tissues that normally contain negligible amounts, such as intramyocellular lipids and hepatocellular lipids. Lipotoxicity refers to the impairment of the insulin signalling cascade via activation of specific second messengers by lipid metabolites which might also involve inflammatory pathways and thereby relates to the inflammation hypothesis. A more detailed description of the possible cellular mechanisms will be given in the following chapters. Chronic excess in protein intake is also associated with insulin resistance, glucose intolerance, and T2DM. Increased availability of aminoacids related activation of nutrient-sensitive kinase mammalian target of rapamycin and its downstream target S6 kinase contribute to amino acid-induced insulin resistance [40].

1.4.2. The inflammation hypothesis

According to the concept of subclinical inflammation adipocytokines released by adipocytes overloaded with triglycerides stimulate inflammatory pathways that interfere with insulin signal transduction in skeletal muscle and the liver, ultimately lead to insulin resistance [41]. However, the cellular mechanisms are yet unknown [42, 43]. Also, inflammation-related processes are involved in the development of diabetes-related comorbidities [44-46]. Cross-sectional studies reported associations between elevated concentrations of high sensitive C reactive protein (hsCRP) and sE-selectin and insulin resistance in patients with T2DM. Relations between proinflammatory cytokines and insulin resistance were also observed in healthy individuals and those with impaired glucose tolerance. It has been suggested that associations between markers of low-grade inflammation and HbA1c might be mediated by glucose-induced oxidative stress and could thereby contribute to early stages of atherogenesis.

lipids especially ceramides also induce intracellular inflammatory pathways in myocytes and hepatocytes linking the lipid overflow and cytokine hypotheses.

1.4.3. The perfusion hypothesis

One alternative concept explaining the mechanisms underlying lipid-induced insulin resistance involved possible effects of lipids on insulin-stimulation of acute and chronic local perfusion. Previous studies suggested that meal-induced increases in insulin have hemodynamic effects, thereby enhancing its access and that of nutrients to myocellular metabolism [47, 48]. Insulin increases muscle microvascular perfusion and facilitates delivery of nutrients and hormones to the interstitium [49]. Animal models of lipid-induced insulin resistance suggested that insulin-mediated microvascular perfusion is already reduced in the prediabetic state and relates to impaired insulin action [50, 51]. Thus, preventing the access of glucose and insulin to myocytes could contribute to lower glucose disposal and would also lead to lower G-6-P concentrations during hyperinsulinemia. This would place abnormal microvascular insulin action as an early event in the development of T2DM [52].

1.4.4. The brain hypothesis

In animal models, it has been shown that insulin stimulation of cerebral insulin receptors play an important role for the regulation of hepatic glucose and lipid metabolism. The application of intranasal insulin transiently elevates insulin concentrations in cerebral fluid in humans, improving the human memory function and exerting anorectic and catabolic effects [53-56]. Administration of intranasal insulin caused a reduction of body fat by inhibiting food intake suggesting that central insulin acts as a negative feedback signal in the regulation of obesity [57]. In lean humans, spontaneous cortical activity increased with the application of intranasal insulin compared to placebo, while in obese humans, these effects were suppressed [58, 59]. This "cerebral insulin resistance" correlated with ectopic lipid deposition in the liver, body weight and plasma concentrations of FFA [60]. Recently it was demonstrated that central insulin might improve hepatic energy metabolism and thereby reduce lipid storage in the liver. Thus central insulin effects might be relevant to the development of obesity and insulin resistance. However, the effects of intranasal insulin on adipose tissue lipolysis have not been investigated yet.

1.4.5. The mitochondrial hypothesis

Recent studies challenge the lipid overflow and cytokine hypotheses with regard to the sequence of events promoting insulin resistance by postulating that “mitochondrial dysfunction” may lead to lipid accumulation and insulin resistance. Tissue insulin resistance could be caused by a defect in oxidative capacity, i.e. impaired mitochondrial fitness, or the principal metabolic defect in skeletal muscle by which ectopic lipids accumulate might also be a defect in muscular fatty acid oxidation rather than oversupply of substrate [61]. Hyperglycemia and impaired inhibition of lipolysis resulting from insulin resistance induced glucolipotoxicity which initiates a vicious cycle of metabolic decompensation, especially in association with a sedentary lifestyle and high-fat diet [62]. Reduced mitochondrial function and lipid oxidation would thus give rise to intramyocellular lipid formation and insulin resistance, causing further impairment of mitochondrial function. Recently, an additional mechanism was proposed in which augmented lipid uptake into mitochondria, leads to a mismatch of increased β-oxidation and decreased TCA and electron transport chain activities, triggering the accumulation of β-oxidative intermediates. Accordingly, insulin sensitivity is closely related to fasting whole body lipid oxidation, but not to intramyocellular lipid levels, in relatives of individuals with T2DM [63], which further supports the notion that impaired lipid oxidation is related to insulin resistance. In conclusion, under conditions of lipid overload, impaired lipid partitioning between cytosol and mitochondria can result in progressive accumulation of lipid metabolites and mitochondrial dysfunction. Regulators of subcellular lipid partitioning and mitochondrial oxidation strongly determine insulin sensitivity but are only partially understood [64].

1.5. Tissue specific pathophysiology of insulin resistance

1.5.1. Pathophysiology of skeletal muscle insulin resistance

Skeletal muscle plays a major role in whole-body energy metabolism, and is responsible for at least 80% of insulin-stimulated glucose disposal during the euglycemic–hyperinsulinemic clamp.

In conditions of insulin resistance, impaired insulin-stimulated glucose transport and/or phosphorylation, together with reduced glycogen synthesis, account for more than 50% of the reduction in whole body glucose disposal [65, 66]. Skeletal muscle contributes to atherogenic dyslipidemia and hepatic insulin resistance [39, 67]. This is consistent with the view that insulin resistant skeletal muscle promotes the conversion of energy from ingested carbohydrate into de novo hepatic lipogenesis and increased lipoprotein production [39, 67].

Early studies employing muscle biopsies to measure metabolite concentrations, arteriovenous metabolite balances across the leg or forearm or isotopic dilution of labelled metabolites already suggested that insulin resistance in patients with T2DM relates to reduced insulin-stimulated muscular glycogen synthesis, glucose phosphorylation and transport [68].

FFA relate to insulin resistance and predict not only T2DM but also cardiovascular mortality [69]. In healthy humans, plasma FFA elevation by continuous lipid infusion for hours induces reversible muscular insulin resistance [70]. The lipid overflow and cytokine hypotheses suggested that intracellular lipid metabolites and inflammatory pathways are involved in the development of insulin resistance.

Increased lipid availability relates to enhanced storage of lipids not only in adipose tissue but also in other organs such as the liver and skeletal muscle, referred to as ectopic lipid deposition. Ectopic lipid accumulation may result from impaired expandability of white adipose tissue (functional lipodystrophy, [71]), lipid oversupply by high caloric intake or from lower energy expenditure but also from increased lipolysis rates in insulin resistant adipose tissue (Figure 1.). Non-invasive proton magnetic resonance spectroscopy (^{1}H MRS) allows for the measurement of myocellular lipids (IMCL) contents. By aims of this technique it was shown that IMCL contents relate to insulin resistance in various sedentary populations [70, 72-74].

Previous studies in rodent models had suggested that increased FFA availability inhibits skeletal muscle glucose uptake due to a metabolic process involving the competition of glucose and fatty acids for substrate oxidation, referred to as "Randle mechanism" [75, 76]. The operation of this glucose-fatty acid cycle would imply that glucose-6-phosphate (G-6-P) accumulates with increasing plasma levels of FFA during development of lipid-induced insulin resistance. To examine the mechanism, by which lipids cause insulin resistance in humans, Roden et al measured skeletal muscle glycogen and G-6-P concentrations every 15 minutes by repetitive and simultaneous carbon-13 and phosphorus-31 (^{13}C and ^{31}P) MRS in healthy insulin sensitive humans during hyperinsulinemia under conditions of either low plasma concentrations of FFA (control conditions) or high plasma concentrations of FFA during lipid infusion [70]. Reduction of muscle glycogen synthesis and glucose uptake by elevated plasma FFA was preceded by a fall of muscle G-6-P concentrations. These results demonstrated that FFA induce insulin resistance in humans by initial inhibition of glucose transport/phosphorylation which is then followed by reduction in both the rate of muscle glycogen synthesis and glucose oxidation.

Noninvasive techniques, mainly MRS, made it possible to examine metabolic pathways in real-time mode also in patients with overt T2DM [77]. Multinuclei (^{13}C, ^{31}P, ^{1}H) MRS studies

identified glucose transport and/or phosphorylation as rate-controlling steps for myocellular insulin-stimulated glucose disposal and glycogen synthesis also in patients with T2DM [78]. Thus, ectopic lipid accumulation in skeletal muscle is associated with elevation of intracellular lipid metabolites and stimulation of inflammatory pathways both interfering with insulin signalling [70].

Possible candidates mediating impaired insulin signalling would be intracellular lipid metabolites and their intracellular targets such as diacylglycerol (DAG) and so-called DAG sensible protein kinase C (PKCθ, PKCε), which could inhibit insulin-receptor-substrate 1 (IRS1) [79, 80]. Ceramides could also induce insulin resistance possibly interfering with protein kinase B (PKB) and AKT [81]. In addition, increased cellular oxidative stress (reactive oxygen species) could induce down regulation of oxidative enzymes [82-84] (**Figure 2.**).

Plasma FFA are frequently increased in obesity and T2DM and were shown to inhibit insulin signalling by increasing serine phosphorylation of IRS1. This could result from various elevated intracellular lipid metabolites (DAG, ceramides, acyl-Coenzyme A [CoA]), incomplete β-oxidation raising acyl-carnitines and/or oxidative stress raising ROS along with activation of atypical PKC isoforms and the pro-inflammatory transcription factor nuclear factor-κβ [42, 85, 86] (**Figure 2.**). However, the cellular and molecular mechanisms responsible for causing muscle insulin resistance in humans are still unclear.

Deeper phenotyping of larger cohorts revealed that the correlation between insulin resistance and IMCL is affected by other factors. It was demonstrated that exercise training concomitantly increased fat oxidation and insulin sensitivity and decreased IMCL contents in obese elderly humans suggesting that exercise training promotes lipid utilization in skeletal muscle thereby reversing insulin resistance [87]. On the other hand, physical fitness, assessed from maximal aerobic capacity (VO_2max), increases both IMCL contents and insulin sensitivity. This phenomenon is referred to as the "training paradox". In spite of high insulin sensitivity, elevation of IMCL in endurance-trained athletes indicates that oxidative capacity confounds the positive relationship between IMCL and insulin resistance [88, 89]. Apparently, IMCL serve as an energy source during intensive aerobic exercise in athletes [90] but reflect an imbalance between energy supply and demand in sedentary insulin-resistant humans. Analysis of a larger population revealed a threshold value of VO_2max, which separates a positive and a negative relationship between IMCL contents and insulin resistance [89]. Insulin-sensitive obese humans, who might be referred to as "fit-fat individuals" likely compensate their increased lipid availability by augmented lipid oxidation resulting in normal IMCL content despite increased body weight [88, 91, 92].

Therefore, in addition to lipid overload due to increased lipolysis rates and dietary intake, the primary cause of IMCL accumulation could reside in impaired lipid oxidation [61]. Accordingly, skeletal muscle insulin resistance not only correlates with increased triglyceride storage as IMCL but also with reduced oxidative capacity [93].

1.5.2. Mitochondrial function in insulin resistant skeletal muscle

A number of studies have provided evidence of abnormalities in mitochondrial number/size, structure and function in the insulin resistant state. It is as yet unclear whether these alterations are causal factors initiation deterioration of insulin signalling or secondary, related to ageing and/or changes in metabolic conditions associated with insulin resistance such as higher FFA levels, hyperglycemia or lower glucose uptake.

In patients with T2DM and poor glycemic control, CS activity is ~14-20% reduced compared to lean glucose tolerant humans. This is consistent with lower skeletal muscle mitochondrial content, and could also reflect impaired mitochondrial activity [94, 95]. Complex I and CS activities were ~40% and ~19% lower, respectively, in patients with T2DM compared to obese and lean glucose tolerant humans [94]. Moreover, mitochondrial size and complex I activity were related to insulin sensitivity [94]. Morphological abnormalities in mitochondria were reported across the whole cell, whereas enzyme activities were mainly assessed in the subsarcolemmal compartment. One study [96] found that complex II activity was reduced only in subsarcolemmal mitochondria of patients with T2DM while cytosolic mitochondria were normal. Others found that complex I and II activities were reduced in T2DM when expressed per unit mass of skeletal muscle but not when related to mitochondrial content [97]. Thus mitochondrial function in T2DM might be comparable to that in glucose tolerant humans of comparable age- and body mass and impairment of respiration in T2DM could only be a result of lower mitochondrial content. However, mtDNA content was ~19% lower, but the ratio of mtDNA to total DNA was not changed in those study participants with T2DM, thus mitochondrial content was not reduced. In this study, patients with T2DM were not compared to young, lean, glucose tolerant humans, thus age-related effects were not determined. No measure of insulin sensitivity was assessed, thus, effects of insulin resistance could not be evaluated.

The finding that improvement of glycemic control in patients with poorly controlled T2DM normalised the expression of genes regulating oxidative phosphorylation suggested that glucotoxicity affect mitochondrial function [98]. Accordingly, mitochondrial function in T2DM was found to be ‘normal’ in one study with glucose-lowering medication being withdrawn

only 1 day before the experiment [97], but was impaired when withdrawal of medication was performed 2 weeks before the experiments [95]. Other endocrine factors that might affect mitochondrial function have been reviewed [99]. Using the phosphocreatine recovery technique, it has been shown that impaired mitochondrial oxidative capacity can occur in patients with T2DM (mean HbA_{1c} 7.3%) despite unchanged IMCL [100]. Increased hepatocellular lipids (HCL) have been shown to relate to hepatic insulin resistance, hepatocellular mitochondrial dysmorphology, depletion of mtDNA and decreased activity of the respiratory chain [39, 101, 102]. However, the relation between IMCL and fATP was yet unclear in patients with T2DM. In summary, mitochondrial function was either unchanged or reduced and inconsistently related to measures of insulin sensitivity in different groups of patients with overt T2DM. In addition to differences in body fat mass and age, variations in circulating insulin and substrate levels limit the conclusions that can be drawn from these data.

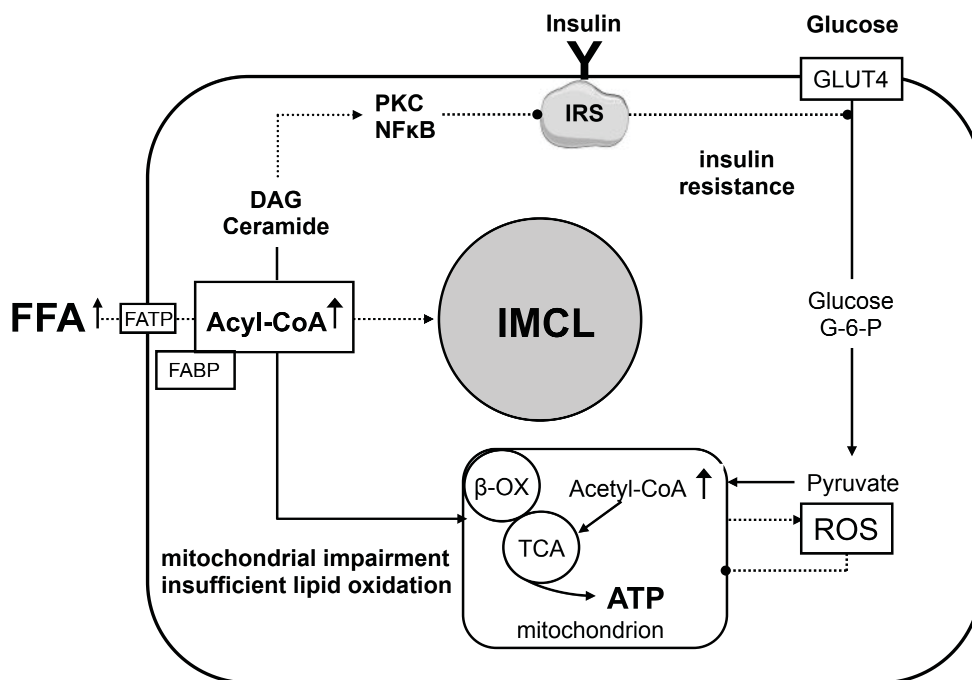

Figure 2.

Free fatty acids (FFA) enter the myocyte via fatty acid transport proteins and increase the acyl-CoA pool. Acyl-CoA enter the mitochondrion for degradation via β-oxidation (β-ox) thereby increasing acetyl-CoA exceeding the capacity of the tricarboxylic acid cycle (TCA) and giving rise to reactive oxygen species (ROS) production. This leads to impaired mitochondrial function and insufficient lipid oxidation. The rising acyl-CoA pool feeds synthesis of triglycerides (IMCL) and its storage as intramyocellular or hepatic lipids and

increases signaling intermediates [diacylglycerol (DAG), ceramides]. The latter activate intracellular mediators [nuclear factor kappa B, (NFκB), protein kinase C (PKC)]. These compounds stimulate serine/threonine phosphorylation of insulin receptor substrates (IRS), resulting in decreased translocation of the glucose-transporter 4 (GLUT4) and phosphorylation to glucose-6-phosphate (G-6-P) [93]

.

Based on the concept that the ratio of the fluxes through the glycolytic and oxidative pathways reflects the plasticity of skeletal muscle energy metabolism [103], a number of studies have shown an increase of glycolytic (e.g. phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase) relative to oxidative (e.g. CS, cytochrome *c* oxidase) enzyme activities in obese and sedentary individuals, and in humans with family history of or with overt T2DM [95, 104-106]. Reduced activation of pyruvate dehydrogenase or impaired inhibition of carnitine palmitoyltransferase-1 (CPT1) complexes by insulin could be responsible for this so called "metabolic inflexibility" [107, 108]. Similarly, the in vivo thermogenic effect of insulin and glucose, as measured by indirect calorimetry, has been reported to be impaired in insulin-resistant conditions, which might reflect 'insulin resistance of the mitochondria' [109, 110].

Ex vivo muscle biopsy studies demonstrated that CS activity and ATP production do not increase in obese individuals with untreated T2DM during prolonged hyperinsulinemic–normoglycemic clamps for 6–8 h [38].

In a recent study, skeletal muscle biopsy samples of obese individuals with poorly controlled T2DM were during fasting and 30 min after oral ingestion of 75 g glucose [97]. The rise in plasma glucose and insulin did not change O_2 flux rates, suggesting a defect in insulin-stimulated mitochondrial function. Likewise, myotubes from lean and obese, but not from T2DM, participants responded to incubation with insulin for 4 h with a ~30-% increase in CS activity. Simultaneous addition of palmitate diminished the stimulatory effect of insulin in myotubes from controls, suggesting that lipid-induced insulin resistance might interfere with insulin-stimulated mitochondrial function [111].

To address this issue, Brehm et al. assessed skeletal muscle mitochondrial activity during short-term lipid induced insulin resistance [25, 112]. They examined glucose disposal, G-6-P, IMCL and fATP during 3 h of FFA elevation in the presence of low plasma insulinemia followed by 3 h of further FFA elevation during hyperinsulinemia and repeated these experiments under control conditions of low plasma FFA concentrations. They found that 3 h of lipid elevation failed to affect ATP synthesis during low insulin levels despite marked reduction of whole body glucose uptake. This suggests that lipid-induced insulin resistance results primarily from mechanisms decreasing glucose uptake rather than from direct

interference of fatty acid metabolites with mitochondrial function. Plasma lipid elevation during hyperinsulinemia resulted in approximately 46% reduced whole-body glucose metabolism and a 70% lower rise of G-6-P without significant changes in IMCL. During hyperinsulinemia, fATP increased by approximately 60% under control conditions and was 24% lower during lipid infusion. Thus, physiologically increased plasma FFA concentrations reduce insulin-stimulated muscle fATP in parallel with induction of insulin resistance. This study again suggests that IMCL are not mediating impairment of insulin signaling, since IMCL remained unchanged during all conditions. Moreover, mitochondrial function remained unchanged during induction of lipid-induced insulin resistance, but was reduced during hyperinsulinemia. This finding provides evidence, that either insulin resistance at the level of the mitochondria might contribute to lower substrate oxidation, or reflects lower substrate uptake due to impaired glucose transport/phosphorylation. It still remained yet unclear, whether this phenomenon was also present in patients with chronic insulin resistance or overt T2DM.

The few available studies investigating insulin stimulated mitochondrial function provide some evidence for mitochondrial insulin resistance (impaired insulin stimulation of mitochondrial activity) as an early abnormality when assessed from in vivo and ex vivo experiments in skeletal muscle mitochondria. Two ex vivo muscle biopsy studies found no increase in CS activity, ATP production and O_2 flux rates in muscle biopsies from obese untreated T2DM during prolonged hyperinsulinemic–normoglycemic clamps or oral glucose loading [38]. Two in vivo studies in severely insulin-resistant relatives of T2DM reported ~85% myocellular insulin stimulated fATP and ~80-100% higher IMCL [24, 35]. One ex vivo study, indicated that insulin stimulation for 6-8 hours of ex vivo skeletal muscle oxidative capacity is impaired in patients with T2DM [38].

The T2DM participants were non-obese, had metabolically well-controlled T2DM and were matched to the controls in terms of physical activity. Furthermore, we tested substrate (glucose)-dependent effects by performing hyperinsulinemic–hyperglycemic clamps,

1.5.3. Mitochondrial function and insulin resistance - cause or consequence?

It is yet unclear whether reduced mitochondrial oxidative capacity is the cause or the consequence of insulin resistance. According to one theory, inherited mitochondrial dysfunction results in accumulation of lipid metabolites which induce insulin resistance [62]. Another concept assumes that lipid abundance due to sedentary life style, high caloric intake

and augmented lipolysis rates promote lipid overload, incomplete lipid oxidation and oxidative stress resulting in acquired damage of mitochondrial proteins, functional impairment of mitochondria and further enhanced deposition of lipid metabolites, which leads to insulin resistance [86, 113, 114]. On the other hand, it is also possible that mitochondrial oxidative capacity, plasticity and biogenesis are dysregulated but not causally related to insulin resistance [115].

To evaluate the causal role of impaired mitochondrial function independent of long term effects of T2DM, overweight or age, normoglycemic but insulin-resistant first-degree relatives of patients with T2DM have been examined in several studies. This group of individuals is characterized by decreased skeletal muscle glucose transport/phosphorylation and glycogen synthesis and lower VO_2max, a marker of oxidative capacity [24, 116-118]. Accordingly, skeletal muscle expression of PGC-1α, which activates genes controlling oxidative metabolism, was reduced in glucose tolerant relatives of T2DM patients [119]. Consistent with this finding, another study reported that mtDNA content was reduced in glucose tolerant relatives of T2DM patients compared to humans without family history of T2DM. Moreover, mitochondrial content related to insulin sensitivity and impaired plasticity of substrate oxidation in response to high-fat diet [106]. Compared to age-matched humans without family history for T2DM, insulin-resistant offspring of patients with T2DM had lower cytochrome *c* oxidase activity (by ~50%) and mitochondrial density (by ~38%), higher IMCL contents (by ~60%), reduced glucose uptake (by ~60%), enhanced inhibitory IRS1-serine phosphorylation (by ~50%) along with reduced insulin-stimulated Akt activation (by~60%) [120]. Petersen et al. applied ^{31}P MRS and showed that insulin-resistant offspring of patients with T2DM have a ~30% lower fasting fATP, ~80% higher IMCL compared to insulin-sensitive humans [24]. These offspring also had reduced insulin-stimulated fATP and Pi uptake into skeletal muscle, which suggests coupling of these processes [35]. These findings are consistent with the concept that impairment of mitochondrial function predisposes these offspring of patients with T2DM to IMCL accumulation and to early development of insulin resistance at younger age. However, in contrast to potentially inherited defects in mitochondrial biogenesis, it is possible that these alterations result from acquired defects such as lipid-mediated interference with insulin signalling. Of note, these studies assessed associations and no longitudinal data were acquired to delineate the sequence of events. In line with this, a recent analysis demonstrated that systemic oxidative stress is related to insulin resistance in relatives of individuals with T2DM even after adjustment for body mass

[121]. Extended longitudinal studies of individuals at risk for T2DM or mechanistic intervention studies would be needed to prove causal relationships.

Lifestyle interventions or medical treatment can improve these perturbations and thereby provide insight into the sequence of events leading to impairment of mitochondrial function and insulin resistance. Lifestyle intervention studies in obese humans and patients with T2DM including either diet [122], diet combined with exercise training [122, 123] or exercise intervention only without weight reduction [124, 125], in equal measure improved insulin sensitivity. Of note, all of these lifestyle interventions improved various features of mitochondrial function in skeletal muscle as assessed from increased enzyme activity comprising tricarboxylic cycle [123, 125, 126], electron transport chain [122, 123, 126] and β-oxidation [125]. Many of the training-related positive effects are mediated by exercise induced activation of AMPK, a key regulator of cellular energy homeostasis. Mitochondrial capacity and content restored in patients with T2DM to values comparable to lean, healthy humans following only 12-weeks of exercise training [124, 127]. Thus, lifestyle intervention in patients with T2DM may overcome perturbations of gene expression may it be inherited or acquired. However, a single nucleotide gene polymorphism encoding for complex 1 of the respiratory chain determined the response of fasting mitochondrial activity and insulin sensitivity to moderate exercise training in glucose tolerant first degree offspring of patients with T2DM [128, 129]. Thus, while lifestyle interventions overall showed improved insulin sensitivity concomitant with enhanced mitochondrial function, genetic background could modulate the efficiency of life style interventions.
Peroxisome proliferator-activated receptor gamma (PPARγ) agonists, the thiazolidinediones, improve insulin sensitivity by several mechanisms including increased uptake and metabolism of FFA mainly in subcutaneous adipose tissue, but also in skeletal muscle [130]. Treatment with pioglitazone for 12 weeks also raised the expression of PGC1-α one main regulator of mitochondrial content and increased lipid oxidation capacity in parallel with lipid storage in white adipose tissue [131]. Another recent study revealed that improvement of insulin sensitivity by pioglitazone was accompanied by activation of AMPK and increased mRNA levels of multiple genes involved in mitochondrial function and fat oxidation [132]. Thus, improvement of oxidative capacity by thiazolidinediones could also underlie the positive effect of thiazolidinediones on insulin sensitivity or both effects occur independently. Accordingly, improvement of glycemic control by rosiglitazone was found in the whole study group, while increase of mitochondrial content and activity was only found in a subgroup of participants

who had higher aerobic capacity already before the treatment [133]. Those participants might have had preserved mitochondrial plasticity or a specific genetic susceptibility to these positive treatment effects [133].
Metformin, one of the most commonly used antidiabetic drugs, exerts reduction of hepatic glucose production at least partially, by activation of AMPK [134]. In vitro results suggest that metformin promotes mitochondrial biogenesis through the activation of PGC-1α via the AMPK pathway [135]. New pharmacological approaches potentially suitable to improve mitochondrial oxidative capacity and glucose uptake include direct activation of AMPK, enhancing both, glucose and lipid oxidation [136, 137], inhibition of pyruvate dehydrogenase [138] and mitochondria-targeted antioxidants [139]. The therapeutic use of antioxidants has not shown beneficial effects in clinical studies.
Markers of cell membrane degradation might reflect consequences of sustained mitochondrial impairment and oxidative stress. Membrane phospholipids are hydrolysed to FFA and phosphodiesters (PDE), which are metabolized to cell membrane degradation products [140]. Therefore, myocellular PDE are elevated in patients with myopathies [141, 142], increase with age [143] and also in response to statin treatment reflecting membrane damage [144]. PDE is thought to relate to fiber atrophy, sarcolemmal damage or structural and functional changes during loss of muscle mass possibly resulting from cumulative oxidative damage. Thus, PDE may serve as marker of biomembrane integrity and prolonged membrane damage. We hypothesized that reduced mitochondrial function secondarily relates to membrane damage. As mentioned above, skeletal muscle insulin resistance relates to lower mitochondrial activity during hyperinsulinemia in insulin resistant offspring of patients with T2DM, resulting in lower postprandial glucose uptake and oxidation in skeletal muscle. Along with hyperinsulinemia, this could shift postprandial energy storage towards de novo lipogenesis in the liver and thereby increase hepatic lipid accumulation [67] (**Figure 1.**).
Another model to assess causal relations between insulin resistance and mitochondrial dysfunction could be mutations of genes encoding for mitochondrial proteins. Most primary mitochondriopathies are associated with maternally-inherited mutations of mtDNA or of nuclear DNA encoding mitochondrial proteins. Diabetes is commonly present in mitochondriopathies and mostly results from impaired glucose-stimulated insulin secretion caused by insufficient increase of ATP in the β-cell [99, 145, 146]. In addition, insulin sensitivity can be decreased in mitochondrial diabetes [147]. Accordingly, mitochondrial dysfunction might at least contribute to insulin resistance. Friedreich's ataxia, a mitochondrial disease is not only

related to reduced mitochondrial function [148], but also to an increased risk of diabetes, and to increased ROS production [147].

On the other hand, long-lasting insulin resistance might affect mitochondrial function and thereby induce irreversible damage of mitochondria. So for example, patients with active acromegaly frequently exhibit hepatic [149] and skeletal muscle insulin resistance [150, 151]. Due to the slow and often insidious onset of the disease, the diagnosis often remains unclear for years after the onset of characteristic symptoms, which may give rise to sustained metabolic alterations [152]. On the other hand, patients with active acromegaly may also feature altered β-cell function either resulting from direct effects of increased growth hormone/insulin like growth factor-1 levels or adaptation to altered glucose and FFA concentrations [153]. These abnormalities could initiate a vicious cycle similar to the muscle in which mitochondrial dysfunction, lipid availability and insulin resistance amplify each other, finally leading to exhaustive β-cell failure [153]. Insulin resistance, impaired glucose tolerance and diabetes secondary to acromegaly can be reversed by the treatment of the underlying disease. It is yet unknown if the defects underlying insulin resistance are completely reversed by normalizing insulin like growth factor-1 levels and the endocrine and metabolic environment or to which extent they may persist after treating acromegaly.

1.5.4. Pathophysiology of hepatic glucose metabolism

Humans with overt T2DM and those with risk factors for T2DM are characterized by hepatic insulin resistance [154]. In overt T2DM, the liver is responsible for the rise of postabsorptive plasma glucose concentrations which results from increased fasting (basal, postabsorptive) EGP [155], due to elevated GNG [156]. After meal ingestion, T2DM patients show impaired suppression of EGP and thus further rising plasma glucose concentrations along with lower glycogen synthesis capacity relating to impaired glucose uptake [22, 155]. Hepatic insulin resistance therefore leads to fasting and postprandial hyperglycemia and is therefore of major interest for the detection of glucose-lowering treatment targets.

1.5.4.1. Hepatic insulin resistance and steatosis

Concepts for the development of hepatic insulin resistance are based on the imbalance between food consumption and energy expenditure leading to ectopic lipid accumulation in the liver. Peripheral insulin resistance gradually raises glucose and insulin plasma concentrations, thereby stimulating hepatic de novo lipogenesis, via upregulation of lipogenic genes including sterol/carbohydrate regulatory element binding protein-1 (CHREBP-

1/SREBP-1) [157]. In adipose tissue of insulin resistant individuals, impaired insulin-mediated inhibition of hormone sensitive lipase raises the release of circulating glycerol and FFA [158], which closely correlate with HCL and insulin resistance of the liver [22]. HCL in turn associates with whole body insulin resistance [159] and is an independent risk factor of cardiovascular mortality [160].

Increased adipose tissue lipolysis can increase EGP in different ways: by increased secretion of the gluconeogenic precursor glycerol or enhanced production of NADH along with additional hepatic β-oxidation of FFA. Moreover, increased de novo lipogenesis is stimulated by raised plasma levels of insulin and glucose. Hepatocellular lipid intermediates like DAG likely play a central role as they are suggested to activate atypical PKC isoforms which stimulate serine phosphorylation of insulin receptor substrate-2 and thereby inhibit insulin signaling at least in rodents [39]. Triglycerides (referred to as HCL) are stored subsequently causing NAFL [39, 161] or secreted into the circulation as large very-low density lipoproteins (VLDL) [162] (**Figure 3.**). If the intake and the synthesis of lipids in the liver surpass the rate of the degradation of lipids by mitochondrial oxidation, the development of steatosis is favored.

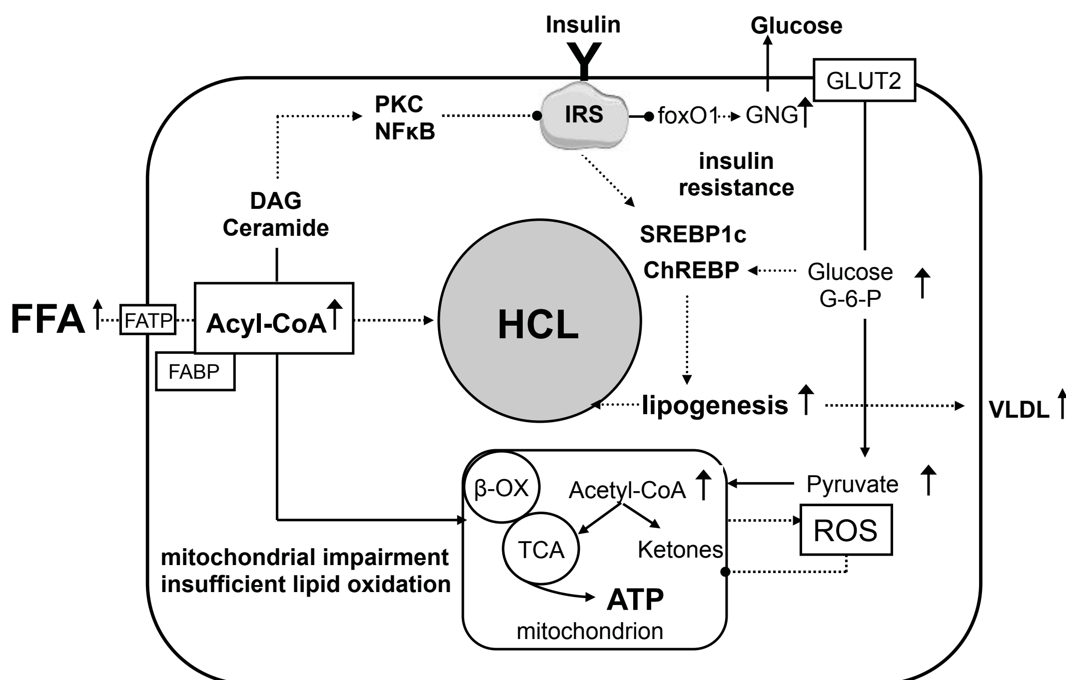

Figure 3.

Free fatty acids (FFA) enter the hepatocyte via fatty acid transport proteins and increase the acyl-CoA pool. Acyl-CoA enter the mitochondrion for degradation via β-oxidation (β-ox)

thereby increasing acetyl-CoA exceeding the capacity of the tricarboxylic acid cycle (TCA) and giving rise to reactive oxygen species (ROS) production. This leads to impaired mitochondrial function and insufficient lipid oxidation. The rising acyl-CoA pool feeds synthesis of triglycerides (TG) and its storage as hepatic lipids and increases signaling intermediates [diacylglycerol (DAG), ceramides]. The latter activate intracellular mediators [nuclear factor kappa B, (NFκB), protein kinase C (PKC)]. These compounds stimulate serine/threonine phosphorylation of insulin receptor substrates (IRS), resulting in decreased translocation of the glucose-transporter 2 (GLUT2) and phosphorylation to glucose-6-phosphate (G-6-P). Glucose activates the carbohydrate responsive element binding protein (ChREBP) and hyperinsulinemia leads to overactivation of sterol regulatory element binding protein 1c (SREBP-1c), both of which stimulate lipogenesis, resulting in increased very-low density lipoproteins (VLDL) and intrahepatocellular triglycerides (HCL). Enhanced glycolysis and β-oxidation supply the Acetyl-CoA pool which diverts to the TCA cycle and into ketogenesis. According to the concept of "selective insulin resistance", forkhead box O1 (FoxO1) is dysregulated, giving rise to increased gluconeogenesis (GNG) [93].

Non-alcoholic fatty liver diseases (NAFLD) include steatosis, steatohepatitis (NASH) and liver cirrhosis. NAFLD is associated with insulin resistance and T2DM. Accordingly, 70% of overweight people and patients with T2DM and 55% of overweight children suffer from a hepatic steatosis (HCL> 5.5 %), whereas the general population present a prevalence of 15% [163]. NAFLD is not a classic liver dysfunction, but rather a dysfunction of the glucose metabolism of patients with T2DM. Reduction of hepatic lipid levels improves glucose metabolism [164]. Therefore understanding the mechanisms underlying NAFLD and development of therapy strategies will not only contribute to the prevention of the progression to liver cirrhosis but also for the treatment and the prognosis of the T2DM. About 10-20% of the patients with steatosis have a progress to NASH, the condition between steatosis and cirrhosis in the spectrum of NAFLD. Age, activity of steatohepatitis, and established fibrosis predispose to cirrhosis, which has a 7- to 10-year liver-related mortality of 12%-25% [165, 166]. Presumably a noteworthy number of cryptogenic cirrhosis could be attributed to end stage NAFLD. NASH is characterized by a macro steatosis, inflammation, pericellular fibrosis and apoptotic hepatocytes, which look like balloons. Risk factors for the development of a NASH are overnutrition and underactivity, insulin resistance, and genetic factors [165, 166]. Lipotoxicity, oxidative stress, cytokines, and other proinflammatory mediators may each play a role in transition of steatosis to NASH [167]. The development of a steatosis to a NASH and cirrhosis and the importance of the liver for the development of an insulin resistance have not yet been determined. However, HCL is a strong marker not only for the insulin resistance of the liver, but also of skeletal muscle.

1.5.4.2. Hepatic mitochondrial function in the insulin resistant state

Mice with primary defects in hepatic mitochondrial function due to targeted mutations developed either reduced [168, 169] or enhanced insulin sensitivity [170] compared to control mice, indicating an evident but unclear relationship between hepatic energy metabolism and insulin resistance. Moreover, the insulin signalling in the liver is differentially regulated, with inhibition of EGP being impaired and stimulation of lipogenesis being increased (selective hepatic insulin resistance), while the insulin-dependent regulation of liver mitochondria is yet unclear [171, 172].

In obese humans hepatic ATP to inorganic phosphate (Pi) ratios were lower [173] and relative hepatic ATP recovery following fructose administration was either decelerated [174] or equal [173] as compared to lean controls. On the other hand, obese glucose tolerant humans had 2-fold higher hepatic lipid oxidation than lean, as assessed from ^{11}C-palmitate PET imaging [175]. Increased lipid oxidation related to increased insulin levels paralleled by adipose tissue insulin resistance, presumably resulting in higher lipid flux to the liver. Although not all of these reports provided data on the presence of hepatic steatosis, it is possible that these obese humans had NAFL. Accordingly, 60-90% of humans with NAFLD are obese [176].

Impaired hepatic lipid oxidation could foster HCL accumulation and hepatic insulin resistance [177], and on the other hand lipid overload might induce oxidative stress and damage of mitochondria in the liver (**Figure 3.**). Thus, mitochondrial adaptation would be particularly important under conditions of increased substrate flux. One recent study focused on obese humans with steatosis, and reported that obese humans with liver fat more than 5.5 % (as assessed from ^{1}H MRS), had two-fold greater hepatic oxidative capacity along with hepatic insulin resistance compared to those with normal liver fat content [178]. Humans with NAFL had 2-fold higher TCA cycle flux rates, 50% higher anaplerosis and 25% higher GNG, which might reflect an adaptive response of liver mitochondria to chronic lipid abundance and higher energy demands. Moreover, HCL correlated with parameters of oxidative and non-oxidative mitochondrial metabolism [178]. Another report studied in liver graft samples showing that mitochondrial content was increased in livers with steatosis compared to normal livers, and the gene expression of mitochondrial proteins was up-regulated [179]. Patients with NAFLD show decreased activity of the enzyme complexes of the respiratory chain [180] and dysmorphologies of the mitochondria coincide with the development of NASH [102]. These data supported the concept, that hepatocellular mitochondrial energy metabolism plays a key role in the progression of NAFL to NASH. Increased lipid flux to the liver might also lead to increased lipid oxidation with production of ROS and induction of oxidative stress [181] which

might deteriorate mitochondrial function and induce inflammatory processes that promote the development of NASH. Accordingly, although NAFL patients show increased mitochondrial β-oxidation [102, 182, 183], the ability to resynthesize ATP after fructose challenge is decreased and negatively related to body mass index (BMI) [174].

Rodent studies confirmed the theory of enhanced hepatic mitochondrial function, ROS production, hepatic insulin resistance in obesity and NAFL, as reflected by elevated TCA flux and β-oxidation due to high-fat diet [184, 185]. On the other hand comparable rates of hepatic lipid oxidation were found in overweight humans with NAFL and healthy lean humans under both basal and insulin-stimulated conditions, as assessed indirectly by plasma 3-hydroxybutyrate levels [186].

Gene expression analyses in the liver of patients with T2DM revealed up-regulated expression of genes involved in OXPHOS, GNG and ROS production in patients with T2DM, which related to obesity, insulin resistance and fasting plasma glucose levels [187]. Intravenous fructose challenge impairs ATP regeneration in obese patients with T2DM [188] suggesting that high dietary fructose intake may impair hepatic energy homeostasis and predispose to liver fat accumulation [188, 189]. In patients with T2DM data on liver mitochondrial function are scarce and conflicting. These data show either impaired [173, 180] or enhanced [175, 178, 183] hepatic mitochondrial function and are derived from indirect methodologies to assess hepatic energy metabolism.

Considering the tight association between liver mitochondrial function and NAFLD pathogenesis, these data indicate that an abnormal hepatic energy metabolism may precede the development of NAFLD in patients with T2DM.

2. Hypotheses

We aimed at assessing the cellular mechanisms underlying the development of lipid-induced insulin resistance in skeletal muscle and the role of mitochondrial activity in skeletal muscle and the liver. We sought to identify potential therapy targets for the prevention and treatment of insulin resistance and fatty liver assuming that this might reduce the mortality related to T2DM. We tested the following hypotheses that are depicted in a scheme on the pathogenesis of insulin resistance (**Figure 4.**).

I. Increased lipid availability impairs insulin signalling via DAG, whereas IMCL and ceramides and effects on local perfusion are not relevant.

I.a. Lipid-induced insulin resistance results from inhibition of myocellular glucose uptake, but not from impaired local perfusion that determines delivery of insulin or glucose to the interstitium (**att. article No. 1**).

I.b. Lipid-lowering therapy improves insulin sensitivity by reduction of plasma FFA independent from changes of IMCL (**att. article No. 2**).

I.c. Increased FFA impair insulin signaling via muscle DAG accumulation while ceramides are not elevated (**att. article No. 3**).

II. The reduction of mitochondrial activity is a consequence of insulin resistance and may promote the development of insulin resistance and steatosis.

II.a. Patients with T2DM have reduction of mitochondrial activity that correlates with disturbed muscle integrity and higher liver fat (**att. article No. 4., 5., 6.**).

II.b. Reduced chronic mitochondrial activity relates to insulin resistance in skeletal muscle (**att. article No. 7**).

II.c. Chronic insulin resistance correlates with reduction of mitochondrial activity (**att. article No. 8**).

III. The reduction of mitochondrial activity in the liver correlates with liver fat content and hepatic insulin resistance.

III.a. Lower hepatic ATP levels correlate with insulin resistance in the liver (**att. article No. 9**).

III.b. Lower hepatic ATP synthesis rates correlate with insulin resistance in the liver (**att. article No. 10**).

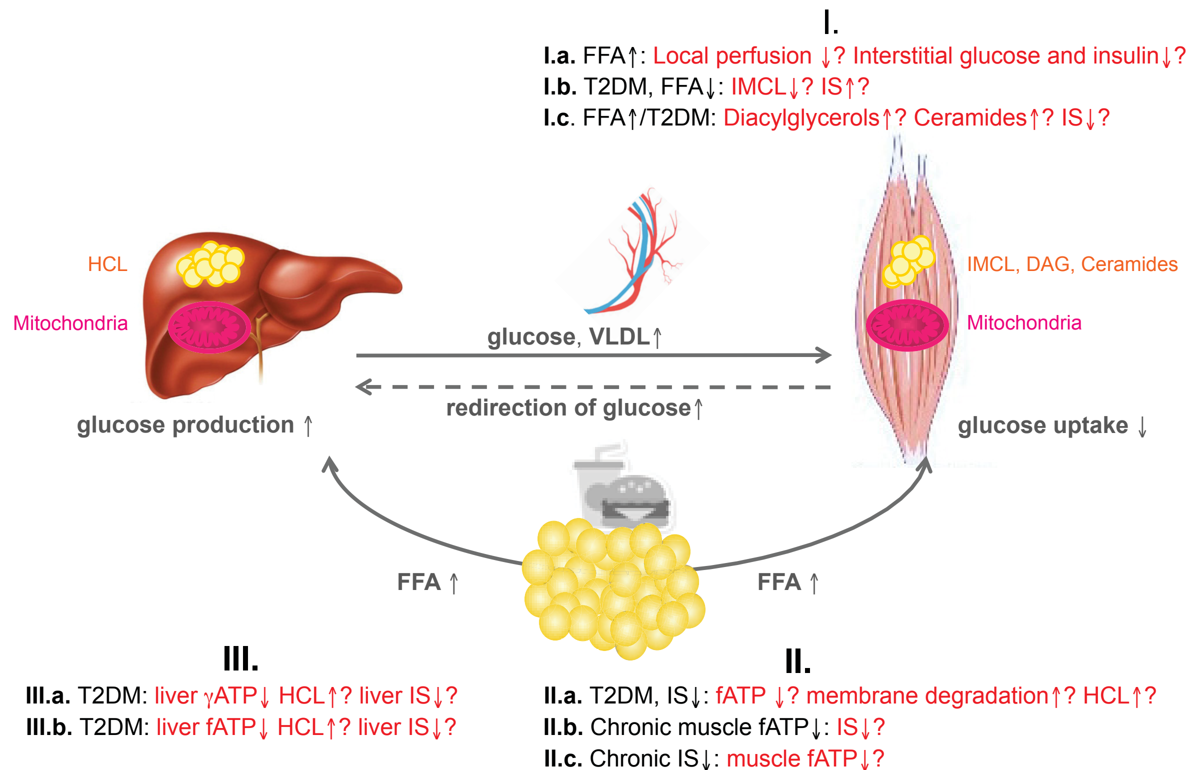

Figure 4.

Hypotheses I-III tested in our studies are summarized in this scheme describing concepts of the pathogenesis of insulin resistance. Prevailing conditions, e.g. increased free fatty acids (FFA↑) derived from dietary intake and released by adipose tissue, type 2 diabetes mellitus (T2DM), decreased ATP synthase flux (fATP↓), γATP or decreased insulin sensitivity (IS↓) are quoted in black, dependent conditions, e.g. hepatocellular lipids (HCL) are quoted in red letters. Myocellular lipid metabolites comprise intramyocellular lipids (IMCL), diacylglycerols (DAG) and ceramides. Very-low density lipoproteins (VLDL).

3. Methods

In these studies we applied gold standard methods of metabolic phenotyping and further developed non-invasive MRS applications of metabolic phenotyping. In the following sections the most relevant methods are explained for better understanding.

3.1. Assessment of dynamic insulin sensitivity

Insulin resistance is defined as the reduced ability of insulin to exert its biological effects on target tissues namely skeletal muscle, liver and adipose tissue. The sensitivity to insulin can be determined by its known individual biological effects, such as glucose clearance in skeletal muscle, inhibition of EGP by the liver and lipolysis by adipose tissue or by its compound action on whole body glucose metabolism. Although not all biological responses are equally sensitive or responsive to insulin, insulin resistance is most frequently referred to as impaired insulin action on glucose metabolism.

Assessment of insulin action yields a concentration-response curve according to Michaelis-Menten kinetics. Insulin resistance is characterized by a shift of the dose-response curve to the right with higher EC_{50} as well as lower insulin responsiveness related to reduction of V_{max}. Thus, insulin sensitivity (and insulin resistance), are continuous variables, making it difficult to define uniform threshold values separating "insulin sensitive" from "insulin resistant" individuals. The World Health Organization (WHO) definition of the metabolic syndrome includes insulin resistance, which is given by values of glucose uptake during the hyperinsulinemic-euglycemic clamp within the lowest quartile of glucose uptake rates of a population. Of note, although being insulin resistant, normoglycemia can be maintained for long periods due to compensatory increases in insulin secretion, resulting in elevation of fasting plasma insulin. In view of that, following the European Group for the Study of Insulin Resistance (EGIR), presence of fasting hyperinsulinemia, defined by plasma insulin within the highest quartile of a glucose tolerant population, indicates insulin resistance [190].
Quantification of insulin sensitivity has become increasingly relevant for metabolic phenotyping in clinical intervention studies, cohort profiling and for the examination of the pathogenesis of insulin resistance. The gold standard method for measurement of whole body and tissue specific insulin sensitivity is the hyperinsulinemic-euglycemic clamp technique. As it is laborious, time consuming and cost intensive, various methods have been developed aiming at easier assessment of insulin sensitivity. The choice of method depends on the number of participants, expected differences and type of study to be undertaken, which has

made it difficult to compare different studies on insulin sensitivity. Interpretation of these methods requires knowledge on several variables and specific protocols, including infusion sampling procedures and hormonal assays.

In most of the included studies, we applied the hyperinsulinemic-euglycemic clamp technique combined with the tracer dilution technique in order to assess tissue specific insulin sensitivity in skeletal muscle and the liver (**att. articles No. 1-7, No. 9, 10**). In some studies, with additional focus on β-cell function, we applied other dynamic tests for the assessment of whole body insulin sensitivity and insulin secretion: the oral glucose tolerance test, and/or the intravenous glucose tolerance test (**att. articles No. 1, No. 7, No. 8**).

3.1.1. The oral glucose tolerance test

The participant rapidly drinks a solution containing 75-g glucose (dextrose diluted in 300 ml flavored water) after 10-12 hours of fasting. Samples are drawn at baseline and at 15- 30 min intervals for 2-3 hours. Plasma glucose increases during the first 60 min and returns back to fasting levels within 2-3 hours in glucose tolerant humans. The underlying principle is comparable to that of the intravenous glucose tolerance test (IVGTT, explained below), despite the fact that the rate of appearance of glucose in plasma is indefinite after oral ingestion. Equations describing the interplay between glucose appearance, insulin secretion and glucose disposal have been developed according to different approaches (e.g. empirically or based on model-derived assumptions). These equations finally yield indices describing insulin sensitivity, which have good correlations with the hyperinsulinemic-euglycemic clamp derived M-value [191, 192].

3.1.2. The hyperinsulinemic-euglycemic clamp test

Due to its high reproducibility and flexibility, the hyperinsulinemic-euglycemic clamp is generally accepted as the "gold standard" for the in vivo assessment of whole body insulin sensitivity in humans [193-195]. The clamp test is used to determine whole body glucose disposal during constant insulin-stimulation. Insulin is infused intravenously to create a sustained hyperinsulinemic condition. Mostly, insulin is infused with 40 mU/min per m^2 body surface area, which corresponds to approximately 1 mU/min per kg body weight and yields plasma insulin concentrations of 50-130 μU/ml [196], but also other doses of insulin can be used according to the research question. In order to rapidly achieve steady-state plasma insulin levels, a short priming dose is applied before the start of the continuous insulin infusion.

Administration of insulin stimulates glucose uptake, which would induce a fall of plasma glucose. This is counteracted by administration of a glucose solution at variable infusion rates, to maintain ("clamp") plasma glucose concentrations at a predefined level within the euglycemic (between 90 and 100 mg/dl), isoglycemic (around the individual fasting plasma glucose), hyperglycemic or hypoglycemic range. Of note, the euglycemic-hyperinsulinemic clamp is generally used to assess whole body insulin sensitivity and was applied in the presented reports. The glucose infusion rate is adjusted according to the prevailing plasma glucose concentrations, which are monitored throughout the test at 5-10 min intervals [196, 197].
The so-called "pancreatic clamp" is performed by simultaneous infusion of somatostatin to completely inhibit endogenous secretion of insulin and of counterregulation via glucagon and growth hormone [198]. Multi-step clamps at different levels of insulinemia can be performed in order to assess whole body glucose metabolism over a broad range of plasma insulin concentrations [199, 200]. A duration of ~100 min has to be taken into account to reach a new steady state with each step of increasing insulin concentrations so that time becomes the limiting factors of multi-step clamp protocols.

As skeletal muscle is responsible for ~80% of the insulin stimulated glucose uptake [201], the rate of glucose infusion rate during steady state required to maintain constant glycemia, expressed as glucose infusion rate (GIR, in ml/h) or as whole-body glucose metabolism (M-value, $mg.kg^{-1}.min^{-1}$) during hyperinsulinemia, reflects the whole body insulin sensitivity [202]. GIR and M are then normalized to body weight in order to yield standardized values for inter- and intraindividual comparison [194, 196]. The so-called “space correction” represents the rate at which glucose was added to or removed from the distribution volume independently of changes in glucose utilization during a defined time interval (mostly 20 min) Thus, space correction allows to correct for fluctuations of plasma glucose concentrations frequently observed even under conditions of steady state and continuous glucose infusion rates [202].
The clamp test provides an intra-individual CV of ~10% [203-206]. Despite the noteworthy inter-individual variability precludes to identify a clear threshold value to discriminate between insulin-sensitive and insulin-resistant subjects, the 95% confidence interval for M-values (4.7-8.7 $mg \cdot kg^{-1} \cdot min^{-1}$) in healthy, non-obese individuals obtained from 18 independent studies [196] indicates insulin resistance at values below 4.7 $mg \cdot kg^{-1} \cdot min^{-1}$. Nevertheless, some limitations have to be acknowledged. This technique is laborious, time-consuming and requires trained and experienced personal. The rapid decline in plasma glucose concentrations may lead to hypoglycemia and counterregulatory endocrine responses [207, 208]. Hyperinsulinemia causes a

compartmental shift of potassium into the cells, possibly leading to hypokalemia, which should be prevented by intravenous or oral potassium administration [209]. The clamp does not allow for differentiation between oxidative and nonoxidative glucose metabolism but this can be derived from combination of the clamp test with indirect calorimetry [210]. Despite the total suppression of EGP during the 40 $mU \cdot m^{-2} \cdot min^{-1}$ hyperinsulinemic-euglycemic clamp in insulin sensitive humans, hepatic insulin resistance can lead to underestimation of insulin sensitivity via sustained glucose release. This limitation has been overcome by the use of labelled glucose infusion for measuring EGP.

3.1.3. Tracer dilution techniques

The liver accounts for the majority of EGP [22], to which the kidneys and the intestine do not contribute significantly. EGP can be assessed from the dilution of exogenous labeled glucose by hepatic glucose release. The decrease of EGP between baseline and clamp conditions, i. e. suppression of EGP, provides a measure of hepatic insulin sensitivity. A suitable tracer for labeling glucose has to fulfill certain criteria with regard to the tracee, (i.e. the unlabeled glucose): both, tracer and tracee should have identical kinetics, transport and metabolism, the tracer should be measurable already in negligible concentrations whole being perfectly distinguishable, e.g. by ionizing radiation or mass differences. Mostly, stable isotopic labeled, [6,6-$^{2}H_{2}$]glucose, and alternatively (rather in former times) radioactively labeled, [3-^{3}H]glucose, have been used. In practice, labelled glucose is given as a primed-continuous infusion initiated at least 120 min before the start, and continued throughout the hyperinsulinemic clamp test. The variable glucose infusion applied to stabilize the plasma glucose at its target, is labeled at the same enrichment as the plasma glucose, in order to prevent dilution of the tracer during the clamp by exogenous glucose according to the HOT-GINF protocol [211, 212]. Finally, in the steady state, only changes of EGP should have impact on the enrichment of glucose by the tracer. About four blood samples are drawn at baseline (before the clamp), after 120 min of continuous tracer infusion, and during hyperinsulinemia (at the end of the clamp) for measuring specific activity or isotopic enrichment in plasma glucose to verify steady state conditions.

At steady state, rates of glucose appearance (Ra) and of disappearance (Rd) are considered to be equal by assuming a homogenous compartment of distribution (1-compartement model of the body glucose pool). Glucose-Ra can be calculated by dividing the tracer glucose infusion rate times tracer enrichment by the percent of tracer enrichment in plasma and subtracting the tracer infusion rate (Steele 1959, [213] and EGP is given by: EGP = R_a – GIR.

In the presented studies, (**att. articles No. 1-7, No. 9, 10**), hepatic insulin sensitivity is measured as the suppression of EGP by insulin by using a continuous infusion of deuterated glucose (D-[6,6-$^{2}H_{2}$]-glucose) (Cambridge Isotope Lab., Andover, Mass., USA; 99% ^{2}H enriched) [214]. The infusion is started two hours before the hyperinsulinemic phase of the clamp by a bolus application for 10 min with a subsequently continuous infusion aiming at enrichment of 2%. All of the infusions used thereafter within the clamp also need to be enriched by 2% of D-[6,6-$^{2}H_{2}$]-glucose. Determination of atom percent enrichment (APE) of ^{2}H and calculation of EGP are done as described before [215].

3.2. Assessment of β-cell function

β-cell function is generally assessed following stimulation by oral or intravenous administration of glucose. Indices derived from changes of insulin and C-peptide levels in serum and glucose concentrations in plasma following ingestion of 75 g glucose, allow for estimates of β-cell function and adaptation of insulin secretion according to prevalent whole body glucose uptake [216].

3.2.1. The intravenous glucose tolerance test

The IVGTT allows assessment of the early and late phase of insulin secretion to assess β-cell function [216]. After baseline blood sampling, a bolus of glucose (1 mg/kg body weight in a 30% solution; Glucosteril, Fresenius Kabi, Bad Homburg, Germany) is injected within 60 sec into the antecubital vein at 0 min and blood samples are obtained at 2-min intervals for the first 10 min and thereafter every 10 min until 60 min. First phase C-peptide secretion is calculated as the incremental area under the curve (iAUC) until 10 min, second phase C-peptide secretion as the iAUC between 10 and 60 min, the total C-peptide secretion as the sum of both. This protocol directly stimulates β-cell function without confounding effects due to gastrointestinal factors typical for OGTT and allows monitoring of dynamics of glucose and insulin concentrations.

3.3. Assessment of features of energy metabolism

Whole body energy metabolism can be assessed from gas exchange and yield an estimate of total energy expenditure. Tissue specific energy metabolism requires the application of non-invasive, localized ^{31}P MRS methods within the organ of interest, or analyses of biopsy samples.

3.3.1. Definitions of mitochondrial function

Mitochondrial content can be assessed from the relative mitochondrial area by electron microscopic imaging or from the copy number of genes encoded by mitochondrial DNA normalized for nuclear encoded genes [217-219]. Other markers such as cardiolipin [124, 220, 221] or enzymatic markers of oxidative phosphorylation, such as citrate synthase (CS), at saturating substrate concentrations implicated in the TCA and the electron transport chain and β-hydroxyacyl-Coenzyme A dehydrogenase to evaluate β-oxidation [94, 221, 222] are equally used as markers for mitochondrial content [34]. Electron microscopic imaging revealed location of muscular mitochondria in subcellular compartments i.e. the intermyofibrillar and subsarcolemmal area. Different methods for mitochondrial isolation allow for separate investigation of the functional properties possibly related to mitochondrial distribution [34]. Mitochondrial fusion and fission determines the mitochondrial network, biogenesis and breakdown of mitochondria as well as migration into subcellular regions of increased energy demand and distribution upon cell division. Regulators of mitochondrial dynamics also influence mitochondrial function via changes in mitochondrial membrane potential and expression of genes involved in oxidative metabolism [34, 223]. Density of the "cristae" of the inner mitochondrial membrane, presence of inclusion bodies and branching of mitochondria can be determined by electron microscopy [34]. Net mitochondrial flux rates are determined by the balance of energy supply and energy demand. Actual ATP production or oxygen consumption at defined metabolic conditions (i.e. fasting, hyperlipidemia, hyperinsulinemia) refer to mitochondrial activity which depends on intrinsic mitochondrial functionality but also on the regulation of metabolic flux rates (mitochondrial fitness, see below) (**Figure 5.**) [34]. Increasing energy demand to (sub)maximum levels by exhaustive exercise (muscle), application of alcohol or fructose (liver), decoupling of the respiratory chain (e.g. myocardiocytes in vitro) or increasing energy supply by application of saturating levels of substrates (state 3) lead to high mitochondrial activity which is limited by mitochondrial content and intrinsic capacity (**Figure 5.**) [34]. Mitochondrial adaptability to environmental conditions as altered energy demand and/or nutrient availability during experimental or lifestyle interventions defines mitochondrial fitness [33] which depends on transcriptional regulators of mitochondrial function under the control of peroxisome proliferator-activated receptor (PPAR) coactivator 1α (PGC-1α) and PGC-1β or AMP activated protein kinase (AMPK) [119, 120] (**Figure 5.**)[34]. A high molar ratio of ATP produced per oxygen consumed (ATP/oxygen ratio) reflects the conversion of energy to ATP rather than heat. Uncoupling of

the respiratory chain via lipids or uncoupling proteins leads to diminished mitochondrial efficiency (**Figure 5.**) [34].

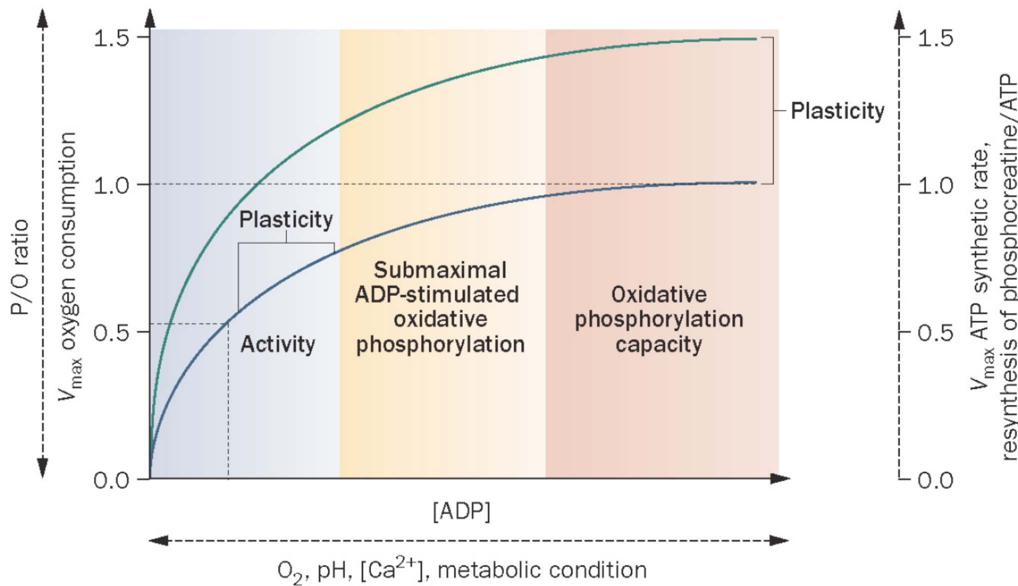

Figure 5.
The fraction of maximum oxygen consumption or ATP synthesis flux rate (fATP, Vmax) is plotted against prevalent ADP concentration and other flux controlling parameters. Mitochondrial activity (blue area) is assessed from fATP at low energy demand (low ADP concentrations) and is determined by prevalent metabolic conditions (e.g. hyperlipidemia). Oxidative phosphorylation capacity (red area) is assessed in ex vivo experiments from fATP or oxygen consumption at maximal energy demand (high ADP concentrations) when oxygen and substrate supply are not flux controlling. Submaximal ADP-stimulated oxidative phosphorylation (orange area) is assessed from resynthesis of ATP or phosphocreatine at increased ADP concentrations and strongly depends on mitochondrial plasticity, coupling (P/O ratio), oxygen and substrates supply and uptake, pH and – in skeletal muscle - calcium release. Interventions (e.g. insulin, exercise training) affecting parameters of mitochondrial function allow for estimating mitochondrial plasticity [34].

When comparing measures of mitochondrial function, the observed effects will depend not only on study design and methods, but also on other confounding factors. Therefore, carefully matched study groups for lifestyle factors (i.e. stable dietary habits, frequency and intensity of physical activity) and for concentrations of substrates and hormones (i.e. glucose and insulin levels in insulin-resistant or diabetic humans) should be mandatory.

3.3.2. *In vivo* assessment of energy metabolism

Despite their advantages, *in vivo* techniques cannot provide an explanation as to whether changed energy metabolism is due to alterations in individual mitochondria, a reduction of mitochondrial density (i.e. number and/or size of normally functioning mitochondria), heterogeneity of O_2 delivery, or decreased energy demand. These aspects require the application of ex vivo methods.

3.3.2.1. Whole body substrate oxidation

The open circuit indirect calorimetry allows to noninvasively determine total energy expenditure, glucose and lipid oxidation by respiratory gas analysis. Following a resting period of 10 minutes, the canopy of the respiratory gas analyzer (e.g. Vmax Encore 29n, Sensor Medics Corp., Homestead, FL, USA) is placed over the volunteers' head to measure the oxygen uptake (VO_2) and the carbon dioxide output (VCO_2) in vivo under resting conditions. The procedure is repeated during the last 30 minutes of the hyperinsulinemic-euglycemic clamp to assess metabolic flexibility of substrate oxidation [224]. The primary parameters VCO_2 and VO_2 allow calculating the corresponding resting energy expenditure (REE) by using the *Weir equation* (REE = $(3{,}941 * V_{O2} + 1.11 * V_{CO2}) * 1.44$) [225].

Open-air spirometry (MasterScreen CPX; Jaeger Viasys Healthcare, Hoech- berg, Germany) was combined with continuous heart rate recording (Sport- testerPE4000; Polar Electro, Oulu, Fin- land). Exercise testing was performed on an electronically braked cycle ergometer (Lode-Excalibur Sport, Groningen, the Netherlands).

3.3.2.2. Tissue specific measures of mitochondrial function with phosphorus magnetic resonance spectroscopy

MRS offers a means of monitoring biochemical fluxes of intracellular metabolites which is non-invasive, direct and quantitative. Therefore, assessments can be performed during fasting and repeated during insulin-stimulation (i.e. during steady-state conditions in the late phase of a hyperinsulinemic-euglycemic clamp test). We applied ^{31}P MRS to assess mitochondrial activity and oxidative capacity as well as for absolute quantification of ATP and Pi concentrations in most of the reports (**att. articles No. 2, 4-10**). Measurements were performed on participants lying supine inside a 3-T spectrometer (Bruker, http://www. bruker.com/) using a 10 cm circular double resonant surface coil for ^{1}H and ^{31}P MRS

measurements, positioned approximately 2 cm into the medial head of the right gastrocnemius muscle or into the liver, avoiding contamination with intercostal skeletal muscle signals.

Application of the magnetization saturation transfer experiment to the exchange between Pi and γ-ATP provides a direct measurement of the unidirectional fATP [226, 227]. Steady-state Pi magnetization is measured with selective continuous wave irradiation of the γ-ATP resonance and this is compared with the magnetization following irradiation placed symmetrically down-field from the Pi frequency. The fractional reduction in Pi magnetization is due to the exchange of saturated γ-ATP with non-saturated Pi nuclei (**Figure 6.**). The respective fractions of Pi magnetization measured after variable interpulse delay lengths are used to calculate the spin lattice relaxation time for Pi. Employing the Forsen and Hoffman equation yields the unidirectional fATP (μmol $[g\ muscle]^{-1}\ min^{-1}$) [227]. We also developed a fast localization ^{31}P NMR technique to quantify fATP in human liver, which yielded an fATP of ~30 μmol $(g\ liver)^{-1}\ min^{-1}$ in healthy young volunteers [228]. It should be noted that the possible contribution of non-mitochondrial ATP turnover needs to be taken into account when making this calculation. The net fATP correlates with measures of oxidative capacity but strongly depends on prevalent metabolic conditions and energy demand.

Intramyocellular concentrations of G-6-P (lmol/l muscle) and Pi were measured from the ratio of the integrated respective peak intensities and β-ATP resonance intensity in spectra without inversion and saturation assuming a constant ATP concentration of 5.5 mmol/l muscle [70].

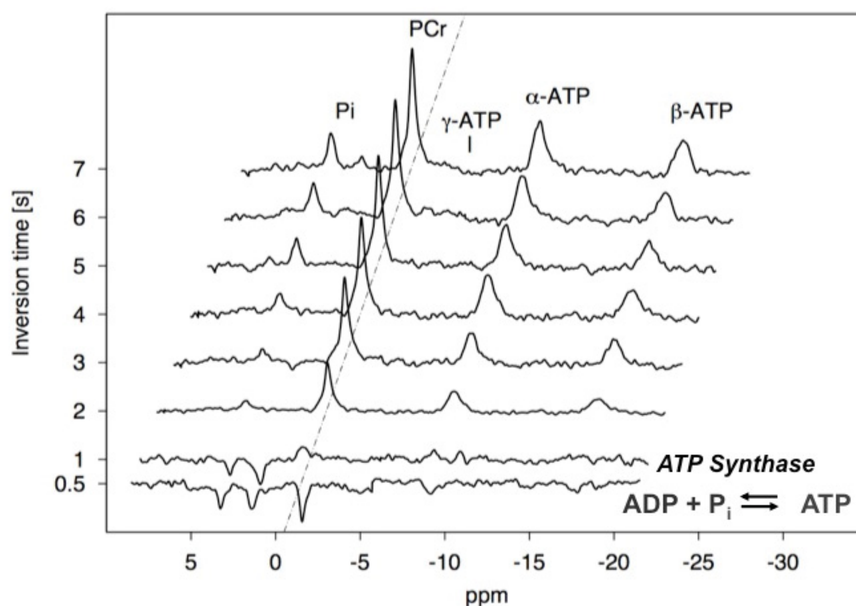

Figure 6.

Assessment of ATP synthesis flux rate applying the saturation transfer experiment during ^{31}P magnetic resonance spectroscopy in one healthy young volunteer. The ^{31}P spectra were obtained during application of selective continuous wave irradiation of the γ-ATP in vivo in the gastrocnemius-soleus muscle complex. Sequential spectra show fractional reductions in inorganic phosphate (Pi) magnetization measured after eight variable interpulse delay lengths ranging from 500 ms (bottom) to 7 s. PCr, phosphocreatine [33].

Tissues that rapidly consume ATP, such as muscle and brain, generate phosphocreatine (PCr) as an energy buffering system for rapid ATP regeneration. Determination of phosphocreatine kinetics using ^{31}P MRS during recovery from a bout of exercise was developed as a tool for estimation of mitochondrial function [229]. During exercise, PCr concentrations decrease and then rapidly recover, yielding a time constant of the recovery rate that is independent of work or power output [230]. This tool shows a high level of sensitivity (**Figure 7.**) and could be used to identify patients with mitochondriopathies [147] and to monitor therapeutic interventions [231]. Notably, this technique does not determine maximal rates of ATP synthesis rates, but rather suprabasal dynamic oxidative capacity, since other energy consuming and producing processes (e.g. transmembrane transportation, protein synthesis, glycolysis) are maintained during phosphocreatine resynthesis. Glycolytic ATP synthesis (anaerobic) and oxidative phosphorylation (aerobic) provide the energy for long-lasting muscular work and immediate ATP buffering by the creatine kinase reaction.

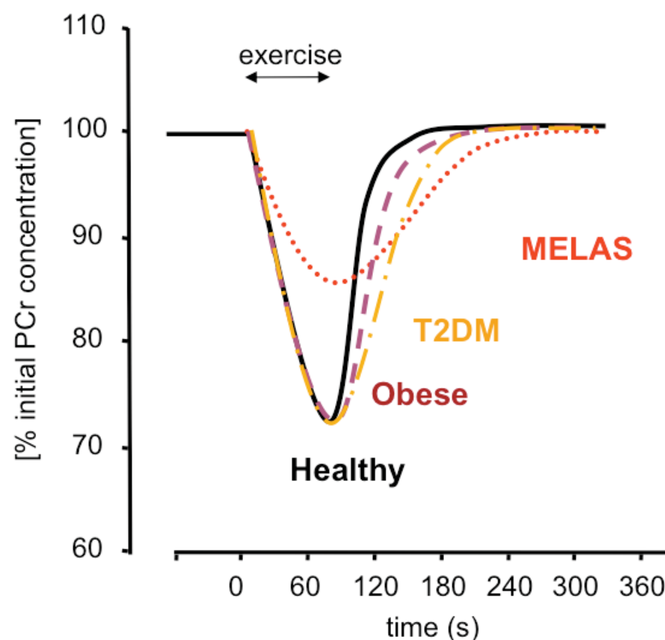

Figure 7.

Time-course of phosphocreatine (PCr) recovery after exercise employing ^{31}P magnetic resonance spectroscopy in healthy volunteers (solid back line), obese volunteers (dashed line) and patients with type 2 diabetes mellitus (T2DM, dotted dashed line) and with mitochondriopathy (Myopathy, encephalopathy, lactate acidosis, stroke-like episodes, MELAS, dotted line) [33].

Absolute quantification of liver phosphorus metabolites is assessed by use of a double resonant surface coil for $^{1}H/^{31}P$, placed over the lateral aspect of the liver as confirmed by magnetic resonance imaging [232] (**Figure 8.**). Multinuclei $^{1}H/^{31}P$ MRS is applied to measure HCL (see below), and to determine absolute concentrations of ATP, Pi,
PDE and fATP in the liver [232].

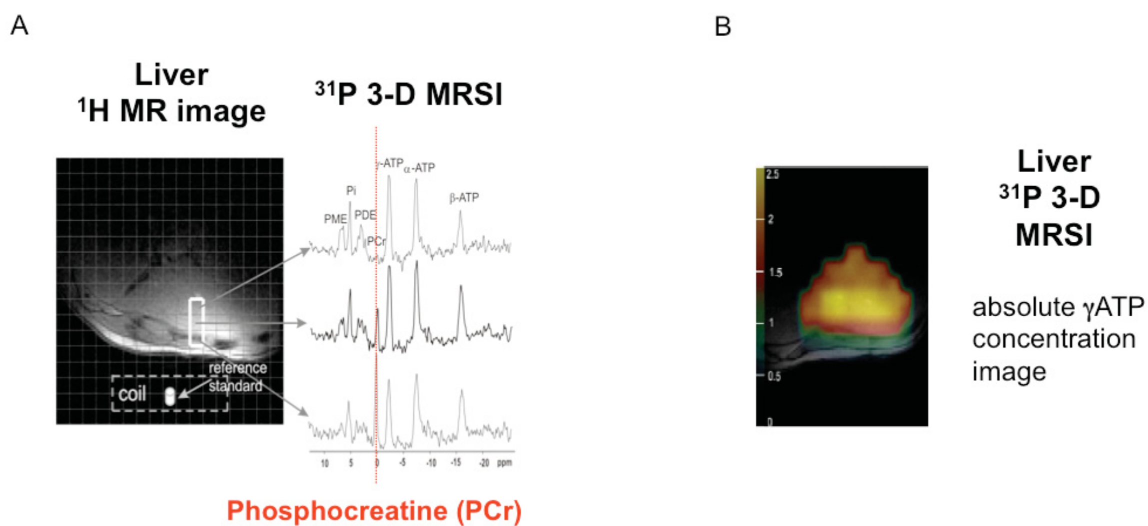

Figure 8.

A: *Left panel: proton-magnetic resonance imaging (^{1}H MRI) to localize the liver and the placement of an extern standard with an established concentration of phosphor (20mM) for quantification of phosphor spectra. Right panel: phosphor spectra are displayed by the white tagged areas. Spectra, which are recorded directly in the liver tissue, do not show signals for phosphocreatine. This is a contrast to spectra originating from the muscle.* ***B:*** *calculation of the average ATP concentration in a three-dimensional phosphorous MRI (^{31}P 3D MRI), marked, depending on the intensity, in yellow* [233, 234].

3.3.3. *Ex vivo* assessment of mitochondrial function

Several approaches have been developed to examine aspects of mitochondrial function, ranging from morphometry and enzymology to functional analysis.

3.3.3.1. Mitochondrial enzyme activities

Enzyme histochemistry can be used to identify impaired mitochondrial capacity through measurement of NADH, ATPase or cytochrome oxidase, and to diagnose deficiencies in mitochondrial proliferation through measurement of succinate dehydrogenase or regulators of mitochondrial biogenesis. The mitochondrial electron transport chain includes coenzyme Q, cytochrome *c* and complexes I–V, which are encoded by both nuclear DNA and mtDNA, and catalyze energy transduction from substrates to the proton gradient for ATP synthesis. A number of approaches for measurement of the corresponding enzyme activities have been reported [235]. Spectrophotometric assays measure enzyme activities per sample protein mass, reflecting enzyme abundance in the presence of substrate excess using specific inhibitors. In general, cytochrome *c*, oxidase II and mitochondrial membrane potential serve as a measure of electron transport chain activity, and the activity of β-hydroxyacyl-CoA dehydrogenase acts as an indicator of β-oxidation [115]. CS activity is a marker of the nuclear expression of genes encoding mitochondrial proteins and a measure of TCA activity. Because expression of the gene encoding CS can increase following acute exercise or insulin stimulation, stimulation of mitochondrial biogenesis might be more reliably determined by measuring mtDNA content relative to nuclear DNA, which is not sensitive to acute metabolic changes [115].

3.3.3.2. Ex vivo mitochondrial ATP production and oxygen consumption

Mitochondrial function can be studied by measuring the activities of enzymes involved in the respiratory chain and substrate oxidation and by more functional methods, which monitor ATP production by bioluminescent techniques or O_2 consumption with high-resolution spirometry (**Figure 9.**). Despite the advantages of ex vivo techniques, a number of limitations need to be considered. First, the isolation procedure separates mitochondria from their physiological environment. This is partly overcome by studying intact or permeabilised cells. Second, the sensitivity of biochemical analyses is affected by the processing of the biopsy samples, fresh muscle being preferable to frozen specimens. Third, maximal enzyme activities do not necessarily reflect metabolic fluxes and may not be sensitive enough to identify subtle

alterations. Because of the limited availability of human tissue biopsy samples, time resolution for detection of dynamic changes is restricted for some ex vivo methods. Of note, only ~3 mg of biopsy sample is needed for high-resolution respirometry. Also, stable preparations permit prolonged steady-state measurements at many different mitochondrial states, not just maximal capacity at saturating substrate concentrations.

ATP production can be quantified in suspensions of isolated mitochondria using a bioluminescent technique that involves luciferin–luciferase ATP monitoring [236]. Recent developments in high-resolution respirometry allow the measurement of O_2 consumption in preparations of permeabilised muscle fibers and isolated mitochondria [237]. This technique also permits the application of multiple substrate–inhibitor titrations, with the aim of characterizing mitochondrial function kinetically.

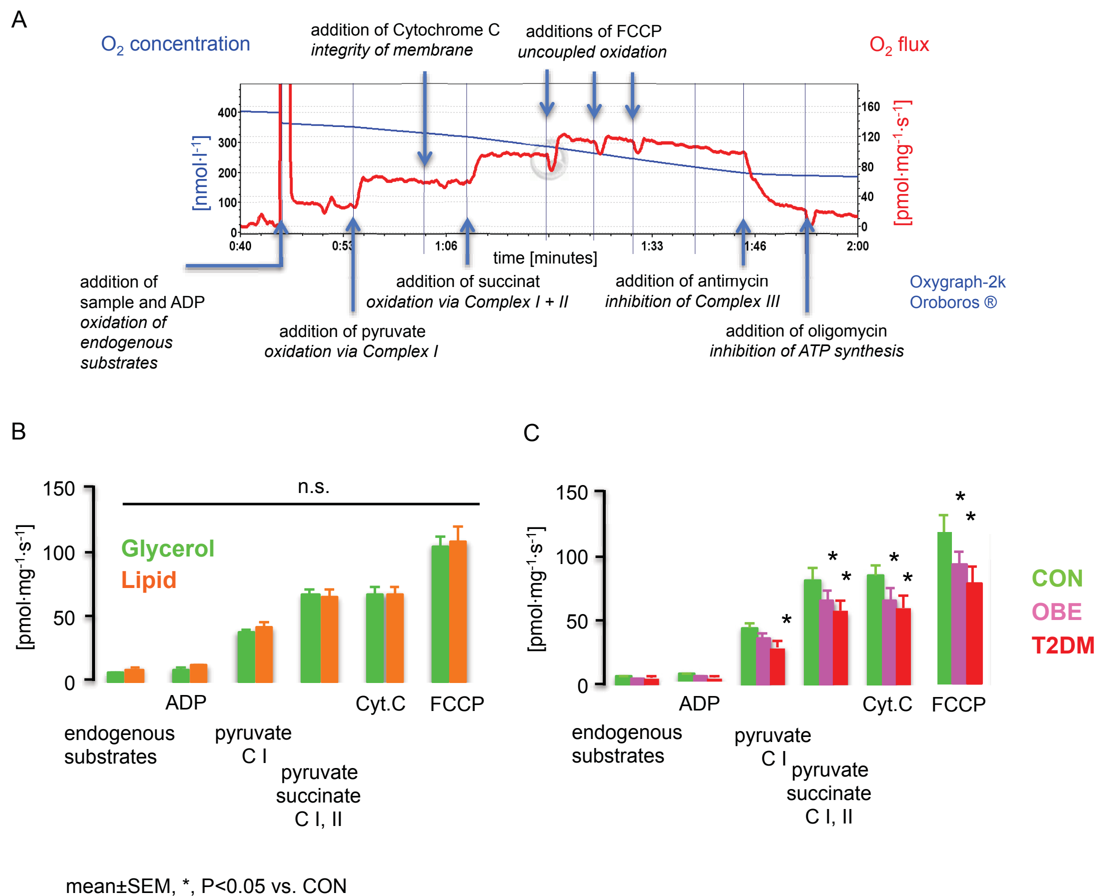

Figure 9.

(A) High resolution respirometry in permeabilised skeletal muscle biopsy sample. Left y-axis (blue line) depicts oxygen concentration within the chamber, right y-axis reflects the change of oxygen concentration with time (red line). Following the time course of the experiment, specific substrates and inhibitors are added to assess oxygen flux reflecting the maximal

*oxidative capacity of different complexes of the respiratory chain and different states e.g. uncoupling by addition of Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP). Oxidative capacity in skeletal muscle biopsy samples supplied by endogenous substrates, after addition of ADP, pyruvate to assess complex I (CI), succinate to assess complex I and II (CII) activity, addition of cytochrome C to assess mitochondrial integrity and after titration of FCCP. Samples were taken after 4 hours of glycerol (green) or lipid (orange) infusion from healthy young controls (B) or at fasting conditions from young healthy controls (CON, green), obese insulin resistant glucose tolerant humans (OBE, pink), or patients with overt type 2 diabetes (T2DM, red). Mean±SEM, *, P<0.05 vs. CON.*

3.3.3.3. Mitochondrial content

CS activity is a marker of the nuclear expression of genes encoding mitochondrial proteins and a measure of TCA activity. Because expression of the gene encoding CS can increase following acute exercise or insulin stimulation, stimulation of mitochondrial biogenesis might be more reliable determined by measuring mtDNA content relative to nuclear DNA, which is not sensitive to acute metabolic changes. Microarray analysis and gene expression profiling studies in muscle biopsies screen for regulators of mitochondrial biogenesis [119, 238].

3.3.4. Assessment of ectopic lipid deposition

Determination of ectopic lipids in humans can be performed by analyses of tissue biopsies with limitations such as invasiveness and small sample size. In vivo measures allow for non-invasive determination of lipid content, which comprises of triglycerides. Ex vivo analyses also allow for the analysis of lipid profiles, i.e. fatty acid composition of different lipid metabolites in different subcellular fraction. Insufficient representation of the whole organ and contamination by surrounding adipose tissue contribute to high variability and overestimation of lipids contents. Noninvasive techniques achieve (semi-)quantitative (ultrasound, CT) or quantitative (^{1}H MRS) assessment of ectopic fat in various body regions. The latter allow for examining of extended tissue volumes and repeated measurements as recently described [239].

3.3.4.1. *In vivo* measures of skeletal muscle and liver lipid content

^{1}H MRS allows quantification of IMCL in soleus or tibialis anterior muscles. The cubic volume of interest within the muscle (1.73 cm3) and the magnetic field is shimmed on the

localized water signal (line width, 2–15 Hz). Localized ^{1}H MRS of the liver is applied to measure HCL as described [37, 72].

3.3.4.2. *Ex vivo* assessment of lipid metabolites

Muscle tissue is homogenized in a buffer solution (20 mM Tris·HCl, 1 mM EDTA, 0.25 mM EGTA, 250 mM sucrose, 2 mM PMSF) containing a protease inhibitor mixture (Roche), and then the sample is centrifuged at 100,000 × g for 1 h. The supernatants containing the cytosolic fraction and the pellet consisting of the membrane fraction are collected. DAG and ceramide concentration were measured as via high liquid chromatography [240].

3.4. Analytical Procedures

Plasma glucose is measured by the glucose oxidase method (Glucose analyzer II, Beckman Coulter, http://www. beckmancoulter.com/). Plasma FFAs are assayed with a microfluorimetric method (Wako Chem USA). Collecting blood into orlistat-containing vials prevents in vitro lipolysis. Plasma triglycerides are measured by a peroxidase-coupled colorimetric assay (Roche). Plasma concentrations of insulin, C-peptide and glucagon are determined by double antibody radioimmunoassay.

4. Results

We addressed the above-mentioned hypotheses in clinical-experimental studies. The main findings are briefly summarized in the following sections. The full texts are attached to this document.

4.1. Mechanisms of lipid-induced insulin resistance in skeletal muscle

The cellular mechanisms underlying the association between increased lipid availability, ectopic lipid deposition in skeletal muscle and reduced glucose uptake have been assessed in healthy young humans with and without lipid infusion, in elderly glucose tolerant humans, and in patients with T2DM.

4.1.1. Increased plasma free fatty acids impair myocellular glucose uptake without affecting regional blood perfusion (att. article No. 1)

Background: Increased lipid availability reduces insulin-stimulated glucose disposal in skeletal muscle, which is generally explained by fatty acid-mediated inhibition of insulin signalling. Insulin increases muscle microvascular perfusion and facilitates delivery of nutrients and hormones to the interstitium [49]. Animal models of lipid-induced insulin resistance suggest that insulin-mediated microvascular perfusion is already reduced in prediabetic states and relates to impaired insulin action [50, 51]. Preventing the access of glucose and insulin to myocytes could contribute to lower glucose disposal and place abnormal microvascular insulin action as an early event in the development of T2DM. It remains unclear whether lipids also impair transcapillary transport of insulin and glucose, which could become rate controlling for glucose disposal.

To challenge this concept, we hypothesized that lipid-induced insulin resistance is induced by inhibiting myocellular glucose uptake and not by interfering with the delivery of insulin or glucose.

Methods: We measured changes in interstitial glucose and insulin in skeletal muscle of healthy volunteers during intravenous administration of triglycerides plus heparin or glycerol (control condition) during physiologic and supraphysiologic hyperinsulinemia, by combining microdialysis with oral glucose tolerance tests and euglycemic-hyperinsulinemic clamp tests.

Results: Lipid infusion reduced insulin-stimulated glucose disposal by ~70% ($p<0.05$) during clamps and dynamic insulin sensitivity by ~12% ($p<0.05$) during oral glucose loading. Dialysate insulin and glucose levels were unchanged or even transiently higher ($p<0.05$)

during lipid than during glycerol infusion, whereas regional blood flow remained unchanged.
Conclusions: This study demonstrates that transcapillary transport of insulin and glucose is not blunted during the onset of lipid-induced insulin resistance under conditions of dynamic and constant hyperinsulinemia. We did not observe increased blood flow under insulin-stimulated conditions, which is in line with studies in healthy humans at comparable and ~20fold higher plasma insulin concentrations [241]. Of note, other studies suggested that meal-induced increases in insulin have hemodynamic effects, thereby enhancing its access and that of nutrients to myocellular metabolism [47, 48]. Here, we report that lipid infusion induced whole-body insulin resistance, while blood flow tended to be greater during constant hyperinsulinemia indicating increased rather than reduced blood flow. Thus, vascular effects of FFA are not rate-controlling for muscle insulin-stimulated glucose disposal.
Limitations: First, we report relative but absolute interstitial glucose and insulin concentrations. This, however, does not affect our conclusions that are based on the time-dependent relative changes. Second, lipids could interfere with analyte measurements using microdialysis [242]. To prevent this phenomenon, we added human albumin to the perfusion medium and used probes with low molecular weight cut-off membranes [242].
Advantages: We applied two gold standard measures of insulin sensitivity, M-value and the OGTT to report mechanisms of FFA-induced insulin resistance with and without contribution of effects on glucose resorption [42].

4.1.2. Reduction of plasma free fatty acids relates to improvement of insulin sensitivity, independent of changes of IMCL (att. article No. 2)

Background: Interventional studies emphasized that statin treatment leads to reduction of cardiovascular events with benefit for patients with T2DM [243]. Statin therapy was shown to improve endothelial function, inhibit smooth muscle cell proliferation, reduce oxidative stress and inflammation [244]. Statins could also contribute to diabetes prevention due to lipid lowering and so-called pleiotropic action. The direct action of statins on insulin sensitivity remains controversial, because beneficial [245], indifferent and unfavorable [246] effects were reported. Statins not only decrease low-density lipoprotein cholesterol (LDL-C) levels but may also interfere with fasting and postprandial metabolism of triglyceride-rich lipoproteins resulting in altered substrate flux to and accumulation of HCL [247, 248], which could subsequently affect muscle glucose metabolism and deposition of IMCL. Simvastatin is one of the most frequently prescribed statins due to its efficacy to reduce LDL-C levels,

tolerability, and reduction of cardiovascular risk and mortality [249]. Its effects on insulin action and metabolism at the maximal recommended dose of 80 mg/day are unclear.

To test the concept that a lipid lowering therapy might improve insulin sensitivity by reduction of ectopic lipid deposition and/or plasma FFA, we performed a randomized controlled trial in patients with chronic insulin resistance.

Methods: We performed a randomized, double-blind, placebo-controlled, single-center study. Twenty patients with T2DM received 80 mg simvastatin or placebo daily for 8 weeks and were compared to ten healthy young humans. Euglycemic-hyperinsulinemic clamp tests combined with D-(6,6-d_2) glucose infusion were employed to assess insulin sensitivity and EGP. ^{1}H MRS was used to quantify intramyocellular and hepatocellular lipids.

Results: High-dose simvastatin treatment lowered plasma total and LDL-C by ~33% and ~48% ($p<0.005$), but neither affected insulin sensitivity nor ectopic lipid deposition in soleus, tibialis anterior muscle and liver nor basal and insulin-suppressed EGP. In simvastatin-treated patients, changes of LDL-C related negatively to changes in insulin sensitivity ($r=-0.796$, p values<0.01). Changes of FFA related negatively to changes in insulin sensitivity ($r=-0.840$, p values<0.01) and positively to plasma retinol-binding protein 4 (RBP4, $r=0.782$, $p=0.008$).

Conclusion: High-dose simvastatin treatment has no direct effects on whole-body or tissue-specific insulin action and ectopic lipid deposition. Reduction of plasma FFA likely mediates alterations in insulin sensitivity in vivo. These results support our concept of a prominent role of FFA for the induction of insulin resistance also in long standing overt T2DM. While ectopic lipid deposition is a good marker of insulin resistance, it may not represent the pool of lipid metabolites directly involved in the mediation of impaired insulin signalling.

Limitations: A number of anti-inflammatory and anti-oxidant mechanisms that potentially contribute to effects of stations on insulin action were not explored in the present study. **Advantages:** We assessed tissue specific insulin sensitivity as well as ectopic lipid deposition in skeletal muscle and the liver while other studies used surrogate markers to estimate these parameters. The study was appropriately designed to answer our questions as prospective, placebo-controlled, randomized trial. Only patients with untreated hypercholesterolemia in need of cholesterol lowering drug treatment according to current practical guidelines were included.

4.1.3. Diacylglycerol-induced activation of protein kinase C impairs muscle insulin signaling (att. article No. 3)

Background: Abnormal insulin-stimulated muscle glucose transport is mainly responsible for muscle insulin resistance [250], but the underlying cellular mechanisms are unclear in humans [42, 79]. While triglyceride accumulation in skeletal muscle and the liver are a good marker for insulin resistance, evidence accumulates that other lipid metabolites impair insulin signaling. To address this question, we performed serial muscle biopsies in healthy, lean subjects before and during a lipid infusion to induce acute muscle insulin resistance. In addition, we assessed lipid and inflammatory parameters that have been previously implicated in causing muscle insulin resistance in obese, insulin resistant glucose tolerant humans and in patients with T2DM.

Methods: Lipid-induced muscle insulin resistance: healthy young lean participants underwent two experimental conditions in random order. A pancreatic clamp was performed (-240 – 0 min) with continuous infusion of somatostatin to inhibit insulin secretion. Insulin was applied to standardize fasting insulin levels and combined with infusion of D-[6,6-$^2H_2$]glucose. A dextrose infusion labelled with D-[6,6-$^2H_2$]glucose according to the hot-glucose infusion protocol was periodically adjusted to maintain normoglycemia. Intravenous infusion of lipids was administered to raise plasma FFA. On another occasion spaced by 4- to 8-week intervals. glycerol dissolved in saline was infused. Chronic insulin resistance: insulin resistant obese humans (n=10) and patients with T2DM (n=10) without severe dyslipidemia were investigated under fasting conditions. All participants underwent a high-insulin-euglycemic clamp (0-150 min) combined with infusion of D-[6,6-$^2H_2$]glucose.

Results: We show that short-term lipid infusion neither affects circulating or myocellular acylcarnitines nor the cytokines interleukin-6, adiponectin, retinol-binding protein 4 or soluble intercellular adhesion molecule-1. Of note, lipid infusion transiently increases total cytosolic DAG, but not ceramides in muscle of lean nondiabetic humans. Subsequently, muscle PKCθ activity increases followed by a rise of serine-1101 phosphorylation of insulin receptor substrate-1, inhibition of downstream insulin signalling and reduction of glucose disposal. Similarly, total and cytosolic DAG and PKCθ activity, but neither ceramides nor circulating or myocellular acylcarnitines and circulating cytokines are elevated in muscle of humans with obesity and T2DM. Particularly, muscle DAG species in the membrane fraction containing C16:0-, C18:0-, C18:1-, C18:2- and C20:4-fatty acids tightly correlate with insulin resistance and PKCθ activity across all individuals, with C18:2 being the most frequent residue.

Conclusion: These results favor a uniform mechanism of short-term and common insulin resistance and may help identifying novel targets for treating obesity and T2DM. T2DM and obesity are characterized by impaired insulin action, i.e. insulin resistance. Increased plasma free fatty acids can induce insulin resistance and are independent risk factors of cardiovascular diseases, but the underlying cellular mechanisms are yet unclear. This study found that muscle DAG tightly correlate with insulin resistance resulting from elevation of free fatty acids or from obesity and T2DM. The rise in DAG species precedes PKCθ activation and impairment of insulin signaling in human muscle. Other lipids such as ceramides or acylcarnitines and circulating adipocytokines are not necessarily higher in human insulin resistance. DAG metabolism might become a promising target to treat insulin resistance.

Limitations: We did not observe the increase of DAG with the development of insulin resistance over the years in single individuals, or improve insulin sensitivity by interventions decreasing DAG to increase the evidence for a causal relation between DAG, PKC activation and impairment of insulin signaling.

Advantages: We performed serial biopsies during lipid-infusion and assessed concomitant changes of lipid metabolites and insulin signaling in subcellular fractions during acute as well as chronic insulin resistance while most previous studies assessed total cellular concentrations. Endurance-trained highly insulin-sensitive humans were included as control group in other studies but paradoxic accumulation of certain DAG species has been reported [251]. We included only sedentary humans and performed clamps to exactly determine insulin sensitivity.

4.2. The role of mitochondrial function in skeletal muscle of insulin resistant humans

The following studies investigated mitochondrial activity during fasting and during insulin stimulation (plasticity) in patients with T2DM, with MELAS syndrome and following acromegaly (inherited versus acquired insulin resistance).

4.2.1. Patients with T2DM have reduced muscle mitochondrial activity and plasticity (att. article No. 4)

Background: Muscular insulin resistance is frequently characterized by blunted increases in G-6-P reflecting impaired glucose transport/phosphorylation. These abnormalities likely relate to excessive IMCL and mitochondrial dysfunction. Mitochondria oxidize fatty acids

into energy, and recent studies have revealed that fat accumulation caused by poorly regulated fatty acid metabolism blocks insulin signaling, thus causing insulin resistance.

We hypothesized that even non-obese patients with well-controlled T2DM have lower fasting and insulin-stimulated mitochondrial activity.

Methods: We measured G-6-P, fATP (i.e., synthesis) and lipid contents of skeletal muscle with $^{31}P/^{1}H$ MRS in ten patients with T2DM and in two control groups: ten sex-, age-, and body mass-matched elderly people; and 11 younger healthy individuals. To rule out effects of glucotoxicity and acute glucose uptake, we compared fATP in patients with T2DM and good metabolic control (mean HbA_{1c} 6.9%) during hyperinsulinemic-hyperglycemic clamp tests with normal G-6-P levels as compared to control participants during hyperinsulinemia.

Results: Although insulin sensitivity was lower in patients with T2DM, muscle lipid contents were comparable and hyperinsulinemia increased G-6-P by 50% (95% confidence interval [CI] 39%–99%) in all groups. Patients with T2DM had 27% lower fasting fATP compared to younger controls (p ¼ 0.031). Insulin stimulation increased fATP only in controls (younger: 26%, 95% CI 13%–42%; older: 11%, 95% CI 2%–25%), but failed to increase even during hyperglycemic hyperinsulinemia in patients with T2DM. Fasting FFA and waist-to-hip ratios explained 44% of basal fATP. Insulin sensitivity explained 30% of insulin-stimulated fATP.

Conclusions: Patients with well-controlled T2DM feature slightly lower skeletal muscle fATP, which occurs independently of glucose transport /phosphorylation and lipid deposition but is determined by lipid availability and insulin sensitivity. Furthermore, the reduction in insulin- stimulated glucose disposal despite normal glucose transport/phosphorylation suggests further abnormalities mainly in glycogen synthesis in these patients. These results add to the evidence suggesting that mitochondrial function is disrupted in T2DM and in insulin resistance, but also suggest that there may be abnormalities in glycogen synthesis. These findings re-emphasize the need for people with T2DM or insulin resistance to reduce their food intake to compensate for the reduced energy needs of their muscles and to exercise to increase the ATP-generating capacity of their muscles.

Limitations: We did not take muscle biopsies in this study to assess lipid-induced effects on insulin signaling but addressed these issues in another study.

Advantages: This study is the first to report non-invasively assessed ATP synthase flux rates in patients with T2DM compared to healthy humans during fasting as well as during insulin stimulation.

4.2.2. Lower mitochondrial activity relates to disturbed muscle membrane integrity and insulin resistance (att. article No. 5)

Background: Myocellular PDE are elevated in patients with myopathies [141, 142], increase with age [143] and also in response to statin treatment reflecting membrane damage [144]. PDE is thought to relate to fiber atrophy, sarcolemmal damage or structural and functional changes during loss of muscle mass possibly resulting from cumulative oxidative damage. Thus, PDE may serve as marker of biomembrane integrity and prolonged membrane damage.

We hypothesized that reduced mitochondrial function secondarily relates to membrane damage. Therefore, we assessed the relationship between PDE content, mitochondrial function and insulin sensitivity in healthy lean and obese humans as well as in patients with T2DM.

Methods: Ten nonobese middle-aged patients with T2DM, 10 healthy humans matched for sex, age and physical activity index (CONm) and 18 young healthy humans (CONy). Myocellular PDE and unidirectional fATP were measured with ^{31}P MRS. IMCL and HCL were quantified with ^{1}H MRS. Insulin sensitivity was assessed from hyperinsulinemic-euglycemic clamp tests.

Results: During fasting, T2DM and CONm had 1.5 fold greater PDE than CONy ($p=0.004$). Stimulation by insulin did not affect PDE in any group. PDE correlated negatively with Rd ($r=-0.552$, $p<0.005$) and fATP ($r=-0.396$, $p<0.05$) and positively with age ($r=0.656$, $p<0.001$) and body mass ($r=0.597$, $p<0.001$). PDE also related positively to HbA_{1c} ($r=0.674$, $p<0.001$) and fasting plasma glucose ($r=0.629$, $p<0.001$) within T2DM and across all participants.

Conclusions: Muscular PDE concentrations associate with age, lower resting mitochondrial activity and insulin resistance, which is determined mainly by body mass and glycemia.

Limitations: No indicator of oxidative stress was determined to prove the role of PDE contents as a biomarker of oxidative stress.

Advantages: The biochemical analysis of individual compounds of PDE is difficult, because ischemia during tissue handling has impact on analysis of these instable compounds [252]. In this explorative study we therefore applied non-invasive in vivo measurement of PDE in a deeply phenotyped cohort.

4.2.3. Muscle ATP synthesis relates negatively to liver fat content (att. article No. 6).

Background: As mentioned above, skeletal muscle insulin resistance relates to lower mitochondrial activity during hyperinsulinemia in insulin resistant offspring of patients with

T2DM, resulting in lower postprandial glucose uptake and oxidation in skeletal muscle. Along with hyperinsulinemia, this could shift postprandial energy storage towards de novo lipogenesis in the liver and thereby increase hepatic lipid accumulation, which secondarily might induce mitochondrial damage in the liver [67] (**Figure 1.**).

Muscle insulin resistance has been implicated in the development of steatosis and dyslipidemia by changing the partitioning of postprandial substrate fluxes. Also, insulin resistance may be due to reduced mitochondrial function. We examined the association between mitochondrial activity, insulin sensitivity and steatosis in a larger human population.

To test this concept, we measured the association between HCL, whole body insulin sensitivity and skeletal muscle fATP in a larger study group, comprising of healthy lean and obese, elderly and young glucose tolerant, with and without family history of T2DM as well as of patients with T2DM.

Methods: We analyzed muscle mitochondrial activity from fATP and ectopic lipids by multinuclei MRS from 113 volunteers with and without T2DM. Insulin sensitivity was assessed from M-values using euglycemic-hyperinsulinemic clamps, and/or from OGTT.

Results: Muscle fATP correlated negatively with HCL and HbA_{1c}. After model adjustment for study effects and other confounders, fATP showed a strong negative correlation with hepatic lipid content, and a positive correlation with insulin sensitivity and fasting C-peptide. The negative correlation of muscle fATP with age, HbA_{1c} and plasma FFA was weakened after adjustment. Body mass, muscle lipid contents, plasma lipoproteins and triglycerides did not associate with fATP.

Conclusion: The association of impaired muscle mitochondrial activity with hepatic steatosis supports the concept of a close link between altered muscle and liver energy metabolism as early abnormalities promoting insulin resistance.

Limitations: The ATP synthesis/hydrolysis cycle at rest is driven by energy demands and thus reflects mitochondrial activity rather than maximal oxidative capacity. Nevertheless, fATP associates with changes in mitochondrial content, coupling and oxygen consumption [227] and tightly correlates with PCR recovery [253] indicating that skeletal muscle with higher maximal oxidative fATP is also metabolically more active at rest.

Advantages: Quantitative and non-invasive assessment of liver fat content and mitochondrial activity of skeletal muscle is reserved to specialized centers. This study comprises a substantial number of deeply phenotyped patients ranging from insulin sensitive to overt T2DM.

4.2.4. Inherited reduction of mitochondrial activity relates to muscle insulin resistance (att. article No. 7).

Mitochondrial diseases frequently lead to diabetes, which is mostly attributed to defective β-cell mitochondria and secretion. Insulin-resistant populations show reduced mitochondrial function and increased ectopic lipid deposition. The sequence of events, particularly the possibility of muscle mitochondrial dysfunction leading to insulin resistance, is yet unclear.

In order to test the concept, that impairment of mitochondrial function can act as a primary event in the induction of chronic insulin resistance, we investigated insulin sensitivity, mitochondrial function and ectopic lipid deposition in a patient with MELAS syndrome.

Methods: We assessed muscular mitochondrial function, lipid deposition in liver and muscle using $^{31}P/^{1}H$ MRS, whole body insulin sensitivity and EGP using hyperinsulinemic-euglycemic clamps combined with isotopic tracer dilution in one female patient suffering from MELAS syndrome and in six control subjects.

Results: The MELAS patient had normal fasting EGP, plasma FFA, and insulin suppression of lipolysis (95%) in comparison with the control subjects. EGP suppression and M-value were markedly lower in the patient than in the control subjects. The patient's respiratory quotient was 0.88, indicating reliance on glucose oxidation during fasting. Resting energy expenditure was lower than predicted. Maximal power and VO_{2max} were reduced. G-6-P did not increase after insulin stimulation, proving impaired muscle glucose transport/phosphorylation (control: +75% vs. baseline, $P<0.001$). Fasting and insulin-stimulated fATPase were lower in the MELAS patient than in the control subjects. The patient performed plantar flexion for 6.4 min, resulting in 60% PCr depletion, demonstrating exhaustive exercising. Postexercise PCr recovery was twice that of healthy volunteers, indicating compromised mitochondrial fitness. Resting ATP concentration and PCr-to-P_i ratio were reduced by 40% compared with those in healthy volunteers studied under identical conditions. HCL, albeit within the normal range (<5%), were higher in the MELAS patient than in the control subjects, whereas IMCLs were similar.

Conclusions: Impairment of muscle mitochondrial fitness contributes to insulin resistance in our patient with MELAS and diabetes, which resembles the mechanism described for first-degree relatives of T2DM patients.

Limitations: However, given the broad spectrum of MELAS defects and heterogeneity of insulin sensitivity in these patients, the present report does not permit the conclusion that MELAS is a typical model for the pathogenesis of T2DM.

Advantages: This study addresses potentially unrecognized features of a very rare disease

that might have clinical impact for the treatment of humans with the MELAS syndrome.

4.2.5. Chronic insulin resistance can lead to reduced muscle mitochondrial activity (att. article No. 8)

Background: Acromegaly generally results from slowly growing monoclonal pituitary adenomas secreting growth hormone. As a result of its slow and often insidious onset, it frequently remains unrecognized for an extended time, which may give rise to sustained metabolic alterations. Although improving, the overall standardized mortality of patients with acromegaly is about 1.5fold higher compared with the general population. Among other factors, the higher prevalence of glucose intolerance and overt T2DM could contribute to the increased mortality.

We tested the hypothesis that insulin resistance during active acromegaly induces irreversible metabolic defects that can be detected even years after successful treatment and normalization of metabolic conditions. In an attempt to assess effects possibly conducted via long term insulin resistance referred to as secondary impairment of mitochondrial function, we investigated these parameters in patients with previous insulin resistance during acromegaly.

Methods: Seven patients with previous acromegaly (AM) and age-/BMI-matched healthy volunteers (CON) underwent oral glucose testing for measurement of insulin sensitivity (OGIS) and ß-cell function (adaptation index, ADAP). Mitochondrial function was assessed from fATP using ^{31}P MRS of calf muscle. IMCL of tibialis anterior and as well as liver (HCL) were measured with ^{1}H MRS.

Results: Despite comparable growth hormone, IGF-1 and insulin sensitivity, AM had 17% higher fasting plasma glucose ($p<0.01$) and 85% lower ADAP ($p<0.01$). fATP was ~25% lower in AM ($p<0.05$) and related positively to ADAP ($r=0.744$, $p<0.01$) but negatively to BMI ($r=-0.582$, $p<0.05$). IMCL in m. tibialis and m. soleus and HCL were not different between groups. IMCL in m. soleus related negatively to insulin sensitivity ($r=-0.799$, $p<0.001$) and IMCL in m. tibialis related positively to plasma IGF-1 ($r=0.726$, $p<0.01$).

Conclusions: In conclusion, patients with a history of acromegaly exhibit reduced insulin secretion and muscular fATP. Previous exposure to high IGF-1, lipid and/or glucose concentrations could be responsible for the alterations in β-cell and myocellular mitochondrial function.

Limitations: The study design does not allow discrimination whether these abnormalities directly result from the previous growth hormone/IGF-1 excess or developed as a long-term consequence of metabolic alterations due to glucose, lipids and adipocytokines [42].

Advantages: This study shows that the sustained disruption of mitochondrial function along with severely impaired β-cell function could contribute to an increased risk of this population for developing diabetes despite successful acromegaly therapy.

4.3. The role of mitochondrial function for the development of steatosis and hepatic insulin resistance.

Development of novel non-invasive MRS techniques allowed us to assess hepatic energy metabolism in humans with T2DM.

4.3.1. Hepatic ATP concentrations relate inversely to liver insulin resistance (att. article No. 9)

Increased HCL relate to insulin resistance and are typical for T2DM. Steatosis and T2DM have been further associated with impaired muscular ATP turnover indicating reduced mitochondrial fitness. Before, various tracer dilution methods were applied to assess surrogate parameters of human hepatic mitochondrial function [254]. However, these methods rely on a range of assumptions, involve whole body protein turnover and do not assess hepatic energy metabolism directly. The time course of ATP re-synthesis upon fructose infusion has been used as more direct marker of hepatic mitochondrial function but apparently becomes less efficient with increasing BMI [174]. Previous liver ^{31}P MRS studies were limited by the inability to estimate absolute concentrations making it necessary to express the intensities of phosphorus signals as ratios to that of another metabolite and/or to the assumed concentration of a metabolite. Consequently, qualitative MRS would fail to detect these alterations in the presences of parallel increases or decreases of metabolites as shown in alcoholic liver disease [255].

We aimed to test the concept that patients with T2DM have reduced hepatic ATP concentration which relates to hepatic and whole body insulin resistance, using a recently developed method for absolute quantification of hepatic phosphorus compounds [233].

Methods: We measured hepatic lipid volume fraction (HLVF) and absolute concentrations of γATP, Pi), phosphomonoesters (PME) and PDE using ^{1}H/^{31}P MRS in T2DM (58±6 years, 27±3 kg/m^2), age- and BMI-matched (mCON) and young lean humans (yCON). Insulin-

mediated whole body glucose disposal and EGP were assessed during euglycemic-hyperinsulinemic clamps.

Results: T2DM had 26% and 23% lower γATP ($p<0.05$). Further they had 28% and 31% lower Pi than mCON and yCON ($p<0.05$). PME, PDE and liver transaminases did not differ between groups. HLVF was not different between T2DM and mCON, but higher ($p=0.002$) than in yCON. T2DM had 13-fold higher EGP during hyperinsulinemia than mCON ($p<0.05$). Even after adjustment for HLVF, hepatic ATP and Pi related negatively to hepatic insulin sensitivity ($r=-0.665$, $p=0.010$, $r=-0.680$, $p=0.007$) but not to whole body insulin sensitivity.

Conclusion: The present study demonstrates that metabolically well-controlled T2DM have markedly lower hepatocellular concentrations of Pi and γATP than both age- and BMI-matched and young lean nondiabetic humans. We conclude that it is highly recommended to use absolute quantification based on external references rather than peak ratios. These data suggest that impaired hepatic energy metabolism and insulin resistance could precede the development of steatosis in T2DM.

Limitations: First, no biopsies were obtained so that exact information on inflammatory status is lacking.

Advantages: Since biopsies are considered not ethical in subjects without any evidence of liver diseases, the application of this novel method to detected alterations in phosphorus metabolites even within the normal range.

4.3.2. Hepatic ATP synthesis relates inversely to liver insulin resistance (att. article No. 10)

Background: Steatosis associates with insulin resistance and may even predict T2DM and cardiovascular complications. Because muscular insulin resistance relates to myocellular fat deposition and disturbed energy metabolism, we hypothesized that reduced hepatic ATP turnover underlies insulin resistance and elevated HCL contents.

Therefore, we aimed to test the concept that patients with T2DM have reduced hepatic ATP synthesis rates.

Methods: We measured hepatic fATP using ^{31}P MRS in patients with T2DM and age- and body mass–matched controls. Peripheral and hepatic insulin sensitivity were assessed with euglycemic-hyperinsulinemic clamps.

Results: Diabetic individuals had 29% and 28% lower peripheral and hepatic insulin sensitivity as well as 42% reduced hepatic fATP than controls. After adjusting for HCL,

hepatic fATP correlated positively with peripheral and hepatic insulin sensitivity but negatively with waist circumference, BMI, and fasting plasma glucose. Multiple regression analysis identified waist circumference as an independent predictor of hepatic fATP and Pi concentrations, explaining 65% ($P = 0.001$) and 56% ($P = 0.003$) of the variations. Hepatocellular Pi primarily determined the alterations in hepatic fATP.

Conclusions: In patients with T2DM, insulin resistance relates to perturbed hepatic energy metabolism, which is at least partly accounted for by fat depots.

Limitations: The saturation transfer experiment provides a measure of total ATP turnover, which includes cycling at the levels of glyceraldehyde-3-phosphate de- hydrogenase and 3-phosphoglycerate kinase of the glycolytic and gluconeogenic pathways. Because fasting EGP was comparable between both groups, despite different degrees of glycemia, a variable contribution of cycling is rather unlikely.

Advantages: This study was the first to non-invasively assess fasting ATP synthesis in human liver of healthy humans and patients with T2DM.

5. Conclusions

The main results of our studies are summarized graphically in **Figure 10**.

1. Increased availability of circulating lipids inhibit insulin signaling via DAG while effects on other lipid metabolites and local perfusion do not play a prominent role.
2. The reduction of myocellular mitochondrial activity results from but may also promote the development of skeletal muscle insulin resistance.
3. The reduction of mitochondrial activity in the liver positively correlates with liver fat content and hepatic insulin resistance.

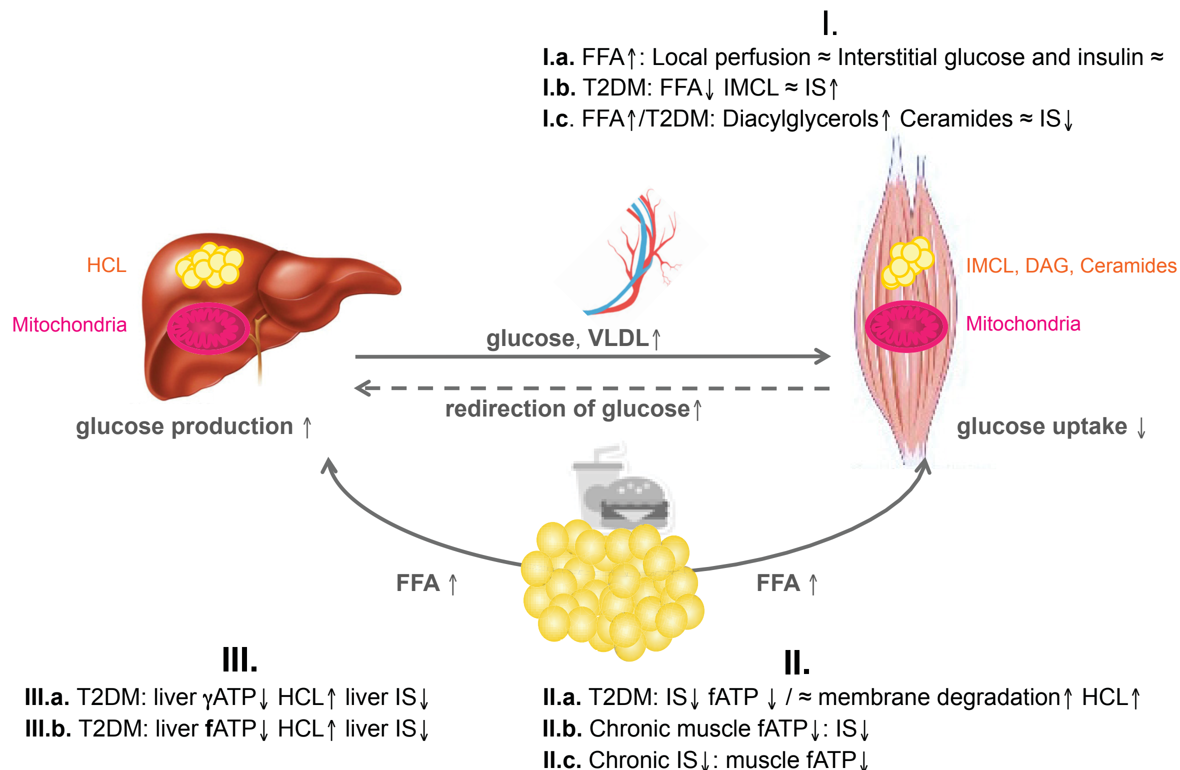

Figure 10.

The results of our studies testing hypotheses I-III are summarized in this scheme describing concepts of the pathogenesis of insulin resistance, introduced in Figure 4. Prevailing conditions, e.g. increased free fatty acids (FFA↑), decreased insulin sensitivity (IS↓), type 2 diabetes mellitus (T2DM) or decreased ATP synthase flux (fATP↓) result in decreased (↓), increased (↑) or unchanged (≈) dependent conditions, e.g. hepatocellular lipids (HCL).

Myocellular lipid metabolites comprise intramyocellular lipids (IMCL), diacylglycerols (DAG) and ceramides. Very-low density lipoproteins (VLDL).

Lipid-induced insulin resistance is mediated by distinct myocellular DAG species. DAG-induced activation of PKCθ leads to serine phosphorylation of insulin receptor substrate 1 and consequently reduces glucose uptake. Lipid-mediated effects on other lipid metabolites and on local perfusion do not play a primary role. Interestingly, levels of free fatty acids correlate negatively with mitochondrial activity in skeletal muscle and reduction of mitochondrial activity is related to insulin resistance, markers of skeletal muscle membrane degradation and liver fat content. The reduction of mitochondrial activity could result from lipotoxic damage of mitochondria, which in turn promote the accumulation of lipid metabolites also containing DAG and thereby contribute to the development of insulin resistance. For patients with overt T2DM the reduction of insulin stimulation of mitochondrial activity (i.e. plasticity) is even more typical and appears independent from intramyocellular glucose concentrations. Finally the substrate flux could be diverted away from the skeletal muscle towards the liver and promote the development of hepatic steatosis. Reduced hepatic mitochondrial activity also relates to hepatic insulin resistance and liver fat content, suggesting that reduced lipid oxidation in the liver promotes steatosis.
In conclusion, DAG-related activation of PKCθ is characteristic of insulin resistance and represents a novel therapeutic target. Another characteristic feature is the impairment of mitochondrial plasticity in skeletal muscle, suggesting another target for the improvement of insulin resistance and fatty liver.

5.1. Mechanisms of lipid-induced insulin resistance in skeletal muscle

IMCL contents relate to insulin resistance in various sedentary populations [72-74]. On the other hand, elevation of IMCL in endurance-trained athletes despite high insulin sensitivity, indicates that IMCL serve as energy source during intensive aerobic exercise in athletes [90] but reflect an imbalance between energy supply and demand in sedentary insulin-resistant humans. Thus, ectopic lipid accumulation may result from lipid oversupply, which is associated with elevation of myocellular triglycerides, not necessarily interfering with insulin signal transduction. In line with the training paradox, endurance trained humans exhibit upregulated muscular adipocyte-specific fatty acid binding-protein which is responsible for intracellular FFA trafficking and possibly allows for increased lipid oxidation [256]. On the other hand, upregulation of myocellular DAG-acyltransferase in mice protects against lipid-induced insulin resistance though it augments triglyceride synthesis in skeletal muscle [257].

Channeling FFA into IMCL resulted in decreased DAG and ceramide levels indicating that the predictive value of IMCL contents strongly depends on lipid flux rates and that other lipid metabolites than triglycerides are more important for the development of insulin resistance. Accordingly, five-hours of lipid infusion resulting in a physiological increase in plasma FFA of ~1 mmol/l induced insulin resistance with reduced glucose transport/phosphorylation without any alterations of IMCL supporting the concept that triglyceride storage is not prerequisite for insulin resistance [25]. Previous studies in rodent models have suggested that muscle DAG species induce inhibition of insulin signaling. Accordingly, the effect of statin therapy on insulin sensitivity did not relate to changes on IMCL. Reduction of fasting FFA following simvastatin therapy improved insulin sensitivity while IMCL levels remained unchanged. Again, rather than IMCL, other lipid metabolites such as DAG inhibit muscular insulin action [258]. Studies in humans have implicated other myocellular lipid metabolites, such as ceramides, acylcarnitines, or adipocytokines in causing lipid-induced muscle insulin resistance. We took a comprehensive view of all of the putative mechanisms. We showed that increasing plasma FFA by lipid infusion, leads to a transient increase in intramyocellular DAG species (C16:0, C18:0, C18:1, C18:2, and C20:4), which were temporally related to PKCθ activation, increased inhibitory IRS1-Ser1101 phosphorylation, and impairment of insulin-stimulated PI3K activation and stimulatory Akt- Ser473 phosphorylation (**Figure 11.**) [259]. Contrary to expectations, lipid-infusion did not result in increases in intramuscular ceramide or acylcarnitine content or changes in adipocytokines. Furthermore, muscle insulin resistance in obese and T2DM patients exhibited similarly increased cytosolic DAG content and PKCθ activation and no relationship with muscle ceramide or acylcarnitine content. Similar to the lipid infusion studies, the DAG species containing either C16:0, C18:0, C18:1, C18:2, or C20:4 acyl showed the strongest relationship with PKCθ activation and insulin resistance in obese and T2DM individuals [259].

Thus, we provided the proof of principle that triglycerides in skeletal muscle are robust markers of insulin resistance, but do not play a direct causal role.

- **In humans with acute insulin resistance due to elevated FFA levels as well as in humans with chronic insulin resistance related to obesity or T2DM, DAG-mediated activation of PKCθ inhibits IRS-1 by inhibitory serine phosphorylation and thereby reduces glucose uptake.**
- **IMCL, ceramides and adipocytokines do not play a prominent role.**
- **DAG mediated activation of PKCθ is a promising target for the therapy of insulin resistance.**

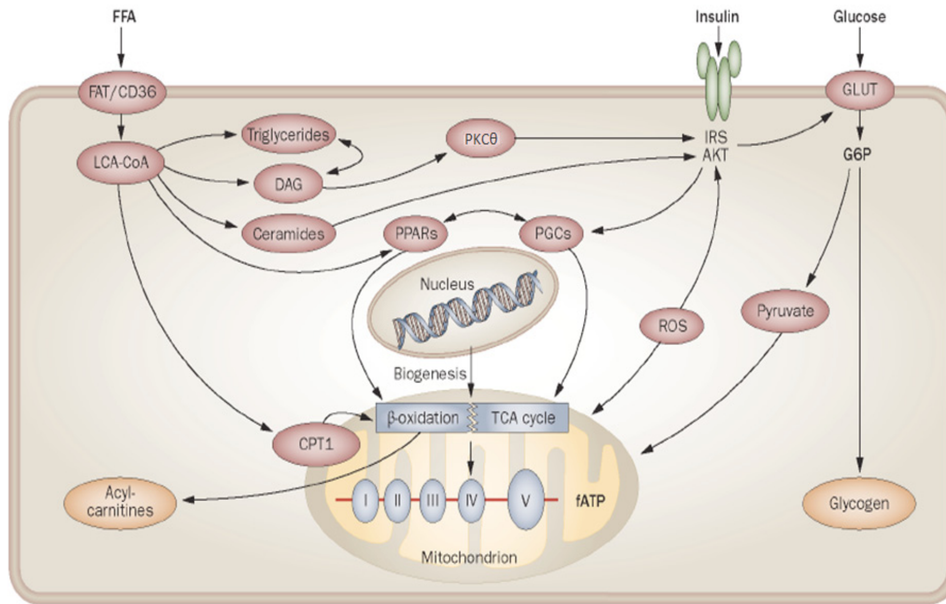

Figure 11.

In the insulin-resistant state, availability of FFA is increased, which raises triglyceride storage and intracellular concentrations of lipid metabolites (DAG, ceramides, LCFA-CoA). DAG and ceramides induce impairment of the insulin signaling pathway via activation of inflammatory messengers (for example, PKCθ), which leads to inhibitory serine phosphorylation of IRS. Glucose transport and phosphorylation is reduced. Stimulation of PGC-1α and PGC-1β, the main regulators of mitochondrial biogenesis and fatty acid oxidation, is induced by insulin in skeletal muscle. FFA activate PPARγ and PPARδ. Stimulation of oxidative capacity, mitochondrial biogenesis and mitochondrial lipid uptake is impaired in the insulin-resistant state. Thus, whole-body lipid oxidation decreases in humans with obesity and insulin resistance as a result of impaired mitochondrial plasticity. It is yet unknown if insulin has direct, rapidly acting effects on mitochondrial function. These defects might reflect dysregulation of the lipid-induced PPAR–PGC-1 interaction after prolonged hyperlipidemia, which could lead to reduced lipid uptake into mitochondria to compensate for lower mitochondrial content and increased lipid availability; lipid-induced uncoupling of the respiratory chain; reduced oxidation of glycolytic substrates, which uncouples fatty acid oxidation rates from TCA cycle rates; and metabolic inflexibility. Abbreviations: CPT1, carnitine O-palmitoyltransferase 1; DAG, diacylglycerol; IRS, insulin receptor substrate; FAT, fatty acid translocase (also known as CD36); fATP, ATP synthase flux rate; FFA, free fatty acids; GLUT, glucose transporter; G-6-P, glucose-6-phosphate; LCA-CoA, long-chain

acyl-CoA; PKCθ, protein kinase C θ; PPAR, peroxisome proliferator-activated receptor; PGC, PPAR coactivator; ROS, reactive oxygen species; TCA, tricarboxylic acid [34].

Effects of increased lipid availability on transcapillary transport of insulin and glucose have been discussed to contribute to the development of insulin resistance. In dogs, measurement of plasma and lymph insulin, as a surrogate of the interstitial compartment, during euglycemic-hyperinsulinemic clamps revealed a tight relationship between lymph insulin and whole-body glucose uptake [260, 261]. After lipid infusion or high-fat diet, they had increased venous but lower lymphatic insulin concentrations upon intramuscular insulin injections [50, 51]. The authors concluded that these conditions prevent insulin from diffusing through the interstitium, maybe due to endothelial dysfunction. Therefore we sought to test if similar effects may contribute to the development of insulin resistance in healthy lean humans. In our experiments lipid infusion reduced glucose uptake without changes in interstitial insulin or glucose concentrations neither after oral ingestion of glucose and endogenous insulin secretion nor after intravenous insulin and glucose infusions. We conclude that transcapillary transport of insulin and glucose is not blunted during the onset of lipid-induced insulin resistance under conditions of dynamic and constant hyperinsulinemia [52].

- **Lipid mediated effects on local perfusion, interstitial glucose, lipid or insulin concentrations, do not play a prominent role in the pathogenesis of insulin resistance.**

5.2. Muscle mitochondrial plasticity in type 2 diabetes mellitus.

Previously, application of different methods to assess parameters of mitochondrial function led to conflicting results and speculations on the role of mitochondrial function for the development of insulin resistance in T2DM [34]. Exercise training concomitantly increased fat oxidation and insulin sensitivity and decreased IMCL contents in obese elderly humans suggesting that exercise training promotes lipid utilization in skeletal muscle thereby reversing insulin resistance [87]. Insulin-resistant relatives of patients with T2DM with markedly higher IMCL contents and lower glucose disposal than insulin-sensitive controls also featured reduced mitochondrial density [120]. Therefore, inherited impairment of mitochondrial function could predispose those genetically susceptible insulin-resistant offspring to accumulation of myocellular lipid metabolites and consequent development of

insulin resistance entailing increased risk for T2DM. However, the role of mitochondrial function for the development of common insulin resistance in patients with T2DM was yet unclear. Several but not all [262] studies suggested "mitochondrial dysfunction" in fasting patients with T2DM, which has been attributed to lower content of muscle mitochondria [94, 97, 100] or altered intrinsic functionality of individual mitochondria [96, 263, 264]. Non-invasive assessment of fasting ATP flux in skeletal muscle determines net mitochondrial activity which results from nutrient oxidation to produce ATP (oxidative phosphorylation) and the prevailing energy demand. Thereby, insulin resistance has now been related to lower mitochondrial activity in elderly humans, in offspring of patients with T2DM, hyperglycemic patients with T1DM, humans with overt T2DM and in patients suffering from mitochondriopathies (**Figure 12.**).

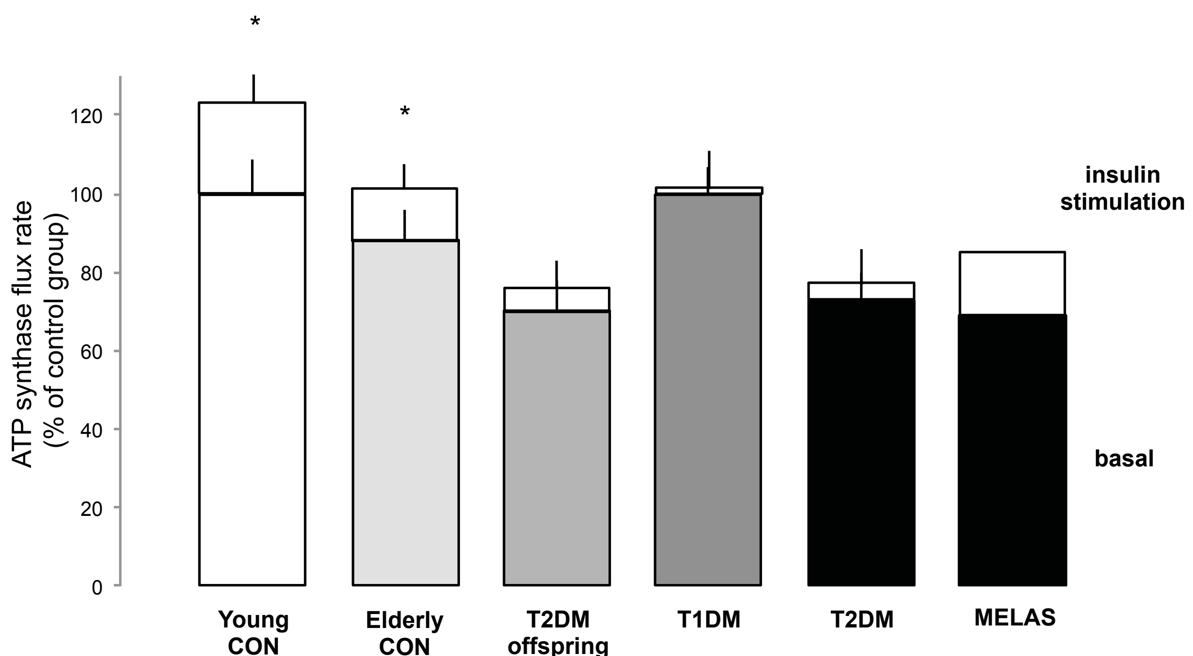

Figure 12.
ATP synthase flux rates (fATP) of various insulin resistant groups are depicted as percentage of the respective control group. fATP was measured in vivo under fasted conditions (basal) and hyperinsulinemic conditions (insulin stimulation, white bar on each column) in non-diabetic participants of different age (white columns): young controls (CON), elderly CON, insulin resistant offspring of patients with type 2 diabetes (T2DM), patients with type 1 diabetes (T1DM), T2DM and one patient with the mitochondriopathy Myopathy,

*encephalopathy, lactate acidosis, stroke-like episodes (MELAS). Data are given as mean±SEM. *, p<0.05 basal vs. insulin stimulation* [34].

Hereditary reduced fasting mitochondrial activity and impaired mitochondrial adaptability to altered metabolic conditions promotes insulin resistance [265]. Also humans with previous insulin resistance due to acromegaly had lower mitochondrial activity. Thus, increase of insulin sensitivity by treatment of the underlying disease can be dissociated from mitochondrial activity and fitness after years of irreversible damage of mitochondria [266]. Chronic insulin resistance and reduction of mitochondrial activity strongly relate to markers of disturbed membrane integrity, supporting the concept that impaired mitochondrial function relates to oxidative stress and results in damage in skeletal muscle [267].

Several groups have demonstrated a relative increase of glycolytic over oxidative enzyme activities during fasting and blunted switch from fat to glucose oxidation during hyperinsulinemia in persons with a family history of T2DM or overt T2DM [95, 104-106]. The inability to adapt fuel oxidation in response to changes in nutrient availability, i.e. metabolic inflexibility [268], has been implicated in the accumulation of IMCL and the development of insulin resistance [95, 104-106]. Based on the concept that impaired plasticity of muscle metabolism underlies altered ratios of the fluxes through the glycolytic and oxidative pathways [103], mitochondrial function was assessed at fasting (basal) and during insulin stimulation in insulin resistant cohorts [33]. While insulin resistance of glucose disposal can be dissociated from fasting mitochondrial activity there is a tight relationship to impaired stimulation of mitochondrial function. This reduction of mitochondrial adaptability is caused by reduced insulin sensitivity of mitochondria and occurs independent from substrate fluxes or substrate availability within the myocyte [262].

- **Mitochondrial activity of skeletal muscle during fasting is negatively related to serum FFA, markers of membrane damage in skeletal muscle, liver fat content and insulin sensitivity.**
- **Reduction of fasting mitochondrial activity can occur in humans with overt T2DM but also in other chronic insulin resistant states e.g. obesity or acromegaly.**
- **Reduced oxidative activity might result from lipotoxic damage of mitochondria that promote the accumulation of lipid metabolites and insulin resistance.**
- **Humans with T2DM mostly feature impaired insulin stimulation of mitochondrial activity (plasticity), which is independent from glucose uptake and might reflect insulin resistance at the level of mitochondria.**

5.3. Hepatic and muscle mitochondria in steatosis and insulin resistance

Increased lipid content in the liver is a strong marker of reduced skeletal muscle and liver mitochondrial function, suggesting that the liver acts as a (protective?) metabolic sink increasing lipogenesis when overall oxidation rates are limited (**Figure 13.**). On the other hand, skeletal muscle triglyceride content does not relate to mitochondrial activity, which might be due to numerous confounding factors, such as physical activity and family history.

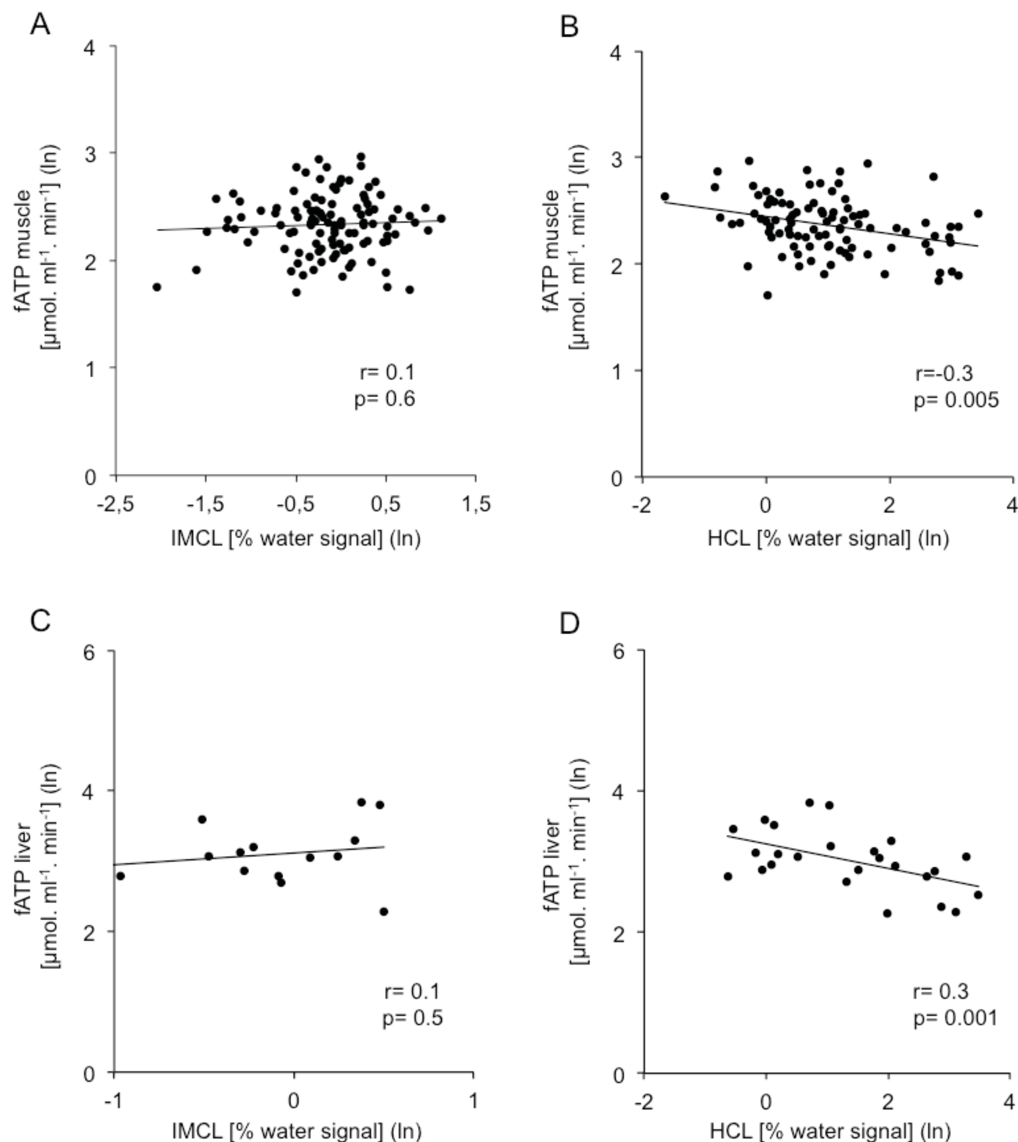

Figure 13.

*Correlation of ATP synthase flux rate (fATP) in skeletal muscle with intramyocellular lipids (IMCL) (**A**) and with hepatocellular lipids (HCL) (**B**) and correlation of fATP in the liver with*

*IMCL (**C**) and with HCL (**D**) in healthy glucose tolerant humans and in patients with type 2 diabetes. Data are from* [64, 234, 262, 265-267]

Liver ATP concentration and ATP synthetic flux rates are reduced in overt T2DM, which relates to insulin resistance, poor glycemic control, increased lipid availability and obesity. This could result from sustained adaptation to increased substrate flux directed from skeletal muscle to the liver, causing irreversible damage via oxidative stress. Reduced energy metabolism could be due to loss of functional hepatocytes or reduction of mitochondrial contents and/or fitness [93]. However, reduction of absolute Pi and γATP concentrations and ATP synthesis rates were found in T2DM but not in age- and BMI- matched healthy humans. In the face of age-dependent decline of whole-body insulin sensitivity across the groups, these data suggest a diabetes-related or even diabetes-specific effect causing the impairment of hepatic energy metabolism. This is corroborated by negative relationship between Pi and γATP and glucometabolic control as assessed from HbA1c and hepatic insulin sensitivity.

- **Reduced glucose uptake and impaired mitochondrial activity and plasticity in skeletal muscle could direct substrate fluxes towards the liver thereby promoting the development of steatosis.**
- **Patients with T2DM have lower absolute concentrations of ATP and lower ATP synthesis rates in the liver.**
- **Independent from liver fat, mitochondrial activity in the liver is negatively related to hepatic insulin sensitivity, body mass and glycemia.**
- **Lipotoxicity and poor glycemic could impair hepatic mitochondrial activity and thereby promote the development of steatosis.**

5.4. Future perspectives

Our findings answered open questions and point at novel therapeutic targets for the improvement of insulin resistance and subsequently for treatment of obesity, T2DM and related disorders such as NAFLD. One promising strategy might comprise dietary interventions aiming at modification the fatty acid composition of DAG species in skeletal muscle, thereby reducing the activation of PKCθ. Another strategy might be to increase the lipid storage capacity of adipose tissue and thereby decrease the extent of ectopic lipid deposition in other tissues thereby lowering DAG pools and improve insulin sensitivity.

It is yet unknown if secondary deteriorations of mitochondrial function are fully reversible and if the improvement of mitochondrial adaptability might increase insulin sensitivity.

Nevertheless, intervention strategies to improve the adaptation of mitochondrial activity in skeletal muscle and liver could also improve insulin sensitivity and lower hepatic fat content. Taken together, DAG-related activation of PKCθ and reduced mitochondrial plasticity are characteristic features of insulin resistance and represent novel targets for the treatment and prevention not only of insulin resistance, but also diabetes related comorbidities and NAFLD.

6. References

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2009 Jühling-Preis der Anna-Wunderlich-Ernst-Jühling-Stiftung

2010 Wewalka-Gedächtnispreis, Österr. Gesellschaft für Gastroenterologie und Hepatologie

2011 Menarini Preis der Deutschen Diabetes Gesellschaft

2012 Karl-Oberdisse-Preis der Nordrhein-Westfälischen Gesellschaft für Endokrinologie und Diabetologie

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Originalarbeiten:

1. Sandtner W, **Szendroedi J**, Zarrabi T, Zebedin E, Hilber K, Glaaser I, Fozzard HA, Dudley SC, Todt H. Lidocaine: A foot in the door of the inner vestibule prevents ultra-slow inactivation of a voltage-gated sodium channel. *Molecular pharmacology*. 2004;66:648-657 **IF: 4.12**
2. Zebedin E, Sandtner W, Galler S, **Szendroedi J**, Just H, Todt H, Hilber K. Fiber type conversion alters inactivation of voltage-dependent sodium currents in murine c2c12 skeletal muscle cells. *American journal of physiology. Cell physiology*. 2004;287:C270-280 **IF: 3.67**
3. Krebs M, Brunmair B, Brehm A, Artwohl M, **Szendroedi J**, Nowotny P, Roth E, Furnsinn C, Promintzer M, Anderwald C, Bischof M, Roden M. The mammalian target of rapamycin pathway regulates nutrient-sensitive glucose uptake in man. *Diabetes*. 2007;56:1600-1607 **IF: 8.47**
4. Meyerspeer M, Kemp GJ, Mlynarik V, Krssak M, **Szendroedi J**, Nowotny P, Roden M, Moser E. Direct noninvasive quantification of lactate and high energy phosphates simultaneously in exercising human skeletal muscle by localized magnetic resonance spectroscopy. *Magnetic resonance in medicine*. 2007;57:654-660 **IF: 3.40**
5. **Szendroedi J**, Sandtner W, Zarrabi T, Zebedin E, Hilber K, Dudley SC, Jr., Fozzard HA, Todt H. Speeding the recovery from ultraslow inactivation of voltage-gated na+ channels by metal ion binding to the selectivity filter: A foot-on-the-door? *Biophysical journal*. 2007;93:4209-4224 **IF: 3.97**
6. **Szendroedi J**, Schmid AI, Chmelik M, Toth C, Brehm A, Krssak M, Nowotny P, Wolzt M, Waldhausl W, Roden M. Muscle mitochondrial atp synthesis and glucose transport/phosphorylation in type 2 diabetes. *PLoS medicine*. 2007;4:e154 **IF: 14.00**
7. Chmelik M, Schmid AI, Gruber S, **Szendroedi J**, Krssak M, Trattnig S, Moser E, Roden M. Three-dimensional high-resolution magnetic resonance spectroscopic imaging for absolute quantification of 31p metabolites in human liver. *Magnetic resonance in medicine*. 2008;60:796-802 **IF: 3.40**
8. Schmid AI, Chmelik M, **Szendroedi J**, Krssak M, Brehm A, Moser E, Roden M. Quantitative atp synthesis in human liver measured by localized 31p

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9. Sunk IG, Demetriou D, **Szendroedi J**, Amoyo L, Raffetseder A, Horl WH, Sunder-Plassmann G, Smolen JS, Bobacz K. Cartilage biomarkers in hemodialysis patients and the effect of beta2-microglobulin on articular chondrocytes. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society*. 2008;16:1336-1342 **IF: 4.66**
10. **Szendroedi J**, Zwettler E, Schmid AI, Chmelik M, Pacini G, Kacerovsky G, Smekal G, Nowotny P, Wagner O, Schnack C, Schernthaner G, Klaushofer K, Roden M. Reduced basal atp synthetic flux of skeletal muscle in patients with previous acromegaly. *PloS one*. 2008;3:e3958 **IF: 3.23**
11. Kacerovsky-Bielesz G, Chmelik M, Ling C, Pokan R, **Szendroedi J**, Farukuoye M, Kacerovsky M, Schmid AI, Gruber S, Wolzt M, Moser E, Pacini G, Smekal G, Groop L, Roden M. Short-term exercise training does not stimulate skeletal muscle atp synthesis in relatives of humans with type 2 diabetes. *Diabetes*. 2009;58:1333-1341 **IF: 8.47**
12. **Szendroedi J**, Anderwald C, Krssak M, Bayerle-Eder M, Esterbauer H, Pfeiler G, Brehm A, Nowotny P, Hofer A, Waldhausl W, Roden M. Effects of high-dose simvastatin therapy on glucose metabolism and ectopic lipid deposition in nonobese type 2 diabetic patients. *Diabetes care*. 2009;32:209-214 **IF: 8.57**
13. **Szendroedi J**, Chmelik M, Schmid AI, Nowotny P, Brehm A, Krssak M, Moser E, Roden M. Abnormal hepatic energy homeostasis in type 2 diabetes. *Hepatology*. 2009;50:1079-1086 **IF: 11.19**
14. **Szendroedi J**, Schmid AI, Meyerspeer M, Cervin C, Kacerovsky M, Smekal G, Graser-Lang S, Groop L, Roden M. Impaired mitochondrial function and insulin resistance of skeletal muscle in mitochondrial diabetes. *Diabetes care*. 2009;32:677-679 **IF: 8.57**
15. Lindstrom J, Neumann A, Sheppard KE, Gilis-Januszewska A, Greaves CJ, Handke U, Pajunen P, Puhl S, Polonen A, Rissanen A, Roden M, Stemper T, Telle-Hjellset V, Tuomilehto J, Velickiene D, Schwarz PE, Acosta T, Adler M, AlKerwi A, Barengo N, Barengo R, Boavida JM, Charlesworth K, Christov V, Claussen B, Cos X, Cosson E, Deceukelier S, Dimitrijevic-Sreckovic V, Djordjevic P, Evans P, Felton AM, Fischer M, Gabriel-Sanchez R, Gilis-Januszewska A, Goldfracht M, Gomez JL, Greaves CJ, Hall M, Handke U, Hauner H, Herbst J, Hermanns N, Herrebrugh L, Huber C,

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Dr. Julia Szendrödi, PhD Düsseldorf, 2016

8. Attached Articles

Article No. 1.

BRIEF REPORT

Lipid-Induced Insulin Resistance Is Not Mediated by Impaired Transcapillary Transport of Insulin and Glucose in Humans

Julia Szendroedi,[1,2] Martin Frossard,[3,4] Nikolas Klein,[3] Christian Bieglmayer,[3,4] Oswald Wagner,[4] Giovanni Pacini,[5] Janette Decker,[1] Peter Nowotny,[1] Markus Müller,[3] and Michael Roden[1,2]

Increased lipid availability reduces insulin-stimulated glucose disposal in skeletal muscle, which is generally explained by fatty acid–mediated inhibition of insulin signaling. It remains unclear whether lipids also impair transcapillary transport of insulin and glucose, which could become rate controlling for glucose disposal. We hypothesized that lipid-induced insulin resistance is induced by inhibiting myocellular glucose uptake and not by interfering with the delivery of insulin or glucose. We measured changes in interstitial glucose and insulin in skeletal muscle of healthy volunteers during intravenous administration of triglycerides plus heparin or glycerol during physiologic and supraphysiologic hyperinsulinemia, by combining microdialysis with oral glucose tolerance tests and euglycemic-hyperinsulinemic clamps. Lipid infusion reduced insulin-stimulated glucose disposal by ~70% ($P < 0.05$) during clamps and dynamic insulin sensitivity by ~12% ($P < 0.05$) during oral glucose loading. Dialysate insulin and glucose levels were unchanged or even transiently higher ($P < 0.05$) during lipid than during glycerol infusion, whereas regional blood flow remained unchanged. These results demonstrate that short-term elevation of free fatty acids (FFAs) induces insulin resistance, which in skeletal muscle occurs primarily at the cellular level, without impairment of local perfusion or transcapillary transport of insulin and glucose. Thus, vascular effects of FFAs are not rate controlling for muscle insulin-stimulated glucose disposal. *Diabetes* **61:3176–3180, 2012**

Skeletal muscle accounts for the majority of glucose uptake after a meal and almost all glucose disposal during hyperinsulinemic-euglycemic clamps (1). In type 2 diabetes (T2DM), muscle insulin resistance predicts postprandial hyperglycemia, but the underlying mechanisms are unclear. Insulin-resistant humans frequently present with increased plasma free fatty acids (FFAs) (2), which can give rise to myocellular diacylglycerols or ceramides and impair insulin signaling (3–5). Insulin increases muscle microvascular perfusion and facilitates delivery of nutrients and hormones to the interstitium (6). Animal models of lipid-induced insulin resistance suggest that insulin-mediated microvascular perfusion is already reduced in prediabetic states and relates to impaired insulin action (7,8). Preventing the access of glucose and insulin to myocytes could contribute to lower glucose disposal and place abnormal microvascular insulin action as an early event in the development of T2DM.

We hypothesized that lipid-induced insulin resistance results from myocellular glucose uptake, but not from impaired delivery of insulin or glucose to the interstitium. We monitored changes of interstitial insulin and glucose in muscle of humans during intravenous triglycerides or glycerol administration under physiologic dynamic (oral glucose tolerance test [OGTT]) and supraphysiologic constant hyperinsulinemic (clamp) conditions.

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J.S. and M.F. contributed equally to this study.

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See accompanying commentary, p. 3063.

RESEARCH DESIGN AND METHODS

The study was registered at www.clinical trials.gov (NCT01482455) and approved by the local ethics committee in accordance with the most recent version of the Helsinki Declaration. Exclusion criteria comprised family history of T2DM or dyslipidemia, glucose intolerance, <19 years of age, BMI >27 kg/m^2, fasting triglycerides >140 mg/dL, total cholesterol >200 mg/dL, serum C-reactive protein >1 mg/dL, clotting disorders, hypersensitivity against study medication, any medication within 2 weeks before the study, alcohol consumption >40 g/d, or smoking.

Eight healthy, male volunteers randomly underwent four studies separated by ≥1 week. After 12 h overnight fasting, three plastic cannulas (Venflon) were inserted into antecubital veins of both forearms for infusions and blood drawing. On day 1 (LIPc), a triglyceride/heparin infusion was administered (0–360 min: Intralipid 20% [Pharmacia AB, Stockholm, Sweden], 90 mL/h; heparin "Immuno," Immuno AG, bolus: 200 IU, 0.2 IU · kg^{-1} · min^{-1}), and a hyperinsulinemic-euglycemic clamp test (Actrapid [Novo Nordisk, Bagsvaerd, Denmark]; 40 mU · m^{-2} body surface area · min^{-1}) was performed (120–360 min). Plasma glucose was maintained at ~5.4 mmol/L using a variable glucose infusion. On day 2 (GLYc), glycerol (in 0.9% saline, 0.7 mg · kg^{-1} · min^{-1}) was infused instead of triglyceride/heparin. Triglyceride/heparin was infused on day 3 (LIPo) and glycerol on day 4 (GLYo) (0–420 min), each followed by a 75-g OGTT (Glucodrink [Roche Diagnostica]; 240–420 min).

Microdialysis. As previously described, a microdialysis probe (cutoff, 20 kDa; shaft length, 70 mm; membrane length, 16 mm; outer diameter, 0.5 mm [CMA, Stockholm, Sweden]) was perfused with Ringer's solution + 5% human albumin (flow rate, 1.5 μL/min) for sampling of interstitial glucose (9). For sampling of interstitial insulin, another probe (cutoff, 100 kDa; shaft length, 70 mm; membrane length, 16 mm; outer diameter, 0.5 mm [CMA]) was perfused with bidistilled water + 5% human albumin (flow rate, 3 μL/min). The microdialysis system was perfused using a microinfusion pump (Precidor; Infors-AG, Basel, Switzerland).

For most analytes, the equilibrium between the respective concentrations in interstitial fluid and perfusion medium is incomplete, so that the measured dialysate concentrations underestimate the absolute interstitial concentrations (10,11). As we aimed to monitor dynamic changes rather than absolute interstitial levels, we report the relative changes of interstitial glucose and insulin of dialysate concentrations compared with baseline (fasting). This avoided time-consuming calibration experiments in addition to the already exhaustive protocols.

Regional blood flow. Muscle blood flow was measured by the laser Doppler flow technique (LDF; Moor Instruments, Devon, U.K.) (12) using a fiber LDF

probe (total diameter, 0.5 mm), inserted 5 cm into m. vastus medialis, 5 cm distant from the microdialysis probe.

Laboratory analyses. Glucose was measured in plasma by a Beckman II glucose analyzer (Beckman Coulter) and in dialysate by a CMA 600 MD analyzer (CMA, Uppsala, Sweden). Serum and dialysate insulin were measured using an ultrasensitive insulin immunoassay (Mercodia, Uppsala, Sweden). Plasma FFAs were assayed microfluorimetrically (Wako USA, Richmond, VA) after collecting blood into orlistat-containing vials (13).

Data analysis. Areas under the plasma glucose and insulin curves ($AUC_{120–360min-serum}$ for clamps; $AUC_{240–420min-serum}$ for OGTT) were calculated using the trapezoidal rule. Fasting insulin sensitivity was assessed from fasting plasma insulin (FPI) and glucose (FPG) as QUICKI (1/[logFPI + logFPG]). During clamps, whole-body insulin sensitivity was calculated as M-value (13). During OGTT, dynamic insulin sensitivity was assessed from the oral glucose insulin sensitivity index (OGIS), a validated index of glucose clearance (14). β-Cell function was determined in relation to glycemia (insulinogenic index) and to insulin sensitivity (disposition index) (14).

Statistical analysis. Data (text, means ± SD; figures, means ± SEM) were analyzed using SPSS 11.0 software (SPSS, Chicago, IL). Statistical comparisons between LIP and GLY were performed using two-tailed, paired Student t tests. Changes of sequential data within experiments were evaluated by repeated-measures one-way ANOVA with post hoc Tukey correction. Linear correlations are Pearson product-moment correlations. $P < 0.05$ was considered to indicate significant differences.

RESULTS

Supraphysiologic hyperinsulinemia

Plasma parameters and insulin sensitivity. Plasma FFAs were similar on both days during fasting (GLYc vs. LIPc, 0.36 ± 0.19 vs. 0.37 ± 0.19 mmol/L), increased within 2 h, and remained elevated during LIPc ($P = 5.10^{-9}$) (Fig. 1*A*). Plasma glucose was comparable on both days during fasting and clamps (5.3 ± 0.2 vs. 5.4 ± 0.9 mmol/L) (Fig. 1*C*). Plasma insulin was also not different at fasting and during clamps (426 ± 155 vs. 514 ± 75 pmol/L) (Fig. 1*E*). The M-value was ~70% lower during LIPc (180–210 min, $P = 0.007$; 210–240 min, $P = 0.011$) (Fig. 2).

Interstitial parameters and muscle blood flow. The change of interstitial glucose was 35% greater in LIPc vs. GLYc (180–240 min, 1.37 ± 0.21 vs. 1.06 ± 0.28, $P < 0.05$)

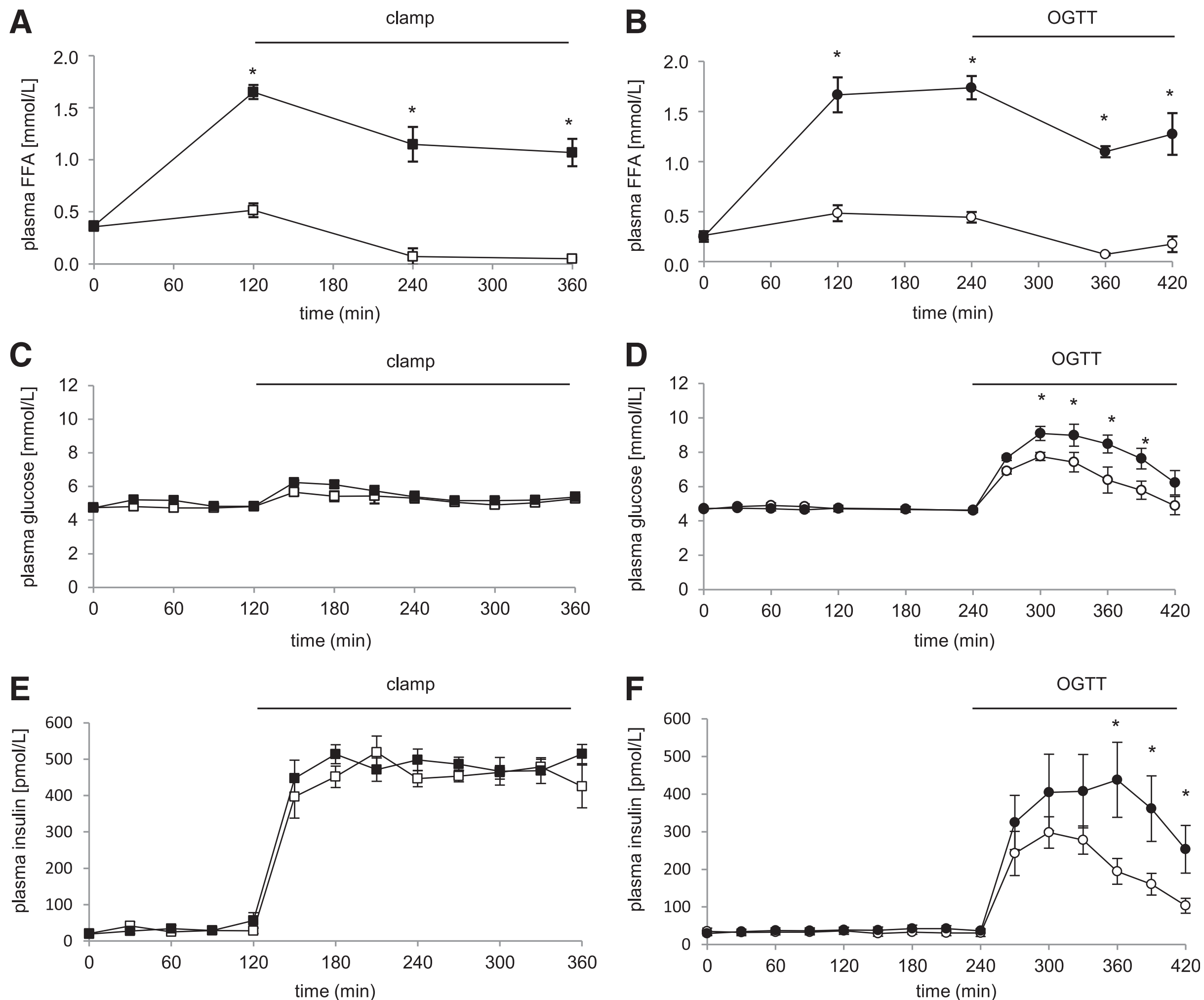

FIG. 1. Time course of plasma concentrations (means ± SEM) of FFAs during hyperinsulinemic-euglycemic clamps (*A*) and during OGTT (*B*), of glucose during hyperinsulinemic-euglycemic clamps (*C*) and during OGTT (*D*), and of insulin hyperinsulinemic-euglycemic clamp (*E*) and during OGTT (*F*). Young, healthy participants received lipid infusion (black symbols) or glycerol infusion (white symbols). Differences in lipid vs. glycerol infusion: *$P < 0.05$.

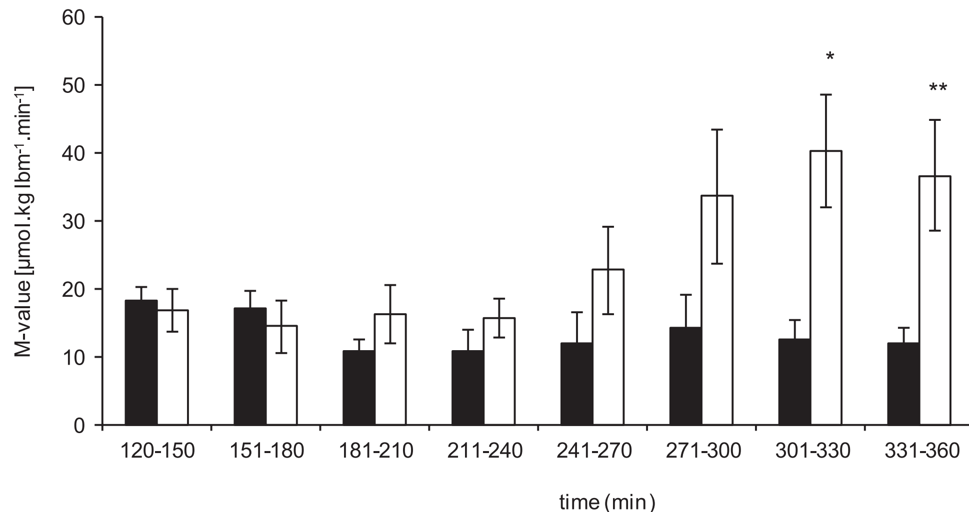

FIG. 2. Whole-body glucose disposal (means ± SEM) during hyperinsulinemic-euglycemic clamps in the presence of lipid infusion (■) and glycerol infusion (□). Differences in lipid vs. glycerol studies: $*P < 0.05$; $**P < 0.01$.

(Fig. 3*A*). The relative increase of interstitial insulin tended to be greater during LIPc compared with GLYc (~1.7- vs. ~1.2-fold, $P = 0.1$) within 60 min into the clamp (Fig. 3*C*). Regional blood flow tended ($P = 0.06$) to be higher during LIPc compared with GLYc without changing above baseline (Fig. 3*E*).

Physiologic hyperinsulinemia

Plasma parameters, insulin sensitivity, and secretion. Plasma FFAs were similar during fasting (0.26 ± 0.12 vs. 0.25 ± 0.14 mmol/L), increased within 2 h of LIPo (0.49 ± 0.23 vs. 1.67 ± 0.50 mmol/L, $P = 3.10^{-5}$), and decreased during OGTT in GLYo ($P = 0.0002$) (Fig. 1*B*). Plasma glucose was comparable during fasting (4.4 ± 0.3 vs. 4.4 ± 0.3 mmol/L) but higher after lipid infusion during OGTT (Fig. 1*D*). Fasting plasma C-peptide (1.27 ± 0.17 vs. 1.21 ± 0.16 ng/mL) and insulin did not differ between interventions (34 ± 27 vs. 29 ± 16 pmol/L). Plasma insulin was higher after 4 h of LIPo, reflecting glucose-induced stimulation of insulin secretion due to lipid-induced insulin resistance (Fig. 1*F*). Fasting insulin sensitivity did not differ (Table 1), whereas dynamic insulin sensitivity was ~12% lower during LIPo. β-Cell sensitivity to glucose and to insulin sensitivity was not different between interventions (Table 1).

Interstitial parameters and muscle blood flow. The relative increase in interstitial glucose was ~64% higher in LIPo during OGTT (300–420 min, 2.4 ± 0.2– vs. 1.4 ± 0.1–fold increase, $P < 0.05$) (Fig. 3*B*). The relative increase of interstitial insulin was ~17% ($P < 0.05$) during lipid infusion and did not change in GLYo (Fig. 3*D*). Regional blood flow relative to baseline did not differ between the interventions (Fig. 3*F*).

DISCUSSION

This study demonstrates that transcapillary transport of insulin and glucose is not blunted during the onset of FFA-induced insulin resistance under conditions of dynamic and constant hyperinsulinemia.

We did not observe increased blood flow under insulin-stimulated conditions, which is in line with studies in healthy humans at comparable and ~20-fold higher plasma insulin concentrations (15). Of note, other studies suggested that meal-induced increases in insulin have hemodynamic effects, thereby enhancing its access and that of nutrients to myocellular metabolism (16,17). Here, we report that lipid infusion induced whole-body insulin resistance, while blood flow tended to be greater during constant hyperinsulinemia, indicating increased rather than reduced blood flow. Similarly, FFA elevation markedly raised retinal and skin blood flow in humans, although the underlying mechanisms remain unclear (18). Nevertheless, obese humans have a reduced activation of lower-limb blood flow in response to insulin (16,17) and impaired postprandial microvascular recruitment (19). Of note, insulin mainly increases the contribution of the nutritive versus the nonnutritive flow route to microvascular blood flow within muscle (6). Thus, in insulin-resistant states, impaired redirection of blood toward the nutritive flow into the capillary bed could lead to bypass of hormones and substrates away from myocytes.

In the absence of lipid or glycerol infusions, interstitial insulin concentrations can rise 10-fold during clamps or OGTT in healthy humans (20). In our lipid infusion study, dialysate insulin increased slightly above baseline only during the clamp, but not during OGTT-induced splanchnic insulin secretion, which is likely due to its substantial removal by the liver. In dogs, measurement of plasma and lymph insulin, as a surrogate of the interstitial compartment, during euglycemic-hyperinsulinemic clamps revealed a tight relationship between lymph insulin and whole-body glucose uptake (21,22). After lipid infusion or high-fat diet, they had increased venous, but lower lymphatic, insulin concentrations upon intramuscular insulin injections (7,8). The authors concluded that these conditions prevent insulin from diffusing through the interstitium, maybe due to endothelial dysfunction. Our study suggests that both FFA and glycerol, which is also a component of triglycerides, could have been responsible for the absent rise in interstitial insulin in both lipid and control studies. But as insulin resistance was seen only during lipid infusion, we conclude that lipids may affect transcapillary transport of insulin into and/or out of the interstitial space but without being rate controlling for insulin action in lean humans.

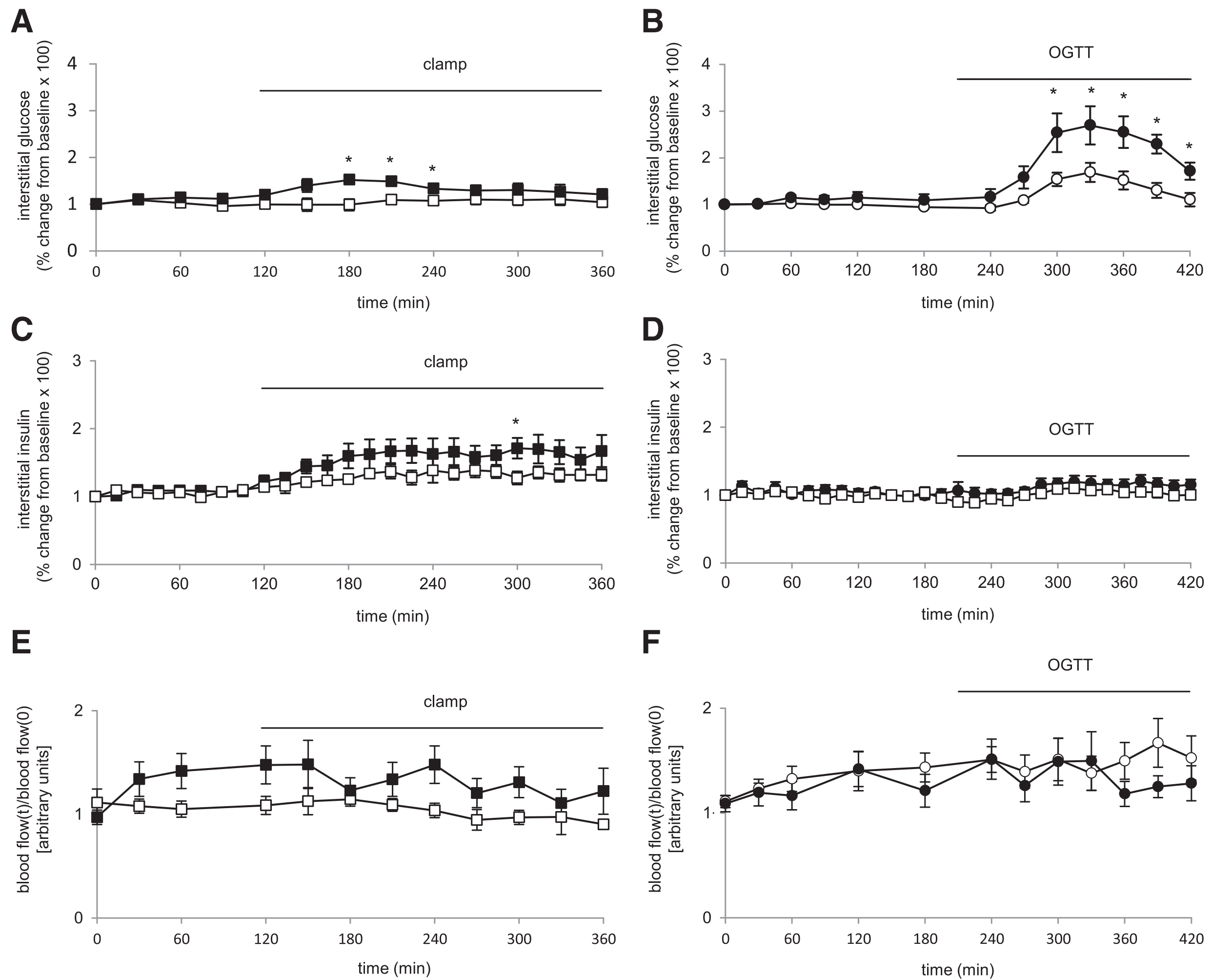

FIG. 3. Time course of changes vs. baseline (means ± SEM) of interstitial glucose during hyperinsulinemic-euglycemic clamps (*A*) and during OGTT (*B*), and changes of interstitial insulin vs. baseline during hyperinsulinemic-euglycemic clamps (*C*) and during OGTT (*D*). Regional blood flow relative to baseline (means ± SEM) during the hyperinsulinemic-euglycemic clamps (*E*) and during OGTT (*F*). Young, healthy participants received lipid infusion (black symbols) or glycerol infusion (white symbols). Differences in lipid vs. glycerol infusion: *$P < 0.05$.

Here we confirm that lipid infusion decreased both M-value and OGIS (23). Plasma FFAs correlate inversely with muscle insulin sensitivity (2), and their elevation impairs muscle glucose transport/phosphorylation within 2 h (5,23). This is likely due to myocellular accumulation of diacylglycerols, which interfere with insulin signaling, at least in rat muscle (4), and supports the evidence for direct myocellular FFA effects.

Increased lipid concentrations can also directly interfere with β-cells and affect insulin secretion. Short-term plasma FFA elevation increases rather than decreases insulin secretion, leading to gradually higher peripheral venous

TABLE 1
AUCs of glucose and insulin as well as surrogate parameters (means ± SD) of dynamic insulin sensitivity and β-cell function during the OGTT mimicking physiologic hyperinsulinemia in the presence of glycerol (GLYo) or lipid (LIPo) infusion

	GLYo	LIPo	*P* value
Plasma glucose (AUC, mmol · L^{-1} · min^{-1})	1,170 ± 189	2,057 ± 465	*P* = 0.0002
Plasma insulin (AUC, pmol · L^{-1} · min^{-1})	37,339 ± 35,986	62,669 ± 36,374	*P* = 0.08
QUICKI	0.52 ± 0.03	0.48 ± 0.02	NS
OGIS (mL · min^{-1} · m^{-2})	416 ± 17	365 ± 21	*P* = 0.007
Insulinogenic index (×10^{-6})	73.75 ± 14.67	101 ± 25	NS
Disposition index (L · min^{-2} · 10^{-3})	36.35 ± 5.72	46 ± 10.42	NS

P compares lipid vs. glycerol infusion.

insulin concentrations (24). In the current study, plasma insulin and glucose concentrations were not different between GLY and LIP during equilibration, indicating no relevant stimulation of insulin secretion. Likewise, dynamic β-cell function as assessed from sensitivity to glucose (insulinogenic index) and to insulin sensitivity (disposition index) did not differ between GLY and LIP. Thus, the higher plasma insulin concentrations during the OGTT in the lipid/heparin study most likely reflect glucose-induced insulin secretion in response to the FFA-mediated peripheral insulin resistance.

Here we monitored the time course of dialysate glucose and insulin, which allowed the assessment of the effectiveness of transcapillary substrate transport independent of differences of muscle perfusion. Nevertheless, this study also has limitations. First, we cannot report absolute interstitial glucose and insulin concentrations without calibration procedures (20), which, however, does not affect our conclusions based on the time-dependent relative changes. It has also been shown that the ratio between interstitial and dialysate analyte concentrations is comparable between various insulin-resistant states and insulin-sensitive humans (10). Second, lipids can interfere with analyte measurements using microdialysis (25). To prevent this phenomenon, we added human albumin to the perfusion medium and used probes with low-molecular-weight cutoff membranes (25). Of note, despite increased plasma FFA or glycerol levels, dialysate glucose and insulin concentrations were stable during the equilibration periods, arguing against any interference of lipids with glucose or insulin measurements. Third, M-value and OGIS are composite measures of whole-body insulin sensitivity so that other tissues, particularly the liver, could have contributed to the observed FFA-induced insulin resistance (23), but skeletal muscle accounts for the vast majority of glucose uptake during such hyperinsulinemic conditions (1). Finally, we did not obtain biopsies to measure FFA effects on insulin signaling because these were demonstrated before (23) and the study design was already demanding.

In conclusion, short-term FFA elevation induces muscle insulin resistance primarily at the cellular level, without impairment of local perfusion or transcapillary transport of insulin and glucose. However, this does not exclude that microvascular alterations contribute to insulin resistance in long-term dyslipidemia and overt T2DM.

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J.S. wrote the manuscript and analyzed data. M.F. researched data, contributed to discussion, and reviewed and edited the manuscript. N.K., C.B., O.W., G.P., and P.N. researched data and reviewed and edited the manuscript. J.D. analyzed data and reviewed and edited the manuscript. M.M. designed the study, contributed to discussion, and reviewed and edited the manuscript. M.R. designed the study, analyzed data, contributed to discussion, and wrote, reviewed, and edited the manuscript. M.R. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Article No. 2.

Effects of High-Dose Simvastatin Therapy on Glucose Metabolism and Ectopic Lipid Deposition in Nonobese Type 2 Diabetic Patients

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OBJECTIVE — Statins may exert pleiotropic effects on insulin action that are still controversial. We assessed effects of high-dose simvastatin therapy on peripheral and hepatic insulin sensitivity, as well as on ectopic lipid deposition in patients with hypercholesterolemia and type 2 diabetes.

RESEARCH DESIGN AND METHODS — We performed a randomized, double-blind, placebo-controlled, single-center study. Twenty patients with type 2 diabetes received 80 mg simvastatin (BMI 29 ± 4 kg/m^2, age 55 ± 6 years) or placebo (BMI 27 ± 4 kg/m^2, age 58 ± 8 years) daily for 8 weeks and were compared with 10 healthy humans (control subjects; BMI 27 ± 4 kg/m^2, age 55 ± 7 years). Euglycemic-hyperinsulinemic clamp tests combined with D-[6,6-d2]glucose infusion were used to assess insulin sensitivity (*M*) and endogenous glucose production (EGP). ^{1}H magnetic resonance spectroscopy was used to quantify intramyocellular and hepatocellular lipids.

RESULTS — High-dose simvastatin treatment lowered plasma total and LDL cholesterol levels by ~33 and ~48% ($P < 0.005$) but did not affect *M*, intracellular lipid deposition in soleus and tibialis anterior muscles and liver, or basal and insulin-suppressed EGP. In simvastatin-treated patients, changes in LDL cholesterol related negatively to changes in *M* ($r = -0.796$, $P < 0.01$). Changes in fasting free fatty acids (FFAs) related negatively to changes in *M* ($r = -0.840$, $P < 0.01$) and positively to plasma retinol-binding protein-4 ($r = 0.782$, $P = 0.008$).

CONCLUSIONS — High-dose simvastatin treatment has no direct effects on whole-body or tissue-specific insulin action and ectopic lipid deposition. A reduction in plasma FFAs probably mediates alterations in insulin sensitivity in vivo.

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Type 2 diabetes is commonly associated with dyslipidemia, which represents a synergistic risk factor for cardiovascular disease (1). High-circulating lipids (free fatty acids [FFAs]) induce insulin resistance because of impaired muscle glucose transport/phosphorylation, and intracellular lipids in muscle (IMCLs) and liver (HCLs) predict insulin resistance (2).

Interventional studies emphasized that statin treatment leads to a reduction in cardiovascular events with benefits for patients with type 2 diabetes (3). Statins could also contribute to diabetes prevention owing to lipid-lowering and so-called pleiotropic action. Statin therapy was shown to improve endothelial function, inhibit smooth muscle cell proliferation, and reduce oxidative stress and inflammation (4). Retrospective analysis of the West of Scotland Coronary Prevention Study (WOSCOPS) revealed that 5 years of treatment with pravastatin reduced diabetes incidence by ~30%. The authors suggested that although lowering of triglyceride levels could influence diabetes incidence, other mechanisms such as anti-inflammatory action may be involved (5). However, pravastatin did not decrease diabetes incidence in another trial including glucose-intolerant humans, suggesting that early inception of statin therapy may be required for effective diabetes prevention (6). Likewise, simvastatin did not affect diabetes incidence in patients with atherosclerosis in the Heart Protection Study (7). In contrast, atorvastatin marginally increased diabetes incidence in the Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT-LLA), which could be explained by statistical variation (8). Thus, the effect of statins on diabetes incidence is still uncertain.

The direct action of statins on insulin sensitivity remains controversial because beneficial (9) and indifferent and unfavorable (10) effects were reported. Statins not only decrease LDL cholesterol but may also interfere with fasting and postprandial metabolism of triglyceride-rich lipoproteins, resulting in altered substrate flux and accumulation of HCLs (11,12), which could subsequently affect muscle glucose metabolism and deposition of IMCLs.

Simvastatin is one of the most frequently prescribed statins because of its efficacy in reducing LDL lipoprotein cho-

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lesterol levels, its tolerability, and its reduction of cardiovascular risk and mortality (7). Its effects on insulin action and metabolism at the maximal recommended dose of 80 mg/day are unclear. Thus, we examined the effects of 80 mg/day simvastatin therapy on *1*) insulin sensitivity, *2*) IMCLs and HCLs, *3*) fasting and insulin-mediated suppression of plasma FFAs, and *4*) β-cell function using euglycemic-hyperinsulinemic clamps combined with stable isotope dilution and nuclear magnetic resonance spectroscopy in hypercholesterolemic, normotriglyceridemic patients with type 2 diabetes.

RESEARCH DESIGN AND METHODS

Twenty patients with type 2 diabetes and hypercholesterolemia were included. Eligibility criteria were known duration of disease of 3–10 years, age 35–75 years, BMI <32 kg/m^2, LDL cholesterol >4.16 mmol/l, triglycerides <2.75 mmol/l, A1C <9%, serum creatinine <1.8 mg/dl, liver transaminases <20% over the upper limit with no active liver disease and creatine kinase <50% above the upper limit, and no evidence of metabolic diseases other than type 2 diabetes. Patients were taking neither lipid-lowering drugs nor other drugs known to interfere with metabolism of statins. The only glucose-lowering drugs allowed were metformin, sulfonylureas, and α-glucosidase inhibitors. Ten age-, sex-, and BMI-matched healthy volunteers (control subjects) were examined only at baseline.

The study had a double-blind, randomized, placebo-controlled and parallel group design. The trial has been registered as a clinical trial. The sample size was calculated using data from our previous studies in diabetic patients who complied with the inclusion criteria of the present study and were examined with identical experimental methods. The false-positive and false-negative error rates tolerated were $Z\alpha = 1.96$ for a two-tailed α of 0.05 and $Z\beta = 0.84$ for a β of 0.2. An increase or decrease of ~20% in the mean values for the primary target variables, insulin-stimulated whole-body glucose disposal (*M* value) and insulin-suppression of endogenous glucose production (EGP), was considered to be physiologically and clinically relevant. The respective mean ± SD values were ~5 ± 1 mg · kg^{-1} · min^{-1} for *M* values (3 ± 0.3 [ref. 13], 8 ± 1 [ref. 14]), and ~0.5 ± 0.1 mg · kg^{-1} · min^{-1} for EGP suppression (13,14). These considerations revealed a sample size of eight as the minimal number of patients receiving simvastatin. Expecting a dropout rate of ~15%, we included 10 participants for each study group.

After a run-in period of 3 weeks, the patients were randomly assigned to treatment with 80 mg daily simvastatin (Merck Sharp & Dohme, Hoddesdon, U.K.) or placebo for 2 months. Glucose metabolism, IMCLs, and HCLs were determined before and after treatment following overnight fasting for at least 12 h. According to previous studies, sulfonylureas (three in the simvastatin group and nine in the placebo group), metformin (five in the simvastatin group and seven in the placebo group), and α-glucosidase inhibitors (two in the simvastatin group and one in the placebo group) were withdrawn at 1 and 3 days before the clamps, respectively (13,14). The study was approved by the local ethics committee, and patients consented to participate.

Glucose metabolism

At 7.00 A.M. patients were transferred to the metabolic unit. A primed infusion of D-[6,6-d2]glucose (3.6 mg/kg body weight × [fasting plasma glucose/90]) followed by a continuous infusion (0.036 mg/min × kg body weight) was started to determine EGP (15). At 9.00 A.M., a primed continuous infusion of 40 mU/min per m^2 body surface area was administered for 150 min to assess insulin sensitivity (*M*) and the ratio of *M* to the prevailing plasma insulin concentration (*M/I*) by hyperinsulinemic-isoglycemic (at baseline fasting plasma glucose [FPG]) clamps in control subjects and to standardize for increased FPG by a euglycemic-hyperinsulinemic (~100 mg/dl) clamp in type 2 diabetic patients. In type 2 diabetic patients, euglycemia was achieved by identical primed continuous insulin infusions as in control subjects, and no additional insulin infusion was required. A 20% dextrose infusion, 2% enriched with D-[6,6-d2]glucose was periodically adjusted to maintain euglycemia (15).

Analytical procedures

Glucose was measured by the glucose oxidase method (Glucose Analyzer II; Beckman Instruments, Fullerton, CA). Atom percent ^{2}H enrichments in glucose were determined by gas chromatography–mass spectrometry (15). FFAs were assayed microfluorimetrically (Wako Chemicals USA, Richmond, VA) in blood samples using orlistat to prevent in vitro lipolysis (15). Triglyceride levels were measured colorimetrically (Roche, Vienna, Austria). Insulin, C-peptide, and glucagon were determined by double-antibody radioimmunoassay (15). Retinol-binding protein (RBP)-4 was assayed nephelometrically using an antiserum to human plasma RBP (code OUVO; Dade Behring, Deerfield, IL) (16).

^{1}H nuclear magnetic resonance spectroscopy

Volunteers were lying supine inside a 1.5-T spectrometer (Magnetom; Siemens, Erlangen, Germany). HCLs were quantified using a breath-hold–triggered single voxel sequence without water suppression applied on the 27-cm^3 volume within the right lateral liver (2). IMCLs were determined in 1.73-cm^3 volumes within soleus and tibialis anterior muscles using water-suppressed PRESS and the AMARES algorithm as implemented in the jMRUI software package. After T_2 relaxation, IMCLs were quantified from the intensity of the $(CH_2)_n = 1.25$ ppm resonance, which was compared with the water resonance intensity obtained from spectra without water suppression.

Calculations and statistics

The computer-solved homeostasis model assessment (HOMA2) was used to derive surrogate parameters of basal β-cell function (HOMA-B) and insulin sensitivity (HOMA-IR). EGP was calculated from the difference between rates of glucose appearance (R_a) (15) and of mean glucose infusion. Statistical analyses were performed using SPSS software (version 6.0; SPSS, Chicago, IL). Data are presented as means ± SD (±SEM in the figures). Comparisons between groups and drug-induced effects were assessed by ANOVA with or without repeated measurements with Tukey post hoc testing. Within-group differences were determined using two-tailed Student's *t* tests. Differences were considered significant at the 5% level for *M*, FFAs, and EGP and at 1% for other parameters to correct for interrelated comparison. Linear correlations are Pearson product-moment correlations and were considered to be significant at the 5% level for *M*, FFAs, and EGP and at 1% for all other relations.

Table 1—*Baseline characteristics of type 2 diabetic patients and matched nondiabetic volunteers*

	Simvastatin (80 mg/day)	Placebo	Control subjects
n (women/men)	10 (3/7)	10 (5/5)	10 (5/5)
BMI (kg/m^2)	28.9 ± 3.5	27.3 ± 3.7	27.4 ± 4
Age (years)	55 ± 6	58 ± 8	55 ± 7
A1C (%)	6.7 ± 0.6	6.7 ± 0.7	5.6 ± 0.2‡
FPG (mmol/l)	8.7 ± 1.3	8.5 ± 1.3	4.9 ± 0.4§
HOMA-B	64 ± 23	69 ± 27	81 ± 17
HOMA-IR	2.7 ± 0.9	2.7 ± 0.8	0.8 ± 0.2‖
Fasting EGP (mg · kg^{-1} · min^{-1})	1.7 ± 0.3	1.7 ± 0.4	1.4 ± 0.4**
TGs (mmol/l)	1.7 ± 0.5	1.9 ± 0.6	1.1 ± 0.4*†
FFAs (μmol/l)	503 ± 229	618 ± 206	613 ± 206
TC (mmol/l)	7.6 ± 2.5	6.6 ± 0.8	5.6 ± 0.9*
TG-to-HDL cholesterol ratio	2.9 ± 1.0	3.3 ± 1.2	1.8 ± 0.8¶
HDL cholesterol (mmol/l)	1.4 ± 0.3	1.4 ± 0.2	1.5 ± 0.2
LDL cholesterol (mmol/l)	5.4 ± 2.3	4.3 ± 0.6	3.6 ± 0.8*
ALT (units/l)	37 ± 13	34 ± 11	26 ± 9
AST (units/l)	25 ± 7	21 ± 4	26 ± 7
GGT (units/l)	37 ± 13	34 ± 11	21 ± 12*

Data are mean ± SD anthropometric and laboratory characteristics of type 2 diabetic patients after allocation to either placebo or simvastatin therapy and healthy control subjects. BMI, FPG, surrogate parameters of basal β-cell function (HOMA-B) and basal insulin sensitivity (HOMA-IR), total triglycerides (TGs), FFAs, total cholesterol levels (TC), HDL cholesterol and calculated LDL cholesterol, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and GGT were determined. **P* < 0.05, control versus simvastatin; ***P* < 0.05, control versus simvastatin and placebo; †*P* < 0.005, control versus placebo; ‡*P* < 0.0005, simvastatin and placebo versus control; §*P* < 0.00001, simvastatin and placebo versus control; ‖*P* < 0.00005, simvastatin and placebo versus control; ¶*P* < 0.01, placebo versus control.

RESULTS

Baseline characteristics

Baseline characteristics of patients with type 2 diabetes after allocation to either placebo or simvastatin therapy and control subjects are shown in Table 1. A1C, FPG, and triglycerides were increased in both diabetic groups, and total cholesterol and LDL cholesterol were slightly higher in the simvastatin group than in control subjects. In type 2 diabetic patients, HOMA-IR was ~3.4-fold higher than in control subjects, whereas HOMA-B was comparable. γ-Glutamyl transpeptidase (GGT) was ~76 and ~62% higher in type 2 diabetic patients than in control subjects ($P = 0.020$ versus simvastatin; $P = 0.062$ versus placebo). Basal EGP was ~21% higher in type 2 diabetic patients (simvastatin 1.7 ± 0.3, placebo 1.7 ± 0.4, and control 1.4 ± 0.4 mg · kg^{-1} · min^{-1}; $P < 0.05$ versus type 2 diabetes). IMCLs in soleus and in tibialis anterior muscles in type 2 diabetic patients were comparable to IMCLs in control subjects (simvastatin 1.4 ± 0.5 and 0.2 ± 0.2, placebo 1.3 ± 0.6 and 0.3 ± 0.2, and control 1.5 ± 0.9 and 0.4 ± 0.4%). In contrast, HCLs were ~3.6-fold higher in type 2 diabetic patients (simvastatin 14.2 ± 8.6, placebo 14.1 ± 5.8, and control 4 ± 4%; $P < 0.001$ versus type 2 diabetes) (Fig. 1*B*). Across the whole study population, HCLs tended to relate positively to FPG ($r = 0.544$, $P < 0.005$), A1C ($r = 0.409$, $P < 0.05$), and GGT ($r = 0.442$, $P < 0.05$) without reaching predefined statistical significance ($P < 0.01$) but related negatively to *M* ($r = -0.386$, $P < 0.05$). IMCLs did not correlate with any other metabolic parameters.

Whole-body metabolism during the clamps

Within 60 min of the clamps, plasma glucose levels reached steady-state conditions before and after treatment (simvastatin 5.7 ± 0.3 and 5.7 ± 0.3, placebo 5.9 ± 0.6 and 5.7 ± 0.2, and control 4.9 ± 0.4 mmol/l) and did not differ within or among the intervention groups. During the last 60 min of the clamps, plasma glucose levels before and after treatment were 5.4 ± 0.3 and 5.4 ± 0.3 mmol/l in the simvastatin group, 5.5 ± 0.3 and 5.4 ± 0.3 mmol/l in the placebo group, and 4.9 ± 0.3 mmol/l in control subjects and did not differ within or among the intervention groups but was lower in control subjects than in type 2 diabetic patients ($P < 0.005$). Plasma insulin concentrations were 580 ± 102 and 609 ± 109 pmol/l in the simvastatin group, 537 ± 80 and 551 ± 94 pmol/l in the placebo group, and 515 ± 58 pmol/l in control subjects and did not differ within or among the intervention groups. *M* values were ~42% lower in type 2 diabetic patients and did not differ among the intervention groups (control 7.4 ± 2.4, simvastatin 4.1 ± 1.9, and placebo 4.5 ± 2.7 mg · kg^{-1} · min^{-1}; $P < 0.005$, type 2 diabetic patients versus control subjects) (Fig. 1*A*). Similarly, the *M*-to-*I* ratio was lower in type 2 diabetic patients [control subjects 0.01 ± 0.005 mg · kg^{-1} · min^{-1} · (pmol/l)$^{-1}$; $P < 0.01$] but not different among intervention groups (Table 2). Insulin-mediated suppression of EGP (Table 2) and FFAs (control 94 ± 5, simvastatin 87 ± 10, and placebo 92 ± 2%) was comparable in all groups. Plasma triglycerides related positively to HOMA-IR ($r = 0.683$, $P = 0.00003$) and negatively to *M* ($r = -0.555$, $P = 0.001$), A1C ($r = -0.539$, $P = 0.002$), and FPG ($r = -0.497$, $P = 0.005$).

Effects of simvastatin on lipid and glucose metabolism

Intervention-related changes of plasma lipids and glucose metabolism are shown in Table 2. At 2 months, plasma total and LDL cholesterol decreased by ~33 and ~48% in the simvastatin group but remained unchanged in the placebo group. There were no significant changes in triglycerides, HDL cholesterol, and fasting FFAs after simvastatin therapy compared with baseline. Nevertheless, the simvastatin group had ~29 and ~35% lower triglycerides and FFAs than the placebo group. In the simvastatin group, the decreases in LDL cholesterol and FFAs were positively associated ($r = 0.774$, $P < 0.001$) but did not relate to changes in triglycerides. Despite no significant changes in *M* after simvastatin treatment, changes in FFAs were negatively correlated with the change in *M* in the simvastatin group ($r = -0.840$, $P = 0.002$), which was weakened by the exclusion of one subject with excessive changes in *M* and FFAs ($r = -0.641$, $P = 0.063$). The relationship between changes in *M* and LDL cholesterol ($r = -0.796$, $P = 0.006$) was completely lost by omission of this subject ($r = 0.242$, $P = 0.531$) (Fig. 2*A*). Adjustment for FFAs disrupted the relationship between the changes in LDL cholesterol and *M* ($r = 0.424$, $P = 0.256$), whereas the association between changes

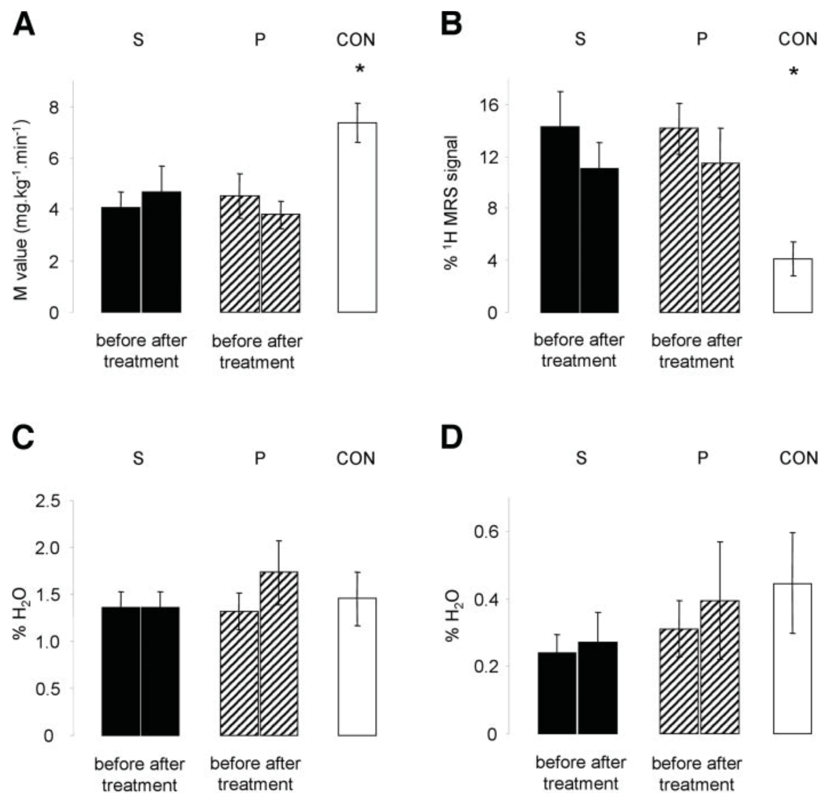

Figure 1— *Whole-body insulin sensitivity (*M *value) (*A*), ectopic lipid deposition in liver (*B*) soleus muscle (*C*), and anterior tibialis muscle (*D*) in patients with type 2 diabetes before and after treatment with 80 mg/day simvastatin (S,* n *= 10, ■) or placebo (P,* n *= 10, ▨), and healthy humans (CON,* n *= 10, □,* P *< 0.005 versus simvastatin and placebo groups).*

in FFAs and *M* remained robust after adjustment for LDL cholesterol ($r = 0.584$, $P = 0.099$). Changes in the *M*-to-*I* ratio after simvastatin treatment also related positively to changes in plasma FFAs ($r = 0.674$, $P = 0.033$). Plasma RBP-4 did not differ between the groups (simvastatin 5.4 ± 0.4 and placebo 5.0 ± 0.5 mg/dl) but tended to relate positively to HOMA-IR ($r = 0.479$, $P = 0.032$). After simvastatin treatment, plasma RBP-4 correlated with the change in FFAs ($r = 0.782$, $P = 0.008$) (Fig. 2*B*). IMCLs and HCLs remained unchanged (simvastatin 1.4 ± 0.6, 0.3 ± 0.3, and 11.0 ± 6.5% and placebo 1.7 ± 1.0, 0.4 ± 0.5, and 11.5 ± 8.0%) (Fig. 1). Changes in insulin sensitivity did not relate to muscle and liver lipids. Also, basal EGP and EGP suppression were not affected by treatment (simvastatin 1.7 ± 0.2 $mg \cdot kg^{-1} \cdot min^{-1}$ [72 ± 14%] and placebo 1.5 ± 0.4 $mg \cdot kg^{-1} \cdot min^{-1}$ [74 ± 12%]).

CONCLUSIONS

Effects on serum lipids

High-dose simvastatin treatment reduced LDL cholesterol by ~48% in agreement with the maximum achievable LDL cholesterol reduction. Increases in HDL cholesterol and decreases in fasting triglycerides and FFAs were not observed in our patients with only slight hypertri-

Table 2—*Effects of simvastatin on lipid profiles and glucose metabolism*

	Simvastatin (80 mg/day)	Placebo
A1C (%)	6.7 ± 0.6 (−0.01 ± 0.3)	6.7 ± 0.6 (−0.01 ± 0.4)
HOMA-B	71 ± 31 (6.8 ± 16.6)	67 ± 29 (−1.3 ± 1)
HOMA-IR	2.7 ± 0.6 (−0.03 ± 0.6*)	3.3 ± 1.2† (0.6 ± 0.5)
M ($mg \cdot kg^{-1} \cdot min^{-1}$)	4.7 ± 3.3 (0.6 ± 2.1)	3.8 ± 1.6 (−0.3 ± 2.0)
M-to-*I* ratio ($mg \cdot kg^{-1} \cdot min^{-1}) \cdot (pmol/l)^{-1}$	0.008 ± 0.005 (0.002 ± 0.01)	0.006 ± 0.003 (−0.001 ± 0.008)
Rate of glucose disappearance ($mg \cdot kg^{-1} \cdot min^{-1}$)	5.3 ± 3.1 (0.0 ± 2.9)	4.0 ± 1.3 (−1.2 ± 1.2)
EGP during clamp ($mg \cdot kg^{-1} \cdot min^{-1}$)	0.48 ± 0.32 (0.29 ± 0.95)	0.39 ± 0.33 (−0.01 ± 0.60)
EGP suppression (%)	72 ± 14 (−3 ± 13)	74 ± 12 (4 ± 16)
TGs (mmol/l)	1.5 ± 0.4* (−0.2 ± 0.5*)	2.1 ± 0.8 (0.3 ± 0.4)
FFAs (μmol/l)	392 ± 130* (−111 ± 205)	600 ± 234 (−18 ± 211)
TC (mmol/l)	5.1 ± 1.0†‡ (−2.5 ± 1.8‡)	6.6 ± 0.8 (0.0 ± 0.6)
TG-to-HDL cholesterol ratio	2.7 ± 1.2 (−0.1 ± 1.2)	3.7 ± 1.7 (0.4 ± 0.7)
HDL cholesterol (mmol/l)	1.4 ± 0.3 (2.9 ± 5.9)	1.4 ± 0.3 (−1.8 ± 7.1)
LDL cholesterol (mmol/l)	2.8 ± 0.9†‡ (−2.6 ± 1.6§)	4.2 ± 0.5 (−0.2 ± 0.4)
ALT (units/l)	40 ± 20 (6 ± 16)	29 ± 12 (2 ± 5)
AST (units/l)	31 ± 15 (6 ± 14)	22 ± 6 (1 ± 5)
GGT (units/l)	39 ± 23 (2 ± 15)	36 ± 8 (2 ± 6)
RBP-4 (mg/dl)	5.0 ± 1.1 (−0.4 ± 0.8)	5.8 ± 1.7 (0.7 ± 0.6)

Data are mean ± SD laboratory characteristics of type 2 diabetic patients after treatment with 80 mg/day simvastatin for 8 weeks or application of placebo; changes compared with baseline are given in parentheses. Surrogate parameters of basal β-cell function (HOMA-B) and basal insulin sensitivity (HOMA-IR), total triglycerides (TGs), whole-body glucose disposal (*M*), FFAs, total cholesterol levels (TC), HDL cholesterol and calculated LDL cholesterol, alanine aminotransferase (ALT), aspartate aminotransferase (AST), GGT, and rate of glucose disappearance were determined. *$P < 0.05$ simvastatin versus placebo; †$P < 0.005$ versus baseline; ‡$P < 0.005$, simvastatin versus placebo; §$P < 0.0005$, simvastatin versus placebo.

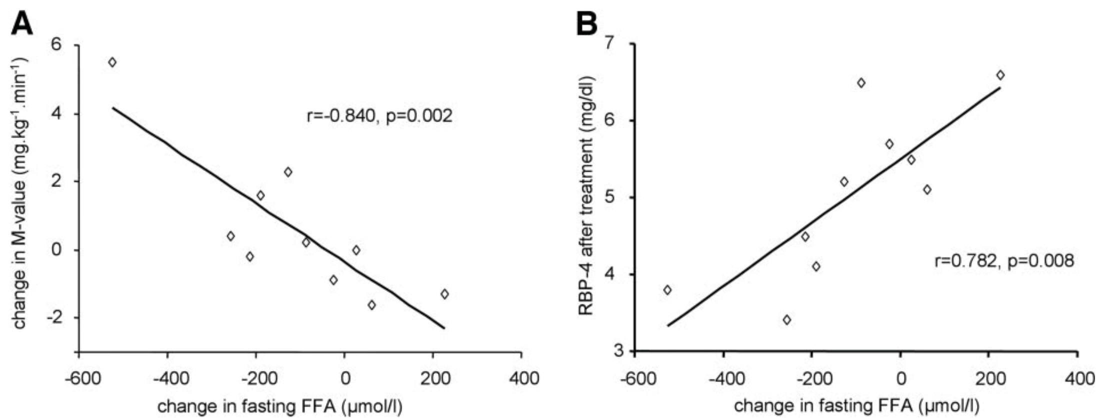

Figure 2— *Correlation of changes in fasting FFAs with the changes in whole-body insulin sensitivity* (M *value)* (A) *and RBP-4* (B) *in patients with type 2 diabetes before and after treatment with 80 mg/day simvastatin (S,* n = *10).*

glyceridemia. Simvastatin might, therefore, exert larger effects on HDL cholesterol and triglycerides in more severe hypertriglyceridemia.

Effects on insulin sensitivity

Simvastatin treatment slightly reduces insulin sensitivity using the quantitative insulin sensitivity check index (17) in line with findings in type 2 diabetes (10). Others reported that simvastatin does not change (18) or increases insulin sensitivity (HOMA-IR) in severely hypertriglyceridemic, hypercholesterolemic patients with type 2 diabetes (9). Only a few studies demonstrated changes in whole-body insulin sensitivity by statin therapy with the use of clamps (10,19). At a dose of 80 mg/day, we found no effect of simvastatin on whole-body insulin sensitivity in nonobese type 2 diabetes with good metabolic control. This finding does not exclude a specific simvastatin effect on hepatic insulin sensitivity. Our patients with type 2 diabetes exhibited marked hepatic insulin resistance indicated by only ~70% EGP suppression. However, simvastatin did not ameliorate EGP suppression in our patients with type 2 diabetes, a result that is in line with the only previous study on pravastatin treatment in familial hypercholesterolemia (20). Statins not only decrease LDL cholesterol but may also interfere with fasting and postprandial triglyceride-rich lipoprotein metabolism, resulting in altered substrate flux and accumulation of HCLs (11,12,21). Our patients exhibited a tight correlation between excessive HCL storage and *M* value similar to that in previous reports (2). Simvastatin did not affect either HCLs or IMCLs in two muscles with different compositions. Also no relationship between changes in insulin sensitivity and ectopic lipids was found.

Effects on parameters influencing insulin sensitivity

According to current paradigms, mechanisms determining insulin sensitivity comprise *1*) circulating FFAs arising from adipocyte lipolysis, lipoprotein secretion, or dietary fat intake, *2*) cytokines from adipose tissue or liver, and *3*) low-grade inflammation. Recently, simvastatin was found to improve FFA composition, fasting lipid fractions, and postprandial plasma triglycerides even in normotriglyceridemic patients (21). In the present study, a reduction in plasma FFAs during the clamp, reflecting insulin-mediated suppression of lipolysis, remained unchanged after therapy. Statins could affect insulin resistance via declining plasma triglycerides in type 2 diabetes. Triglyceride levels were negatively related to *M* at baseline and changes in fasting FFAs were found to induce considerable effects on insulin sensitivity. Accordingly, evidence is accumulating that intracellular long-chain fatty acyl CoA and diacylglycerol inhibit muscular insulin action by stimulating serine phosphorylation of insulin receptor substrate-1 rather than IMCLs (22).

Statins may further affect inflammatory markers (4), which could relate to changed adipocytokines. Circulating RBP-4, produced mainly by adipocytes, is related to whole-body insulin sensitivity and is elevated in insulin-resistant states (23), but its role remains controversial (16). Here we show that serum RBP-4 relates to a surrogate of fasting insulin sensitivity and to changes in plasma FFAs upon simvastatin therapy. Nevertheless, serum RBP-4 did not relate to whole-body insulin sensitivity as assessed from the euglycemic clamp and simvastatin did not affect RBP-4.

Effects on fasting β-cell function

High-dose lipophilic statins may induce unfavorable pleiotropic effects including impairment of insulin secretion (24,25). The proposed mechanism suggests that these statins inhibit the glucose-induced elevation of free [Ca^{2+}] in cytoplasm, thereby diminishing insulin secretion. However, other studies reported increased or unchanged fasting insulin (9,10). We found no changes in either fasting insulin or HOMA-B during simvastatin therapy.

Some limitations of this study need to be considered. First, the number of participants per treatment group is low but was based on a sample size calculation considering that increases of whole-body and hepatic insulin sensitivity by ~20% represent a clinically relevant treatment effect. Second, only patients with untreated hypercholesterinemia in need of cholesterol-lowering drug treatment according to current guidelines were included. Thus, this trial comprised a typical but preselected population, which does not allow extrapolation of the results to normolipidemic type 2 diabetic or nondiabetic populations. Third, the extensive metabolic characterization revealed a high number of parameters assessed so that the level of significance was adjusted to correct for interrelated comparison. Nevertheless, despite the extensive metabolic characterization by gold-standard

techniques, a number of anti-inflammatory and antioxidant mechanisms that potentially affect insulin action were not explored in the present study. As a result, the issue of whether a possible dissociation exists among different pleiotropic effects of statins cannot be completely resolved. Finally, different glucose-lowering drugs were used in both groups and withdrawn before the clamp. However, antidiabetic medication did not have any impact on whole-body and hepatic insulin sensitivity and patients taking thiazolidinediones or insulin were not included in this study.

Thus, this study shows that even high-dose simvastatin treatment that effectively reduces LDL cholesterol does not directly improve either whole-body or hepatic insulin sensitivity or intracellular lipid deposition in near normotriglyceridemic patients with type 2 diabetes.

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No other potential conflicts of interest relevant to this article were reported.

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Article No. 3.

Role of diacylglycerol activation of PKCθ in lipid-induced muscle insulin resistance in humans

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Muscle insulin resistance is a key feature of obesity and type 2 diabetes and is strongly associated with increased intramyocellular lipid content and inflammation. However, the cellular and molecular mechanisms responsible for causing muscle insulin resistance in humans are still unclear. To address this question, we performed serial muscle biopsies in healthy, lean subjects before and during a lipid infusion to induce acute muscle insulin resistance and assessed lipid and inflammatory parameters that have been previously implicated in causing muscle insulin resistance. We found that acute induction of muscle insulin resistance was associated with a transient increase in total and cytosolic diacylglycerol (DAG) content that was temporally associated with protein kinase (PKC)θ activation, increased insulin receptor substrate (IRS)-1 serine 1101 phosphorylation, and inhibition of insulin-stimulated IRS-1 tyrosine phosphorylation and AKT2 phosphorylation. In contrast, there were no associations between insulin resistance and alterations in muscle ceramide, acylcarnitine content, or adipocytokines (interleukin-6, adiponectin, retinol-binding protein 4) or soluble intercellular adhesion molecule-1. Similar associations between muscle DAG content, PKCθ activation, and muscle insulin resistance were observed in healthy insulin-resistant obese subjects and obese type 2 diabetic subjects. Taken together, these data support a key role for DAG activation of PKCθ in the pathogenesis of lipid-induced muscle insulin resistance in obese and type 2 diabetic individuals.

lipotoxicity | insulin signaling

Decreased insulin-stimulated muscle glycogen synthesis, attributable to reduced insulin-stimulated glucose transport activity (1–3), plays a major role in the pathogenesis of type 2 diabetes (T2D) but the cellular and molecular mechanisms responsible for this abnormality remain unknown (4–6).

Increased intramyocllular lipid content is a strong predictor of muscle insulin resistance in sedentary adults and children (7–9). Previous studies in lipid-infused rodents have implicated lipid-induced increases in muscle diacylglycerol (DAG) content, leading to activation of PKCθ and subsequent decreased insulin signaling at the level of insulin receptor substrate (IRS)-1 tyrosine phosphorylation (10, 11). Other studies have implicated increases in muscle ceramide (12) and acylcarnitine content attributable to incomplete fatty acid oxidation as causal factors in muscle insulin resistance (13). Alternatively, inflammation and alterations in plasma and tissue adipocytokines have also been implicated in causing lipid-induced muscle insulin resistance (14). Translating these findings to humans has been problematic and has led to inconsistent results (15, 16). However, these studies did not provide a comprehensive analysis for all of these putative pathways under conditions of acute and chronic insulin resistance. Furthermore, it is possible that study-related differences regarding age and physical activity of the respective control groups could have contributed to the discrepant results.

In this study, we took a comprehensive view of all of the putative mechanisms that have been proposed to cause lipid-induced muscle insulin resistance by measuring these parameters in serial muscle biopsies in healthy, young, lean, sedentary humans before and during a lipid infusion to induce acute muscle insulin resistance. In addition, we also examined these same parameters in healthy insulin resistant obese subjects, as well as in insulin-resistant patients with T2D.

Results

Lipid-Induced Muscle Insulin Resistance. ***Plasma metabolites and hormones.*** Plasma fatty acid (FA) levels were comparable at baseline before glycerol (GLY) and lipid (LIP) infusion (LIP: 0.46 ± 0.07 mmol/L; GLY: 0.41 ± 0.05 mmol/L). Mean plasma FA concentrations increased during LIP (2.30 ± 0.14 mmol/L at 360 min vs. 0 min; $P < 0.001$) and remained unchanged during GLY (0.31 ± 0.06 mmol/L at 360 min vs. 0 min: not significant; LIP vs. GLY at 360 min: $P < 0.001$). Thus, during low-insulin clamps, fasting plasma insulin was replaced and lipolysis was not suppressed, as assessed from steady-state plasma FA levels during GLY. During high-insulin clamps, plasma FA increased during LIP (1.65 ± 0.10 mmol/L; $P < 0.001$: 510 min vs. 0 min) and decreased during GLY (0.10 ± 0.04 mmol/L; $P < 0.01$: 510 min vs. 0 min; $P < 0.001$: GLY vs. LIP at 510 min). Plasma

Significance

Muscle insulin resistance is a major factor in the pathogenesis of type 2 diabetes, but the underlying cellular mechanisms are yet unclear. This study found that muscle diacylglycerol content was temporally associated with protein kinase Cθ activation and impairment of insulin signaling in human skeletal muscle. A similar relationship between muscle diacylglycerol content and protein kinase Cθ activation was observed in insulin-resistant obese and type 2 diabetic individuals. In contrast, we observed no relationship between other putative mediators of muscle insulin resistance including ceramides, acylcarnitines, or circulating adipocytokines. These data support the hypothesis that diacylglycerol activation of protein kinase Cθ and subsequent impairment of insulin signaling plays a major role in the pathogenesis of muscle insulin resistance in humans.

Author contributions: J.S., G.I.S., and M.R. designed research; J.S., T.Y., E.P., C.K., M.M., D.Z., T.J., J.M., C.H., P.N., and M.R. performed research; G.I.S. contributed new reagents/analytic tools; J.S., D.Z., G.I.S., and M.R. analyzed data; and J.S., G.I.S., and M.R. wrote the paper.

The authors declare no conflict of interest.

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triglycerides increased during LIP and remained unchanged during GLY compared with baseline (290 ± 25 vs. 67 ± 13 mg/dL; $P < 0.001$). Plasma insulin concentrations were comparable at baseline (9.5 ± 3.3 vs. 13.3 ± 4.3 pmol/L) and after 4 h of LIP and GLY (9.8 ± 2.9 vs. 11.2 ± 2.6 pmol/L) but increased to 60 ± 2 and 61 ± 4 pmol/L (LIP and GLY) during the high-dose insulin clamp. C-peptide was suppressed to undetectable levels in nearly all subjects within 120 min of the clamp.

Glucose metabolism, energy expenditure, and substrate oxidation. Insulin-stimulated whole-body glucose disposal was 61% lower ($P < 0.001$) during LIP than during GLY (3.8 ± 1.3 vs. 9.7 ± 3.8 $mg \cdot kg^{-1} \cdot min^{-1}$) (Fig. S1). During LIP, glucose disposal was 50% and 63% lower in females and males ($P < 0.005$ and $P < 0.001$ each vs. GLY), respectively, without difference between the sexes. The respiratory quotient (RQ) did not change during LIP (0.79 ± 0.02) but increased during GLY (0.98 ± 0.03; basal vs. clamp; $P < 0.005$). During insulin stimulation, lipid oxidation fell by 32 ± 9% and 78 ± 22% in LIP and GLY ($P < 0.001$ vs. fasting), remaining higher in LIP ($P < 0.01$ vs. GLY). LIP reduced nonoxidative glucose use by 49% ($P < 0.05$ vs. GLY). Total plasma acylcarnitines did not change after 4 h of LIP (10.9 ± 0.9 vs. 12.1 ± 1.5 μM) and decreased by 45% after 4 h of the GLY (9.7 ± 0.8 vs. 5.4 ± 0.4 μM; $P < 0.001$).

Myocellular lipid metabolites. During LIP, total muscle DAG doubled at 2.5 h (287 ± 45 nmol/g; $P < 0.005$ vs. baseline) and decreased at 4 h (215 ± 23 nmol/g; $P < 0.05$ at 2.5 vs. 4 h), remaining higher compared with baseline ($P < 0.005$, baseline vs. 4 h). Total DAG did not change during GLY. During LIP, total membrane DAG rose by 80% at 2.5 h ($P < 0.01$ vs. basal) and remained 40% higher at 4 h compared with baseline ($P < 0.05$, baseline vs. 4 h) (Fig. 1*A*). Total membrane DAG did not change during GLY (Fig. S2*A*). Certain membrane DAG species (C18:1, C16:0; C16:0, C20:4; C18:2, C18:0; C18:1, C18:0; C18:1, C18:1; C18:1, C18:2; C18:2, C18:2; C16:0, C18:2) were increased during LIP (Fig. 2*A*) but remained unchanged during GLY (Fig. S3*A* and Tables S1 and S2). In LIP, total cytosolic DAG content increased by 80% at 2.5 h ($P < 0.01$ vs. basal) (Fig. 1*A*), decreased by 36% at 4 h ($P < 0.05$ vs. 2.5 h), and remained higher compared with baseline ($P < 0.01$). During GLY, total cytosolic DAG did not change (Fig. S2*A*). Certain cytosolic DAG species increased during LIP (C16:0, C20:4; C18:2, C18:0; C18:1, C18:1; C18:1, C18:2; C18:2, C18:2; C16:0, C18:2; Fig. 2*B*) but remained unchanged during GLY (Fig. S3*B* and Tables S1 and S2).

Total ceramides (Fig. 1*A*) and individual ceramide species (Fig. S4 *A* and *B*) did not change during LIP or GLY. Likewise, we observed no changes in myocellular acylcarnitines after 4 h of LIP or GLY (207 ± 27 vs. 181 ± 18 or 133 ± 5 nmol/g; $n = 7$), with a tendency for lower acylcarnitines during GLY ($P = 0.051$: basal vs. 4 h GLY; $P = 0.071$: GLY vs. LIP after 4 h).

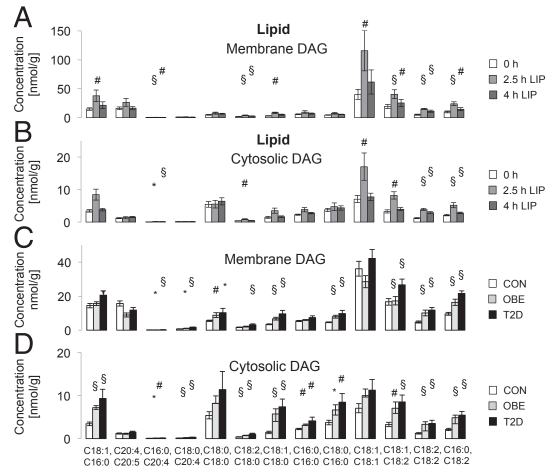

Fig. 2. Concentration of membrane (*A*) and cytosolic (*B*) DAG species in healthy, lean controls (CON) ($n = 16$) at baseline (white columns), after 2.5 h (light gray columns), and after 4 h (dark gray columns) of lipid infusion. (*C* and *D*) Membrane (*C*) and cytosolic (*D*) DAG species concentrations in CON (white columns; $n = 16$), in young obese humans (OBE) (light gray columns; $n = 10$), and in elderly obese patients with T2D (black columns; $n = 10$). Data are given as means ± SEM. *$P < 0.05$; #$P < 0.01$; §$P < 0.001$ (2.5 or 4 h of lipid infusion vs. 0 h and OBE and T2D vs. CON).

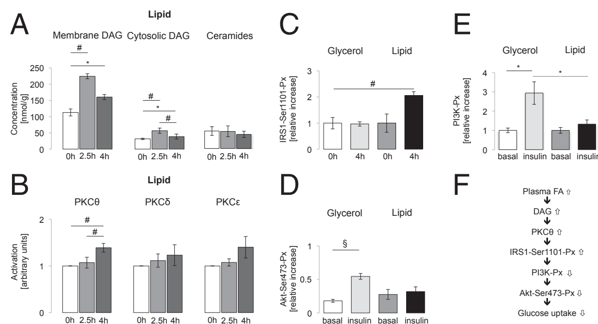

Fig. 1. (*A*) Myocellular DAG concentrations in the membrane and cytosolic fraction and myocellular ceramide concentrations during lipid infusion in young lean healthy controls (CON) ($n = 10$). (*B*) Activation of myocellular PKCθ, -δ, and -ε during lipid infusion in CON ($n = 10$–14). (*C*) Phosphorylation of serine 1101 residue at IRS1-Ser1101-Px at baseline and its relative increase after 4 h glycerol (white and light gray columns) or lipid (dark gray and black columns) infusion in young lean healthy controls (CON) ($n = 7$). (*D* and *E*) PI3K-Px (*E*) and membrane/cytosolic ratio of Akt-Ser473 phosphorylation (Akt-Ser473-Px) (*D*) at baseline and after 4.5 h of glycerol (light gray column) or lipid (black column) infusion during insulin stimulation for 30 min in CON ($n = 7$). Data are given as means ± SEM. *$P < 0.05$; #$P < 0.01$; §$P < 0.001$. (*F*) Increased plasma FAs lead to myocellular accumulation of DAGs and consequent IRS1-Ser1101-Px, impaired PI3K-Px, and blunted insulin stimulation of Akt-Ser473-Px.

Activation of myocellular PKCθ. Translocation of PKCθ from cytosol to plasma membrane, reflecting activation of PKCθ, was not altered at 2.5 h but increased after 4 h of LIP ($P < 0.05$: LIP vs. GLY; $P < 0.005$: basal and 2.5 h vs. 4 h of LIP; Fig. 1*B*). GLY did not affect activation of PKCθ (Fig. S2*B*). Activation of PKC isoforms δ and ε did not occur (Fig. 1*B* and Fig. S2*B*).

Myocellular insulin signaling. Phosphorylation at serine residues 1101 of insulin receptor substrate 1 (IRS1-Ser1101-Px) increased twofold ($P < 0.005$) at 4 h of LIP but did not change during GLY (Fig. 1*C*). Hyperinsulinemia increased the membrane/cytosol ratio of Akt phosphorylation at serine 473 (Akt-Ser473-Px) by threefold during GLY ($P < 0.001$ basal vs. insulin) but not during LIP (Fig. 1*D*). Accordingly, hyperinsulinemia stimulated phosphoinositide-3 kinase phosphorylation (PI3K-Px) by 184% ($P < 0.05$) during GLY but not during LIP (Fig. 1*E*).

Circulating adipokines and adhesion molecules. Plasma concentrations of interleukin (IL)-6 (2.09 ± 1.21 vs. 1.80 ± 1.02 pg/mL), adiponectin (7.7 ± 2.2 vs. 8.2 ± 1.0 μg/mL), retinol-binding protein (RBP)4 (21.3 ± 5.7 vs. 21.6 ± 4.6 μg/mL), and soluble intercellular adhesion molecule (sICAM)-1 (142 ± 26 vs. 153 ± 142 ng/mL) were comparable after 4 h of LIP and GLY.

Plasma and Tissue Parameters in Insulin-Resistant Obese and Insulin-Resistant T2D Subjects. ***Plasma metabolites and hormones.*** Fasting plasma FA concentrations were comparable in lean healthy human subjects (CON) (0.43 ± 0.05 mmol/L), obese subjects

(0.50 ± 0.05 mmol/L), and in T2D patients (0.68 ± 0.06 mmol/L) and decreased during hyperinsulinemic–euglycemic clamps to 0.09 ± 0.02 in obese and to 0.13 ± 0.03 mmol/L in T2D (P < 0.001 vs. baseline). Fasting plasma triglycerides were higher in obese and T2D (112 ± 15 and 172 ± 15 mg/dL) compared with CON (97 ± 7 mg/dL; P < 0.05). During hyperinsulinemic–euglycemic clamps, plasma insulin concentrations were similar in all groups (control: 78 ± 7 pmol/L; obese: 73 ± 5 pmol/L; T2D: 60 ± 5 pmol/L).

Glucose uptake, energy expenditure, and substrate oxidation. Physical activity was comparable between obese, T2D, and CON (Baecke index: 2.6 ± 0.6, 2.5 ± 0.7, 2.4 ± 0.5). Insulin-stimulated glucose disposal was 78% lower in obese (10.6 ± 3.8 vs. 1.6 ± 0.7 $mg \cdot kg^{-1} \cdot min^{-1}$; P < 0.005) and 88% lower in T2D (1.5 ± 1.0 $mg \cdot kg^{-1} \cdot min^{-1}$; P < 0.001) compared with CON (Fig. S5). RQ did not change during the hyperinsulinemic–euglycemic clamps compared with fasting (obese: 0.79 ± 0.02 vs. 0.87 ± 0.04; T2D: 0.71 ± 0.06 vs. 0.88 ± 0.02). At baseline, lipid oxidation was 22% and 36% lower in obese and T2D participants than in CON. During the clamp, nonoxidative glucose utilization was 76% and 41% (P < 0.001) lower compared with CON. Plasma acylcarnitines did not differ between the groups (CON: 10.9 ± 0.8 µM; obese: 8.9 ± 0.9 µM; T2D: 11.3 ± 0.8 µM).

Myocellular lipid metabolites. Total DAGs were 1.5- and 2-fold higher in obese and T2D participants compared with CON (P < 0.001 vs. CON) and did not differ between obese and T2D. Membrane DAGs were not increased in obese but 30% higher in T2D than in CON and obese (P < 0.001) (Fig. 3*A*). Cytosolic DAGs were 85% and 120% greater in obese (P < 0.001 vs. CON) and in T2D (P < 0.005 vs. CON) (Fig. 3*A*). Specific membrane (Fig. 2*C* and Tables S1 and S2) and cytosolic DAG species (Fig. 2*D* and Tables S1 and S2) were increased in obese and T2D patients. Neither total DAG or subcellular fractions differed between female and male participants. Total ceramides (Fig. 3*A*) and ceramide species (Fig. S4*C*) were comparable in all groups. Acylcarnitines did not differ between the groups [CON: 203 ± 25 nmol/g (n = 7); obese: 176 ± 22 nmol/g (n = 10); T2D: 175 ± 22 nmol/g (n = 10)]; however, given the limited number of samples in each group, a type 2 error cannot be excluded.

Correlation analyses of basal biopsy samples of the whole study population revealed that total cytosolic DAG correlated negatively with insulin sensitivity (M value) (r = −0.566; P < 0.001) but positively with body mass index (BMI) (r = 0.509; P < 0.005). After adjustment for BMI, C18:0 and C18:2 (r = −0.441; P < 0.05), C16:0- and C18:2-containing (r = −0.479; P < 0.05) cytosolic DAG negatively correlated with M value. In the membrane fraction, C18:0 and C20:4 (r = −0.416; P < 0.05), C18:0 and C18:2 (r = −0.492; P < 0.05), C18:1 and C18:2 (r = −0.403; P < 0.05), C18:2 and C18:2 (r = −0.410; P < 0.05), as well as C16:0- and C18:2-containing (r = −0.619; P < 0.005) DAG correlated negatively with M value, even after adjustment for BMI. C20:4- and C20:5-containing membrane DAG correlated negatively with BMI and positively with M value. Adjustment for M value abolished the associations of DAG species with BMI. Some cytosolic DAG species [C18:0, C20:4 (r = 0.379; P = 0.025); C18:2, C18:0 (r = 0.417; P = 0.013); C18:1, C18:2 (r = 0.340; P = 0.045); and C16:0, C18:2 (r = 0.348; P = 0.041)] and the following membrane species correlated with age: C18:2, C18:0 (r = 0.480; P = 0.004); C18:2, C18:2 (r = 0.445; P = 0.007); C16:0, C20:4 (r = 0.352; P = 0.038); C18:0, C20:4 (r = 0.352; P = 0.037); C18:1, C18:2 (r = 0.394; P = 0.019); and C16:0, C18:2 (r = 0.0365; P = 0.031). Adjustment for HbA_{1c} and M value abolished these correlations.

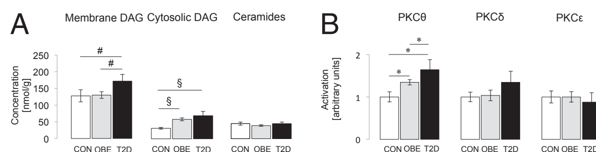

Fig. 3. Myocellular concentration of membrane and cytosolic DAGs and myocellular total ceramide concentrations (*A*) and activation of PKCθ, -δ, and -ε (*B*) in young lean healthy controls (CON) (white columns; n = 16), in young obese humans (OBE) (dark gray columns; n = 10), and in elderly obese patients with T2D (black column; n = 10). Data are given as means ± SEM. *P < 0.05; #P < 0.01; §P < 0.001.

Activation of myocellular PKCθ. Activation of PKCθ was 35% and 64% (P < 0.05 vs. CON) higher in obese and T2D than in CON and 22% (P < 0.05) greater in T2D than in obese. In contrast, there were no differences in activation of PKCδ and PKCε between groups (Fig. 3*B*). PKCθ activation correlated negatively with M value (r = −0.575; P < 0.005) but positively with plasma FA (r = 0.428; P < 0.05), BMI (r = 0.436; P < 0.05), total cytosolic (r = 0.441; P < 0.05), and membrane DAG (r = 0.410; P < 0.05) and with specific DAG species (Table S3). C18:2- or C20:4-containing DAG exhibited the strongest relationships with PKCθ activation and were increased in both the lipid infusion studies, as well as in the insulin-resistant obese subjects and T2D subjects. In contrast to the strong relationship between muscle DAG content, PKCθ activation, and muscle insulin resistance, there was no relationship between muscle insulin resistance and total or any specific ceramide species in skeletal muscle.

Circulating adipokines and adhesion molecules. In obese and T2D, plasma concentrations of IL-6 (1.84 ± 1.02 and 1.70 ± 0.68 pg/mL), RBP4 (29.5 ± 10.3 and 33.2 ± 65.4 µg/mL), and sICAM-1 (191 ± 64 and 245 ± 58 ng/mL) were comparable to CON (1.78 ± 1.16 pg/mL; 21.4 ± 5.3 µg/mL; 145 ± 47 ng/mL). Plasma adiponectin concentrations were slightly lower in obese and T2D [3.70 ± 1.83 and 2.78 ± 0.85 µg/mL; P < 0.05 vs. CON (7.9 ± 1.4 µg/mL)].

Discussion

This study found that increasing plasma FA, by lipid infusion, leads to a transient increase in intramyocellular DAG species (C16:0, C18:0, C18:1, C18:2, and C20:4), which were temporally related to PKCθ activation, increased IRS1-Ser1101-Px, and impairment of insulin-stimulated PI3K activation and Akt-Ser473-Px (Fig. 1*F*). In contrast, lipid-induced muscle insulin resistance in these subjects was not associated with increases in intramuscular ceramide or acylcarnitine content or changes in circulating adipocytokines. Furthermore, muscle insulin resistance in obese and T2D individuals exhibited similarly increased cytosolic DAG content and PKCθ activation and no relationship with muscle ceramide or acylcarnitine content. Similar to the lipid infusion studies, the DAG species containing either C16:0, C18:0, C18:1, C18:2, or C20:4 FA showed the strongest relationship with PKCθ activation and insulin resistance in obese and T2D individuals.

Short-term elevation of plasma FA concentrations similarly reduced M values in both male and female volunteers, which argues against sex-related differences in the metabolic response to an acute lipid infusion (15, 16). The decrease in M values induced by lipid infusion could mostly be attributed to decreased insulin-stimulated rates of nonoxidative glucose metabolism, which is consistent with previous studies demonstrating lipid-induced reductions in insulin-stimulated glucose transport/phosphorylation activity, leading to reductions in muscle glycogen synthesis (3, 7, 17, 18). The early blunting of the increase in glucose-6-phosphate during combined lipid and insulin infusion (1) argues against the putative role of lipid-induced reductions of pyruvated dehydrogenase activity and glucose oxidation as being responsible for the observed insulin resistance (19). It has been postulated that impaired lipid oxidation (20), interference of lipids with insulin-stimulation of ATP synthesis (21), or relative deficiency of glycolytic precursors of the tricarboxylic acid cycle leading to accumulation of acylcarnitines (13) could underlie

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lower glucose uptake. However, plasma and myocellular acylcarnitines were not increased during the lipid infusion or in the insulin-resistant obese and T2D subjects, and mitochondrial ATP synthesis only decreased after induction of muscle insulin resistance (22), indicating that altered oxidative capacity is not a prerequisite for lipid-induced muscle insulin resistance. Overreplacement of insulin is unlikely to underlie differences of acylcarnitines between lipid and glycerol infusion protocols because plasma insulin concentrations were low and similar in both protocols.

Previous studies in rodent models have implicated increases in myocellular lipid metabolites, such as ceramides and DAGs, with subsequent inhibition of insulin signaling as causal factors in the pathogenesis of lipid-induced muscle insulin resistance (5, 6, 10, 11, 23). Studies in humans have been less conclusive because of conflicting results of studies with different experimental protocols, resulting in varying plasma FA concentrations and compositions (15, 16, 24). By performing serial muscle biopsies before and during a lipid infusion, we found that total muscle DAG content increased transiently within 2.5 h and subsequently declined 4 h after starting the lipid infusion. These data are consistent with previous rodent studies demonstrating a transient increase in muscle DAG content during a lipid infusion (11). This transient increase in muscle DAG content may explain the inability of some previous studies that failed to observe an increase in muscle DAG content in relation to muscle insulin resistance during a lipid infusion (25). It is also possible that higher lipid oxidation rates or increased conversion of DAG to triglyceride by increased DAG acyltransferase (DGAT) activity in physically active persons might have prevented a lipid-induced increase in muscle DAG content (26). In this regard, the present study was comprised of only sedentary participants, thereby avoiding possible confounding effects resulting from different levels of physical activity on lipid-induced muscle insulin resistance.

In this study, we infused somatostatin, to inhibit endogenous insulin secretion, and insulin, to maintain fasting plasma insulin concentrations, to avoid any potential confounding effects mediated by lipid-induced endogenous insulin secretion. Nevertheless, even subtle changes of plasma insulin levels can affect endogenous FA release (27). We cannot distinguish between endogenous and exogenous FA but during this short-term lipid-induced muscle insulin resistance; the majority of the FA is provided by the infused lipid. Moreover, DAG did not increase during glycerol infusion experiments; thus, FAs from endogenous sources do not seem to play a major role in our model of short-term muscle insulin resistance. Chronic insulin resistance was examined in fasting muscle biopsies obtained from insulin resistant obese and T2D subjects; thus, endogenous FAs played a prominent role for the increased muscle DAG content in these individuals. Although steady-state plasma FA and insulin concentrations did not change during the glycerol infusion in the low dose hyperinsulinemic–euglycemic clamp study, we cannot rule out that insulin might have been overreplaced in these experiments. This, together with the absence of growth-hormone replacement, might account for lower FA flux rates during the low-dose hyperinsulinemic–euglycemic clamp study with glycerol infusion (28). However, replacement of plasma insulin was the same during glycerol and lipid infusions; thus, any effects of potentially suppressed endogenous flux rates of plasma FA on RQ and M values during the high-insulin clamp are rather unlikely.

This study also reports for the first time to our knowledge the temporal sequence of changes in muscle DAG composition and localization and insulin signaling in humans. Lipid infusion increased mainly DAG containing C18:0, C18:1, or C18:2 FAs and in addition membrane DAG with C16:0 and C20:4 FAs. Although composition of DAG species likely reflects the availability of FAs from circulating sources, we found that C16:0-, C18:1-, C18:2-, and C20:4-containing DAG were also increased in obese and T2D subjects, with C18:2 representing the most abundant residue in DAG species.

The rise in myocellular DAG content during the lipid infusion preceded the membrane translocation of PKCθ, which was observed at 4 h of lipid infusion in the absence of changes in PKCβ, PKCε, and PKCδ activities. These data are consistent with previous studies in rodents that have shown that lipid infusion leads to a transient increase in intramyocellular DAG content, PKCθ activation, and muscle insulin resistance (10, 11). In vitro activation of different nPKC isoforms vary in response to different DAG species with different time courses and competitive effects of specific DAG species (29). This might explain the difference between the present and previous findings of increased activity of membrane-associated PKCβII and PKCδ isoforms but unchanged PKCθ, PKCε, and PKCζ after a 6-h lipid infusion combined with a hyperinsulinemic-euglycemic clamp (15). Furthermore, the present study also found that lipid-induced muscle insulin resistance was associated with increased myocellular IRS1-Ser1101-Px at 4 h of lipid infusion, which was associated with blunted insulin-stimulation of PI3K activation and Akt-Ser473-PX in the membrane/cytosolic fraction. These data are consistent with a previous in vitro study demonstrating that increased IRS1-Ser1101-Px will lead to reduced insulin-stimulated Akt-Ser473-PX (30).

Previous lipid infusion studies in humans provided evidence for impaired IRS1-Tyr-Px and PI3K-Px combined with reduced (31) or unchanged Akt-Px but did not assess lipid metabolites or nPKC activation (32). Other studies failed to show impairment of insulin-stimulated IRS1-Tyr-Px, PI3K-Px IRS1, and Akt-Ser473-Px despite reduction of insulin-stimulated glucose uptake (16, 17). This inconsistency could result from differences in study protocols, such as combined lipid and insulin infusions, duration and dosage of lipid infusion, and timing of muscle biopsies, as well as from individual variation of the study participants (e.g., physical activity, age, and glucose tolerance).

Lipid infusion has also been shown to increase circulating cytokines and in turn induce a proinflammatory status and insulin resistance (33). It has been proposed that saturated FAs can activate the innate immune system and inflammatory pathways via binding to toll-like receptor (TLR)4, which in turn leads to an increase in myocellular ceramide content, which will lead to insulin resistance by direct inhibition of Akt (23). In this study, myocellular concentrations of total, as well as ceramide species, were comparable before and after lipid infusion and not related to the induction of muscle insulin resistance. This is consistent with some (15, 16) but not other studies in humans (34). Although it is possible that the low content of saturated FAs in the lipid infusion used in the current study might explain this discrepancy, a recent study found that saturated fat-induced insulin resistance can occur independently of TLR4 activation and increased in tissue ceramide content (35). Finally, markers of systemic inflammation, IL-6, adiponectin, RBP4, and sICAM-1, which have all been implicated in causing insulin resistance in obese and T2D (14) individuals, remained unchanged during the lipid infusion, thus dissociating these factors from lipid-induced muscle insulin resistance in this study (24).

This study also found that increased muscle DAG content and PKCθ activation was strongly associated with muscle insulin resistance in obese and T2D individuals, independent of dyslipidemia. Enzymes involved in DAG synthesis (adipose triglyceride lipase) are negatively related to insulin sensitivity (36), whereas enzymes controlling DAG hydrolysis (hormone-sensitive lipase) or conversion to triacylglycerols are decreased in obese humans (36). On the other hand, obese individuals with normal DAG content but increased triacylglycerols had higher muscle DGAT expression (37). Thus, individual lipolytic control in skeletal muscle could modulate lipotoxic effects and thereby explain the difference between reports of unchanged (38, 39) or increased

total muscle DAG in obese humans (40). Previous studies found that the degree of DAG saturation associated positively (39), negatively (41), or not (38) with insulin resistance. We found that several cytosolic and membrane DAG species containing C16:0, C18:0, C:18:1, C18:2, and C20:4 were associated with both BMI and M value. After adjustment for BMI, the correlations were more pronounced in the membrane fraction and mainly driven by unsaturated FAs (C:18:1, C18:2, and C20:4) at one or two binding sites. Increased DAG species containing C:18:1, C18:2, and C20:4 were also associated with acute induction of muscle insulin resistance during the lipid infusion. We also found that C20:4, C20:5 membrane DAG species correlated positively with insulin sensitivity, which is consistent with previous findings in fat-fed animals (42). Polyunsaturated FAs of muscle phospholipids, which were not measured in the present study, have previously been shown to positively correlate with insulin sensitivity and have been suggested to increase membrane fluidity and increased movement of lipid drafts and insulin signaling mediators (43).

This study also addressed alternative mechanisms that have been proposed to cause muscle insulin resistance. We found no relationship between myocellular acylcarnitine concentrations and muscle insulin resistance during the lipid infusion or in insulin-resistant obese and T2D subjects, thus dissociating acylcarnitines from muscle insulin resistance under these conditions. Plasma acylcarnitines can be increased in insulin resistant humans, specifically during hyperglycemia, which has been attributed to intracellular deficiency of glycolytic precursors needed for complete lipid oxidation (44). The participants in the current study were nondiabetic or metabolically well-controlled T2D, which might explain the normal plasma acylcarnitines. Finally, we found no differences in plasma concentrations of IL-6, RBP4, and sICAM-1 in our obese and T2D subjects, which is in accordance with some (45) but not all (46) studies. Only plasma adiponectin concentrations were slightly lower in obese and T2D subjects compared with control subjects.

In summary, by performing sequential biopsies during a lipid infusion, we were able to delineate the temporal sequence of events responsible for acute lipid-induced insulin resistance in human skeletal muscle. We found that raising plasma fatty acid concentrations led to a transient increase in myocellular DAG content (mostly C18:0, C18:1, or C18:2 species) at ∼2.5 h, which was temporally related to PKCθ activation, increased IRS1-Ser1101-Px, and decreased insulin activation of AKT2. We found a similar strong relationship between muscle DAG content and PKCθ activation with muscle insulin resistance in obese and T2D subjects. In contrast, we observed no relationship between myocellular acylcarnitine content or ceramide content, thus dissociating these metabolites from muscle insulin resistance under these conditions. Taken together, these data support the hypothesis that DAG activation of PKCθ plays an important role in mediating lipid-induced insulin resistance in human skeletal muscle and identifies this pathway as a relevant therapeutic target to prevent and reverse muscle insulin resistance in humans.

Experimental Procedures

Participants gave written informed consent before inclusion, which was registered (Clinicaltrials.gov Identifier no. NCT01229059) and approved by the local institutional ethics board of Heinrich Heine University. They underwent a screening procedure including clinical examination, and a standardized 75-g oral glucose tolerance test. We included the following: (*i*) young (20–40 y) lean (BMI of 20–25 kg/m^2) glucose-tolerant subjects without family history of diabetes; (*ii*) age-matched glucose-tolerant insulin-resistant obese subjects; and (*iii*) obese subjects with T2D (Table S4). Exclusion criteria were smoking, medication affecting insulin sensitivity, lipid metabolism or immune system (acute or common) diseases, and a history of cancer. Female subjects were studied between days 5–9 of their menstrual cycle. For 3 d before every study day, participants refrained from physical exercise, consumed isocaloric diet, and fasted for 12 h before the start of the experiments. Subjects with T2D were on treatment with metformin (n = 8) or sitagliptin (n = 1). Glucose-lowering medication was withdrawn for 1 wk before the study.

Lipid-Induced Muscle Insulin-Resistance Study. The protocol is depicted in Fig. S6. Healthy young lean participants underwent two experimental conditions in random order. A pancreatic clamp was performed (−240 to 0 min) with continuous infusion of somatostatin (0.1 μg·kg^{-1}·min^{-1}; UCB Pharma) to inhibit insulin secretion. Insulin (Actrapid; Novo Nordisc) was applied to standardize fasting insulin levels (6 mU·m^{-2} body surface per minute, −240 to 0 min) and combined with infusion of D-[6,6-$^2H_2$]glucose (−360 to +150 min) as previously described (22). Intravenous infusion of lipids (lipid infusion: n = 36; 20% (vol/vol) Intralipid; Fresenius Kabi) was administered (−240 to −230 min: 10 mL/h; −229 to +150 min: 90 mL/h) to raise plasma FAs. On another occasion spaced by 4- to 8-wk intervals, 2.5% (wt/vol) glycerol dissolved in 0.9% saline (glycerol infusion: n = 24; Fresenius Kabi) was infused (−240 to −230 min: 10 mL/h; −229 to +150 min: 90 mL/h).

Obese and T2D Subject Studies. The design is depicted in Fig. S7. Common insulin resistance was investigated in insulin resistant obese (n = 10) and patients with T2D (n = 10) without severe dyslipidemia (triglyceride levels >300 mg/dL). Insulin resistance was defined by oral glucose insulin sensitivity values below 462.8 mL·min^{-1}·m^{-2}, as previously reported (47). All participants underwent a high-insulin euglycemic clamp (0–150 min) combined with infusion of D-[6,6-$^2H_2$]glucose (−180 to +150 min).

Hyperinsulinemic–Euglycemic Clamp Studies. A primed-continuous intravenous infusion of D-[6,6-$^2H_2$]glucose (98% enriched; Cambridge Isotope Laboratories) at (3.6 mg·kg^{-1} body weight) × (fasting glucose [mg/dL])/90 [mg/dL] for 5 min and at 0.036 mg·min^{-1}·kg^{-1} body weight was started before the start of the high-insulin clamp (40 mU·m^{-2} body surface per minute, 0 to +150 min) during the studies on lipid-induced insulin resistance (−360 min) and 180 min before the start of the high-insulin euglycemic clamp in all studies (−180) until the end of the experiments (+150 min).

Indirect Calorimetry. Indirect calorimetry was performed using V_{max} Encore 29n (CareFusion) at baseline (−330 to −300 min) and during steady-state high-insulin clamp conditions (120 to 150 min). Physical activity index was assessed according to Baecke et al. (48).

Metabolites and Hormones. Blood samples were chilled and centrifuged, and supernatants were stored at −20 °C. Blood glucose was measured using the glucose oxidase method (EKF biosen C-Line glucose analyzer; EKF Diagnostic). Serum triglycerides were analyzed by enzymatic assays on a Hitachi analyzer (Roche Diagnostics) and free FAs with the microfluorimetric method [intraassay coefficient of variation (CV), <1%; interassay CV, 2.4%; Wako] after prevention of lipolysis using orlistat (21). High-molecular-weight adiponectin was determined using the Adiponectin (Multimeric) ELISA kit from ALPCO Diagnostics. Intra- and interassay CVs were 5.1% and 8.2%. Plasma concentrations of TNFα and IL-6 were determined by Quantikine HS (TNFα and IL-6) and Quantikine (IL-1ra) ELISA kits from R&D Systems, as described (49). For assessment of acylcarnitines, 50 μL of plasma or ∼200 mg of tissue was homogenized and centrifuged. The supernatant was passed through a reversed-phase cartridge (Sep-Pak C18; 500 mg; Waters), and the eluate was concentrated to 100 μL and injected into an API 6500 Qtrap (ABSCIEX). The limit of detection is 1×10^{-6} nmol acetylcarnitine injection, corresponding to 3 pmol/g in tissue and 4.5×10^{-4} pmol/μL in plasma, with a CV of 22%. The CV is 12% at 1×10^{-5} nmol and 0.3% at 1×10^{-4} nmol acetylcarnitine injections.

Skeletal Muscle Biopsy Samples. All study participants had muscle biopsies at baseline. Twenty-three of the CON underwent biopsies at baseline and during LIP after 2.5 and 4 h, 11 of those also during glycerol infusion. Thirteen CON had muscle biopsies at baseline and after 4.5 h of lipid and glycerol infusion after 30 min of the high-insulin clamp to examine insulin signaling during short-term insulin resistance. The region above the musculus vastus lateralis was anesthetized with local anesthetics (Xylocain 2%) (24), a muscle biopsy of ∼70–400 mg was obtained using a Bergstrom needle. Samples were immediately stored in liquid nitrogen and then frozen at −80 °C.

Muscle tissue was homogenized in a buffer solution (20 mM Tris·HCl, 1 mM EDTA, 0.25 mM EGTA, 250 mM sucrose, 2 mM PMSF) containing a protease inhibitor mixture (Roche), and samples were centrifuged at 100,000 × g for 1 h. The supernatants containing the cytosolic fraction and the pellet

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consisting of the membrane fraction were collected. DAG and ceramide concentration were measured as previously described (50).

Membrane translocation for PKC isoforms was assessed as described (51). PKC translocation was expressed as ratio of membrane over cytosol bands on the same film.

For phosphorylation of IRS1-Ser1101, the membrane was probed with anti phospho IRS1-Ser1101 first and then stripped and probed again with antibodies for total IRS-1 (Cell Signaling). The membrane and cytosolic fractions were prepared using differential centrifugation to assess Akt-Ser473-Px (Cell Signaling). IRS1-associated PI3K activity was determined in muscle extracts after immunoprecipitation with IRS1 antibody/agarose conjugate overnight at 4 °C.

Calculations and Statistics. All statistical analyses were performed using SPSS 6.0 software (SPSS). Data are presented as means ± SD throughout the text and as means ± SEM in the figures. Statistical comparisons between study groups were performed using ANOVA and repeated-measurements ANOVA with Tukey post hoc testing when appropriate. Within-group differences were determined using two-tailed Student *t* tests. Differences were considered significant at the 5% level.

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Supporting Information

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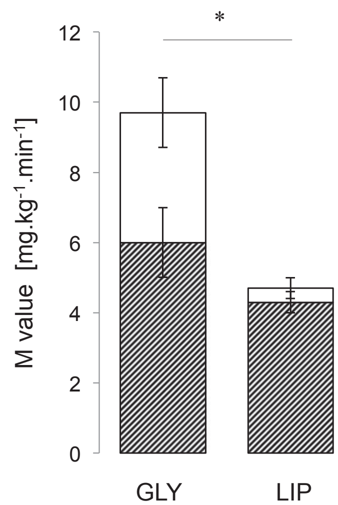

Fig. S1. Whole-body glucose disposal in lean healthy young humans after 4 h glycerol (GLY) or lipid (LIP) infusion (n = 23), glucose oxidation (striped), and lipid oxidation (plane). M value was reduced during lipid infusion ($P < 10.10^{-7}$). Data are given as means ± SEM.

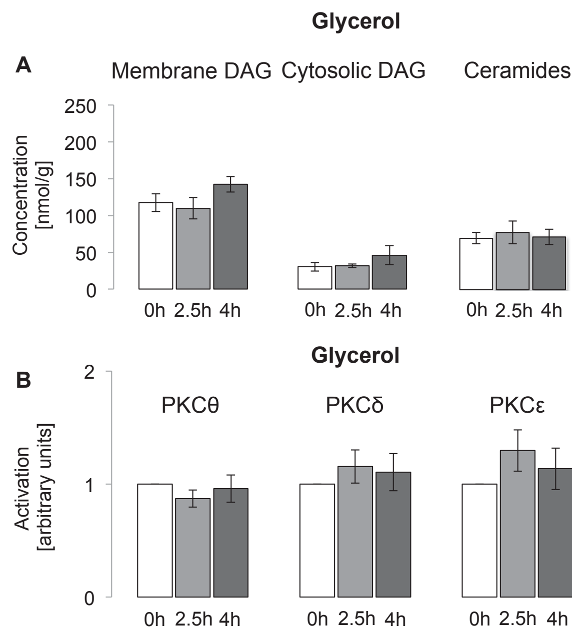

Fig. S2. (*A*) Myocellular diacylglycerol (DAG) concentrations in the membrane and cytosolic fraction and ceramide concentrations during glycerol infusion in young lean healthy controls (CON) (n = 10). Myocellular ceramides did not change during lipid infusion. (*B*) Activation of myocellular PKCθ, PKCδ, and PKCε during glycerol infusion in young lean healthy controls (n = 10–14). Data are given as means ± SEM.

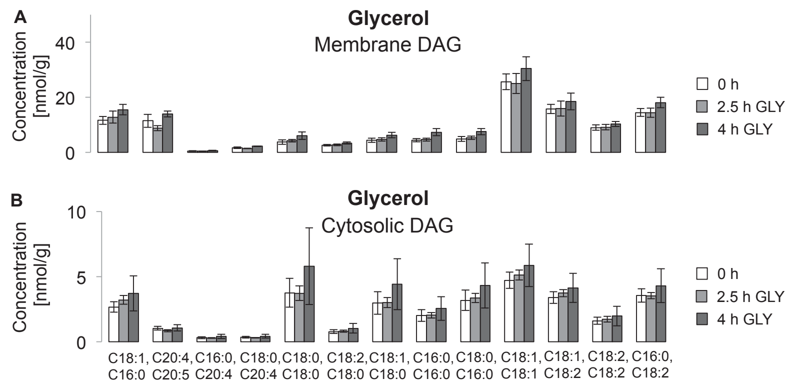

Fig. S3. Membrane (*A*) and cytosolic (*B*) fractions of myocellular DAG species concentrations in young lean healthy humans (CON) ($n = 10$) at baseline (white columns) and after 2.5 h (light gray columns) and after 4 h (dark gray columns) of glycerol infusion.

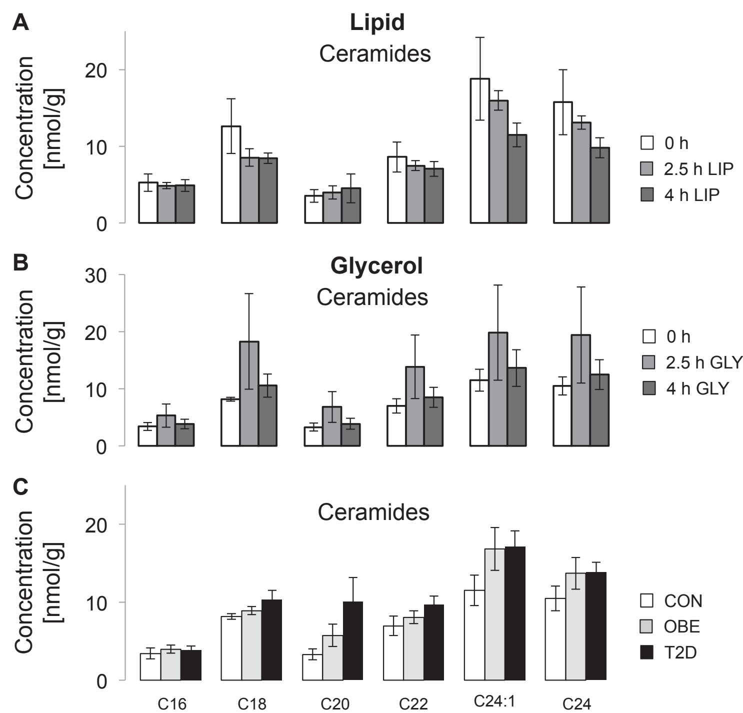

Fig. S4. (*A* and *B*) Ceramide species in young lean healthy humans (CON) ($n = 7$) at baseline (white columns) and after 2.5 h (middle gray columns) and after 4 h (dark gray columns) of lipid (*A*) or glycerol (*B*) infusion. (*C*) Ceramide species concentrations in CON (white columns; $n = 7$), in young obese humans (OBE) (light gray columns; $n = 10$), and in elderly obese patients with type 2 diabetes (T2D) (black columns; $n = 10$). Data are given as means ± SEM.

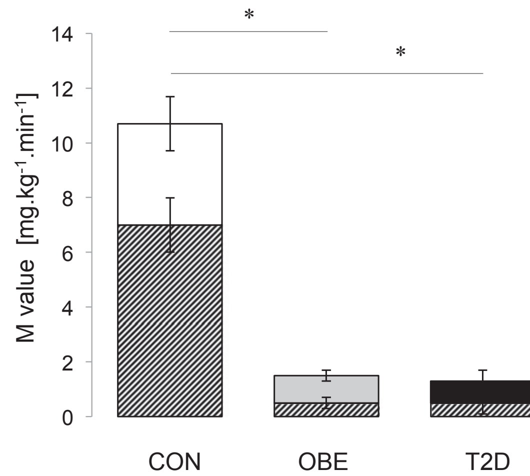

Fig. S5. Whole-body glucose disposal in lean healthy young humans (CON) ($n = 36$), in young obese humans (OB) (gray columns; $n = 10$), and in elderly obese patients with T2D (black columns; $n = 10$) (glucose oxidation, striped; lipid oxidation, plane). Compared with healthy humans, glucose disposal was reduced by 78% in OB ($P < 0.005$) and by 88% in T2D ($P < 0.001$). Data are given as means ± SEM.

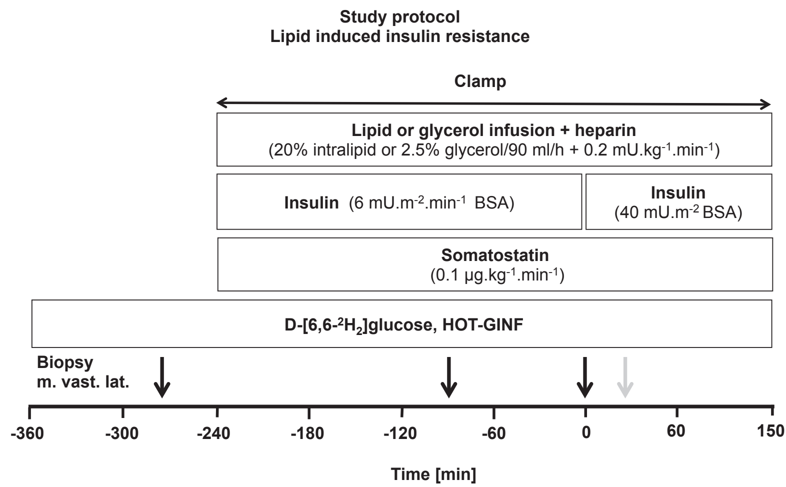

Fig. S6. Study protocol for short-term insulin resistance.

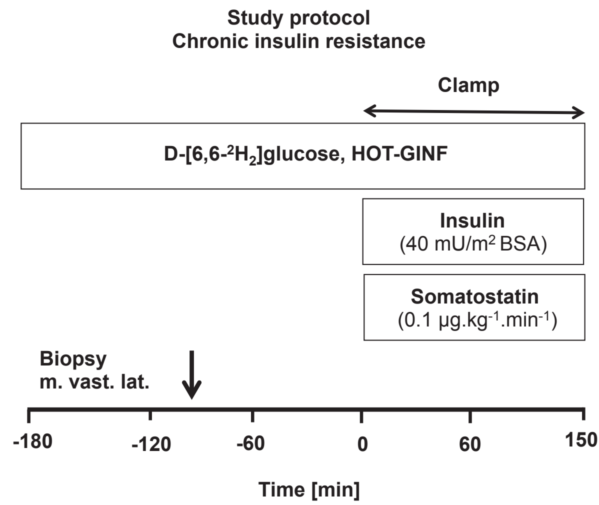

Fig. S7. Study protocol for common insulin resistance.

Table S1. Myocellular DAG species in short-term and common insulin resistance

Study group and condition	C18:1, C16:0			C20:4, C20:5			C16:0, C20:4			C18:0, C20:4			C18:0, C18:0			C18:2, C18:0			C18:1, C18:0		
	Basal	2.5 h	4 h	Basal	2.5 h	4 h	Basal	2.5 h	4 h	Basal	2.5 h	4 h	Basal	2.5 h	4 h	Basal	2.5 h	4 h	Basal	2.5 h	4 h
CON																					
Glycerol																					
Cytosol, nmol/g																					
Mean	2.67	3.21	3.71	1.04	0.85	1.06	0.31	0.29	0.41	0.35	0.31	0.43	3.76	3.73	5.81	0.77	0.81	1.04	2.98	3.00	4.41
SD	0.99	0.74	3.04	0.33	0.19	0.55	0.17	0.05	0.36	0.17	0.05	0.34	2.72	1.28	6.59	0.37	0.17	0.85	2.13	0.86	4.38
Membrane, nmol/g																					
Mean	11.64	12.82	15.56	11.49	8.77	13.98	0.48	0.46	0.69	1.73	1.45	2.24	3.78	4.24	6.11	2.62	2.72	3.42	4.38	4.74	6.32
SD	3.18	4.30	4.28	5.34	1.78	2.42	0.20	0.11	0.31	0.64	0.19	0.23	1.83	0.93	3.09	0.58	0.62	0.81	1.70	1.26	2.19
Lipid																					
Cytosol, nmol/g																					
Mean	3.15	8.24	3.96	1.44	1.59	1.64	0.07	0.11	0.11	0.12	0.14	0.12	5.17	5.92	4.24	0.37	0.74	0.45	1.32	3.47	1.48
SD	1.27	5.59	1.90	0.74	1.12	1.14	0.04	0.04	0.06	0.06	0.06	0.04	4.29	5.18	2.17	0.18	0.27	0.32	1.39	3.78	1.16
Membrane, nmol/g																					
Mean	14.48	27.64	19.70	15.74	20.07	17.78	0.18	0.35	0.29	0.78	1.16	0.94	5.55	6.98	6.66	1.75	3.34	2.60	3.48	6.37	4.91
SD	6.46	18.19	17.63	7.48	12.86	7.65	0.08	0.11	0.17	0.38	0.31	0.49	1.83	3.37	2.02	0.78	1.05	1.27	1.41	2.68	3.77
OBE																					
Cytosol, nmol/g																					
Mean	7.21			1.15			0.12			0.21			8.18			0.70			5.76		
SD	1.38			0.44			0.04			0.05			5.61			0.28			3.76		
Membrane, nmol/g																					
Mean	15.66			9.01			0.24			1.12			8.86			2.12			6.85		
SD	3.51			3.84			0.07			0.41			4.57			0.80			2.71		
T2D																					
Cytosol, nmol/g																					
Mean	9.35			1.51			0.15			0.29			11.41			1.04			7.40		
SD	7.13			0.67			0.07			0.16			14.01			0.65			6.06		
Membrane, nmol/g																					
Mean	20.68			11.78			0.30			1.67			10.41			3.28			9.65		
SD	8.15			5.15			0.10			0.90			7.98			1.56			6.84		

Table S2. Myocellular DAG species in short-term and common insulin resistance

	C16:0, C16:0			C18:0, C16:0			C18:1, C18:1			C18:1, C18:2			C18:2, C18:2			C16:0, C18:2		
	Basal	2.5 h	4 h	Basal	2.5 h	4 h	Basal	2.5 h	4 h	Basal	2.5 h	4 h	Basal	2.5 h	4 h	Basal	2.5 h	4 h
CON																		
Glycerol																		
Cytosol, nmol/g																		
Mean	2.01	2.04	2.55	3.18	3.37	4.32	4.71	5.13	5.86	3.39	3.73	4.13	1.61	1.73	1.97	3.55	3.54	4.30
SD	1.09	0.48	2.03	1.91	0.80	3.86	1.53	0.85	3.63	1.09	0.64	2.48	0.67	0.48	1.67	1.27	0.52	2.91
Membrane, nmol/g																		
Mean	4.47	4.68	7.37	4.94	5.32	7.59	25.63	25.03	30.43	15.76	15.95	18.54	9.03	9.20	10.29	14.49	14.41	18.11
SD	1.34	1.13	2.87	2.00	1.15	2.57	6.30	7.23	9.76	3.66	5.57	6.83	2.26	2.09	2.05	3.09	3.42	4.30
Lipid																		
Cytosol, nmol/g																		
Mean	2.15	3.42	2.36	3.51	4.87	2.93	6.64	16.30	8.79	3.00	6.45	4.26	1.18	3.03	3.15	1.92	3.99	2.91
SD	0.70	1.99	0.66	2.43	5.03	1.29	3.55	12.95	6.21	1.26	2.66	2.23	0.53	0.51	1.81	0.75	1.77	0.91
Membrane, nmol/g																		
Mean	5.39	7.91	6.60	4.75	6.24	6.10	36.15	79.51	48.21	16.69	32.24	22.40	4.80	13.02	10.33	9.64	19.66	13.72
SD	1.78	4.02	3.77	1.32	2.35	2.65	21.37	61.92	42.50	9.62	13.91	13.40	2.84	4.33	5.32	4.35	6.60	6.96
OBE																		
Cytosol, nmol/g																		
Mean	3.11			6.66			9.97			7.08			3.33			4.86		
SD	0.97			3.88			4.85			4.50			1.97			1.84		
Membrane, nmol/g																		
Mean	6.04			7.93			28.45			17.32			10.13			16.38		
SD	1.01			2.57			11.20			7.78			5.34			5.64		
T2D																		
Cytosol, nmol/g																		
Mean	4.14			8.45			11.28			8.51			3.51			5.44		
SD	2.94			6.63			8.15			5.35			2.56			2.91		
Membrane, nmol/g																		
Mean	7.40			9.77			42.18			26.67			11.90			21.56		
SD	3.63			6.54			17.14			11.25			4.82			5.25		

Table S3. Myocellular DAG species correlate with PKCθ activation

DAG species	Cytosolic fraction *r*	Cytosolic fraction *P*	Membrane fraction *r*	Membrane fraction *P*
C16:0, C20:4	0.521	<0.005	0.447	<0.05
C18:0, C20:4	0.461	<0.01	0.362	<0.05
C18:2, C18:0	0.581	<0.005	0.428	<0.05
C18:1, C18:2	0.552	<0.005	0.417	<0.05
C18:2, C18:2	0.591	<0.005	0.532	<0.005
C16:0, C18:2	0.570	<0.005	0.529	<0.005

Pearson correlation coefficients (*r*) between myocellular DAG species and PKCθ activation across lean healthy humans ($n = 11$), young insulin-resistant obese humans ($n = 10$) and elderly obese humans with T2D ($n = 10$).

Table S4. Anthropometric data of study participants

	CON	OBE	T2D
n (male/female)	36 (24/12)	10 (5/5)	10 (5/5)
Age, y	28 ± 1	29 ± 2	59 ± 3*
Body mass index, kg/m^2	22.4 ± 0.4†	41.4 ± 1.9	35.6 ± 1.2
Fasting glucose, mg/dL	86 ± 3	77 ± 3	131 ± 17*
Fasting free FA, mmol/L	0.52 ± 0.13	0.50 ± 0.18	0.76 ± 0.15
Fasting triglycerides, mg/dL	106 ± 13	137 ± 24	188 ± 20*
Hemoglobin A_{1c}, mmol/mol	33 ± 0	34 ± 1	54 ± 5*

Data are given as means ± SEM. FA, fatty acids.

*$P < 0.05$ vs. lean healthy controls (CON) and insulin resistant OBE.

†$P < 0.05$ vs. insulin resistant obese and patients with T2D.

Article No. 4.

Muscle Mitochondrial ATP Synthesis and Glucose Transport/Phosphorylation in Type 2 Diabetes

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ABSTRACT

Background

Muscular insulin resistance is frequently characterized by blunted increases in glucose-6-phosphate (G-6-P) reflecting impaired glucose transport/phosphorylation. These abnormalities likely relate to excessive intramyocellular lipids and mitochondrial dysfunction. We hypothesized that alterations in insulin action and mitochondrial function should be present even in nonobese patients with well-controlled type 2 diabetes mellitus (T2DM).

Methods and Findings

We measured G-6-P, ATP synthetic flux (i.e., synthesis) and lipid contents of skeletal muscle with $^{31}P/^{1}H$ magnetic resonance spectroscopy in ten patients with T2DM and in two control groups: ten sex-, age-, and body mass-matched elderly people; and 11 younger healthy individuals. Although insulin sensitivity was lower in patients with T2DM, muscle lipid contents were comparable and hyperinsulinemia increased G-6-P by 50% (95% confidence interval [CI] 39%–99%) in all groups. Patients with diabetes had 27% lower fasting ATP synthetic flux compared to younger controls ($p = 0.031$). Insulin stimulation increased ATP synthetic flux only in controls (younger: 26%, 95% CI 13%–42%; older: 11%, 95% CI 2%–25%), but failed to increase even during hyperglycemic hyperinsulinemia in patients with T2DM. Fasting free fatty acids and waist-to-hip ratios explained 44% of basal ATP synthetic flux. Insulin sensitivity explained 30% of insulin-stimulated ATP synthetic flux.

Conclusions

Patients with well-controlled T2DM feature slightly lower flux through muscle ATP synthesis, which occurs independently of glucose transport /phosphorylation and lipid deposition but is determined by lipid availability and insulin sensitivity. Furthermore, the reduction in insulin-stimulated glucose disposal despite normal glucose transport/phosphorylation suggests further abnormalities mainly in glycogen synthesis in these patients.

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Abbreviations: bfATP, basal ATP synthetic flux; BMI, body mass index; CI, confidence interval; CONo, age, sex, and BMI-matched control volunteer(s); CONy, young healthy control volunteer(s); EGP, endogenous glucose production; ΔEGP, suppression of EGP; FFA, free fatty acid; G-6-P, glucose-6-phosphate; HCL, hepatocellular lipids; ifATP, insulin-stimulated ATP synthetic flux; IMCL, intramyocellular lipids; M, insulin-stimulated whole-body glucose disposal; MRS, magnetic resonance spectroscopy; Pi, inorganic phosphate; SD, standard deviation; T2DM, type 2 diabetes mellitus

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Introduction

Skeletal muscle insulin resistance is characteristic in the elderly as well as in persons at increased risk of type 2 diabetes mellitus (T2DM) and those with overt T2DM. In these groups, the content of intramyocellular lipids (IMCL) is frequently increased and related to insulin resistance [1,2]. However, this relationship disappears during exercise training, which increases both IMCL and insulin sensitivity in parallel [3,4]. Rather than IMCL, intracellular metabolites of free fatty acids (FFAs), such as long-chain fatty acyl coenzyme A and diacylglycerol, inhibit insulin action by stimulating phosphorylation of serine residues of insulin receptor substrate-1 (IRS-1) [5]. This suggests that IMCL do not directly contribute to insulin resistance but accumulate as a consequence of increased lipid availability from augmented lipolysis and excess dietary fat supply and/or of impaired mitochondrial lipid oxidation [6].

In severe obesity and moderately controlled T2DM, insulin resistance has been linked to abnormal mitochondrial function of skeletal muscle as assessed by in vitro and ex vivo examination in biopsies [7–9]. Application of magnetic resonance spectroscopy (MRS) made it possible to non-invasively examine myocellular mitochondrial function by measuring rates of ATP synthesis ("synthetic flux") in humans [10]. Insulin resistant elderly people and first-degree relatives of patients with T2DM have impaired muscle ATP production and elevated IMCL [11,12]. These studies raised the questions of (i) whether lipid accumulation in skeletal muscle and/or insulin resistance promotes mitochondrial dysfunction and, if so, (ii) whether insulin stimulation can overcome such alterations. In this context, we recently showed that short-term elevation of plasma FFAs profoundly inhibits insulin-stimulated glucose transport/phosphorylation and mitochondrial function, reflected by diminished increase in intramyocellular glucose-6-phosphate (G-6-P) and ATP synthesis, independently of ectopic muscle fat contents [13].

Thus, this study examines relationships between basal and insulin-stimulated mitochondrial function, glucose disposal, and ectopic lipids in patients with T2DM and healthy controls. We employed multinuclear MRS to quantify ATP synthetic flux, intracellular phosphorus metabolites (G-6-P and inorganic phosphate [P_i]), IMCL, and hepatocellular lipids (HCL) in combination with the stable isotope dilution technique to assess glucose fluxes during normoglycemic– and hyperglycemic–hyperinsulinemic clamps.

Methods

Volunteers

Thirty-one volunteers were included in this study: (i) patients with metabolically well-controlled T2DM, (ii) sex-, age-, body mass index (BMI)-matched older volunteers without diabetes (CONo) (age: 95% CI 52–63 y), and (iii) younger individuals without diabetes (CONy) (age: 95% CI 25–28 y). Their anthropometrical and laboratory characteristics are summarized in Table 1. All participants were recruited by means of public advertisement and underwent a complete medical history, clinical examination, and lab tests to exclude cardiovascular, hepatic, renal, and thyroid diseases. Patients with diabetes on insulin treatment or presenting with islet cell antibodies and diabetes-related complications were excluded. Five patients with T2DM had a confirmed family history of diabetes. The individuals in the control groups (CON) had neither a family history of diabetes nor were taking any medication on a regular basis. Normal glucose metabolism was confirmed by a standard oral 75 g glucose tolerance test. None of the participants performed intense exercise on a regular basis. Physical activity was assessed with a self-administered questionnaire of habitual physical activity [14]. This questionnaire discriminates between three features of physical activity: (i) physical activity at work (ii) sport during leisure time and (iii) physical activity during leisure time excluding sports. Average physical activity was calculated as the mean value across these features. The protocol was approved by the local institutional ethics board, and informed consent was obtained from each volunteer after explanation of the purpose, nature, and potential complications of the study.

Experimental Protocol

All participants were on an isocaloric diet, refrained from any physical exercise for 3 d prior to the study day, and fasted for 12 h before the start of the experiment. Glucose-lowering medication was withdrawn for at least 3 d before the experiment. All studies were started at 6:30 a.m. with the insertion of catheters (Vasofix; Braun, http://www.bbraun.com/index.cfm) in the antecubital veins of both arms for blood sampling and infusions. A primed-continuous infusion for 5 min ([3.6 mg fasting glucose (mg/dl)/90 (mg/dl)]/[min $\times$ kg body weight]); and for 480 min (0.036 mg/[min $\times$ kg body weight]) of D-[6,6-$^2H_2$]glucose (98% enriched; Cambridge Isotope Laboratories, http://www.isotope.com/cil/index.cfm) was performed until the end of the clamp test to determine endogenous glucose production (EGP). At 7:00 a.m., all participants were transferred to the Magnetic Resonance Unit to measure IMCL, G-6-P, and flux through ATP synthase in calf muscle using $^1H/^{31}P$ MRS. After basal measurements, the clamp test was started at 10:00 a.m. to create conditions of normoglycemia (approximately 5.5 mmol/l glucose) and hyperinsulinemia (approximately 500 pmol/l insulin). Insulin (Actrapid; Novo Nordisk, http://www.novonordisk.com/) was administered as a primed-continuous infusion (40 mU/[m^2 body surface area $\times$ min]) from 0 to 360 min. A 20% dextrose infusion labeled with D-[6,6-$^2H_2$]glucose (2% enriched) according to the hot glucose infusion (hot GINF) protocol was periodically adjusted to maintain normoglycemia [15]. Whole body insulin sensitivity was assessed from whole-body glucose disposal (M) during the last 30 min of the clamp. ^{31}P MRS measurements (G-6-P, P_i, ATP synthesis) were repeated between 120 min and 240 min. Four of the patients with T2DM (three males and one female, mean values $\pm$ SD: age, 62 $\pm$ 2 y, BMI, 27.5 $\pm$ 3 kg/m^2; M, 4.8 $\pm$ 2.5 mg/(min $\times$ kg body weight) underwent an additional hyperglycemic (approximately 9.5 mmol/l glucose) –hyperinsulinemic (approximately 500 pmol/l insulin) clamp test on a separate day to examine the combined effect of insulin and glucose on G-6-P and ATP synthesis.

Magnetic Resonance Spectroscopy

Measurements were performed on participants lying supine inside a 3-T spectrometer (Bruker, http://www.bruker.com/) using a 10 cm circular double resonant surface coil for ^1H and ^{31}P measurements, positioned approximately

Table 1. Clinical Characteristics of Volunteers

Variable	T2DM	CONo	CONy
n (female/male)	5/5	5/5	2/9
Age (y)	59 ± 6 (44–66)[a]	57 ± 6 (46–65)[a]	27 ± 2 (21–28)
BMI (kg/m^2)	27 ± 3 (22–32)[b]	26 ± 3 (20–30)	23 ± 2 (20–26)
Waist-to-hip ratio	0.95 ± 0.19 (0.83–1.03)[b]	0.90 ± 0.1 (0.71–1.01)	0.81 ± 0.1 (0.69–0.87)
Physical activity index	2.7 ± 0.4 (2.5–3.1)	2.9 ± 0.4 (2.8–3.8)	2.8 ± 0.3 (2.4–3.3)
PW	2.7 ± 0.7 (1.6–3.8)	2.8 ± 0.6 (2.4–3.8)	2.3 ± 0.5 (1.3–3.0)
PS	2.9 ± 0.7 (2.1–4.0)	3.0 ± 0.9 (1.8–4.3)	2.8 ± 0.9 (2.0–4.3)
PL	2.6 ± 0.6 (1.9–3.8)	2.8 ± 0.7 (1.8–3.8)	3.2 ± 0.5 (2.5–4.0)
HbA1c (%)	6.9 ± 0.7 (6.0–7.9)[a,c]	5.5 ± 0.3 (5.3–6.0)	5.2 ± 0.2 (4.8–5.4)
FPG (mmol/l)	8.6 ± 1.5 (6.9–12.4)[d]	5.1 ± 0.6 (4.6–5.7)	4.6 ± 0.5 (4.3–5.2)
FPI (pmol/l)	75 ± 32 (13–112)	62 ± 17 (40–98)	61 ± 17 (53–102)
TG (mmol/l)	1.25 ± 0.5 (0.3–1.7)	1.4 ± 0.6 (0.7–2.2)	0.85 ± 0.3 (0.5–1.3)
FFAs (mmol/l)	0.53 ± 0.18 (0.27–0.85)	0.49 ± 0.29 (0.09–0.89)	0.31 ± 0.25 (0.03–0.78)
HDL (mmol/l)	1.6 ± 0.3 (1.2–2.3)	1.6 ± 0.3 (1.2–2.1)	1.6 ± 0.3 (1.3–2.0)
LDL (mmol/l)	3.4 ± 0.8 (2.3–4.3)	3.8 ± 0.9 (2.5–6.0)[e]	2.6 ± 0.4 (1.7–3.2)
ALT (U/l)	29 ± 11 (14–41)[e]	21 ± 7 (6–33)	18 ± 5 (13–32)

Anthropometric and laboratory characteristics, expressed as means ± SDs (ranges), of patients with T2DM, CONo controls, and CONy controls. Physical activity index was assessed according to Baecke et al. [14] and is given as the mean of the indices for physical activity at work (PW), sport during leisure time (PS) and physical activity during leisure time excluding sport (PL). The physical activity index ranges from 2.4 to 3.0 with higher values indicating higher activity. BMI, waist circumference, fasting plasma glucose (FPG) and insulin (FPI), triglycerides (TG), FFAs, high- and low-density lipoproteins (HDL, LDL), and alanine amino transferase (ALT) were determined.
[a] $p < 0.001$ versus CONy.
[b] $p < 0.005$ versus CONy.
[c] $p < 0.001$ versus CONo.
[d] $p < 0.001$ T2DM versus CONo and CONy.
[e] $p < 0.01$ versus CONy.
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2 cm into the medial head of the right gastrocnemius muscle as described previously [13].

^{31}P MRS

^{31}P spectra were acquired at baseline (–100 to –20 min) and during the last 80 min of the clamp. Rates of basal and insulin-stimulated skeletal muscle ATP synthetic flux (bfATP and ifATP, respectively) were assessed with ^{31}P MRS using the saturation transfer experiment applied to the exchange between P_i and ATP [11,13,16]. Intramyocellular concentrations of G-6-P (μmol/l muscle) and P_i were measured from the ratio of the integrated respective peak intensities and β-ATP resonance intensity in spectra without inversion and saturation assuming a constant ATP concentration of 5.5 mmol/l muscle [2].

^{1}H MRS

IMCL in soleus muscle was determined by ^{1}H MRS as described [1]. The cubic volume of interest within the muscle (1.73 cm^3) and the magnetic field was shimmed on the localized water signal (line width, 2–15 Hz). On a separate day and after a 12-h fast, localized ^{1}H MRS of the liver was acquired to measure HCL as described [17].

Analytical Procedures

Plasma glucose was measured by the glucose oxidase method (Glucose analyzer II, Beckman Coulter, http://www.beckmancoulter.com/). Plasma FFAs were assayed with a microfluorimetric method (Wako Chem USA, http://www.wakousa.com/). In vitro lipolysis was prevented by collecting blood into orlistat-containing vials. Plasma triglycerides were measured by a peroxidase-coupled colorimetric assay (Roche, http://www.roche.com/home.html). Plasma concentrations of insulin, C-peptide and glucagon were determined by double antibody radioimmunoassay [15]. Plasma lactate concentrations were measured enzymatically (Roche) [17].

Calculations and Statistics

Basal rates of glucose appearance (R_a) were calculated by dividing the tracer D-[6,6-$^2H_2$] glucose infusion rate times tracer enrichment by the percent of tracer enrichment in plasma and subtracting the tracer infusion rate [18]. R_a was calculated using Steele's nonsteady-state equations [19]. EGP was calculated from the difference between R_a and mean glucose infusion rates. Suppression of EGP (ΔEGP) by insulin is given as the percent change of EGP at 240 min from fasting. Suppression of plasma FFAs by insulin was calculated analogous to EGP.

All statistical analyses were performed using SPSS 6.0 software (SPSS, http://www.spss.com). Data are presented as means ± standard deviations (SDs) throughout the text and in the figures. Statistical comparisons between study groups were performed using ANOVA and repeated measurements ANOVA with Tukey post hoc testing when appropriate. Within-group differences were determined using two-tailed Student's t tests. Non-parametric correlations are Spearman correlations (*R, P*). Multiple linear regression analysis was performed for the dependent variable, bfATP, including the following basal metabolic parameters: C-peptide, alanine aminotransferase, HbA1c, fasting plasma glucose, basal FFAs, basal EGP, M, HCL, IMCL, basal G-6-P, activity index, BMI, waist-to-hip ratio, and age. Regression analysis for the dependent variable, ifATP, was done using the following independent variables: ΔEGP, suppression of FFAs and increase in G-6-P during clamp, M, HCL, IMCL, activity index, BMI, waist-to-hip ratio, and age. Differences were considered significant at the 5% level.

Results

Baseline Characteristics

Volunteers in the T2DM group were nonobese, normolipidemic, and metabolically well-controlled with stable HbA1c values over the course of the last two years (Table 1). Basal EGP was not different from controls (T2DM, 1.8 ± 0.4 mg/[kg body weight × min]; CONo, 1.9 ± 0.3 mg/[kg body weight × min]; CONy, 1.7 ± 0.6 mg/[kg body weight × min]; $p = 0.858$).

Whole Body Metabolism During Normoglycemic-Hyperinsulinemic Clamps

Fasting plasma glucose was higher in patients with T2DM but normalized during the first 30 min of the clamps (Figure 1). In patients with T2DM, fasting plasma C-peptide was 61% higher than in both control groups (T2DM, 1.2 ± 0.3 nmol/l; CONo, 0.8 ± 0.1 nmol/l; CONy, 0.7 ± 0.1 nmol/l; $p < 0.001$ versus T2DM). Nevertheless, fasting plasma insulin was comparable in all groups (Table 1). During the clamps, mean concentrations of glucose and insulin were 5.5 ± 0.5 mmol/l and 514 ± 96 pmol/l, respectively, and did not differ between the groups (Figure 1). Fasting plasma lactate was lower ($p = 0.005$) in the CONy group than in patients with T2DM (T2DM, 1.26 ± 0.41 mmol/l; CONo, 1.01 ± 0.25 mmol/l; CONy, 0.80 ± 0.25 mmol/l). During the clamp, lactate concentrations remained unchanged in patients with T2DM, gradually increased in the CONo participants ($p = 0.075$) and increased in the CONy participants by 56% ($p = 0.017$) (T2DM, 1.30 ± 0.77 mmol/l; CONo, 1.29 ± 0.39 mmol/l; CONy, 1.24 ± 0.11 mmol/l). Insulin-mediated suppression of FFAs was comparable in all groups (T2DM, 92 ± 6%; CONo, 95 ± 4%; CONy, 90 ± 8%), although patients with T2DM had slightly higher plasma FFAs at 60, 180, and 210 min; $p < 0.05$ (Figure 1).

Insulin-stimulated whole body glucose disposal (M) was 126% and 54% higher in CONy and CONo participants than in patients with T2DM, respectively ($p < 0.001$, $p = 0.010$) (Figure 2). Insulin-stimulated suppression of EGP was more pronounced in CONy participants than in patients with T2DM (ΔEGP: T2DM, 88% ± 8%; CONo, 91% ± 21%; CONy, 107% ± 14%; $p < 0.032$ T2DM versus CONy).

Intracellular Metabolites

IMCL did not differ between the groups (T2DM, 1.0% ± 0.5%; CONo, 0.9% ± 0.5%; CONy, 0.9% ± 0.3%; $p = 0.808$) (Figure 2), whereas HCL were 4- to 7-fold higher in patients with T2DM (T2DM, 14.1% ± 8.3%; CONo, 3.3% ± 4.4%; CONy, 2.2% ± 1.8%; $p < 0.001$ versus T2DM).

Intramyocellular G-6-P concentrations were similar during fasting (0.15 ± 0.057 mmol/l muscle) and increased by 50% during the clamp in all groups (0.22 ± 0.071 mmol/l muscle: T2DM, $p = 0.044$ versus baseline; CONo, $p = 0.009$ versus baseline; CONy, $p = 0.002$ versus baseline). Likewise, intramyocellular P_i concentrations were comparable at baseline (2.9 ± 0.4 mmol/l muscle) and increased 17% during the clamp in all groups (3.4 ± 0.4 mmol/l muscle: T2DM, $p = 0.005$ versus baseline; CONo, $p = 0.001$ versus baseline; CONy, $p = 0.004$ versus baseline).

Rates of Muscle ATP Synthesis

bfATP was 27% lower in patients with T2DM than in CONy participants (T2DM, 8.6 ± 1.9 μmol/[g muscle × min]; CONy, 11.8 ± 3.3 μmol/[g muscle × min] $p < 0.031$) and did not differ from CONo participants (10.4 ± 2.6 μmol/[g muscle × min]; $p = 0.293$ versus T2DM and $p = 0.505$ versus CONy) (Figure 3). ifATP rose by 26% and 11% in the CONy participants (14.8 ± 4.3 μmol/[g muscle × min], $p = 0.003$ versus basal) and the CONo participants (11.5 ± 1.7 μmol/[g muscle × min], $p = 0.023$ versus basal), but not in patients with T2DM (9.5 ± 2.7 μmol/[g muscle × min], $p = 0.256$ versus basal). Significant associations between ATP synthesis rates and physiological variables are shown in Table 2 and Figure 4. Fasting plasma insulin levels neither related to bfATP, ifATP, nor to M. C-peptide related negatively to ifATP ($R = -0.458$, $p = 0.010$) and positively to M ($R = 0.550$, $p = 0.001$). Adjustment for M disrupted the association between ATP synthetic fluxes and C-peptide levels. Adjustment for sex, age, BMI, and waist-to-hip ratio did not disrupt the negative correlations of bfATP and ifATP with fasting FFAs ($R = -0.533$, $p = 0.007$; $R = -0.408$, $p = 0.042$) and of ifATP to HCL ($R = -0.415$, $p = 0.044$). bfATP correlated positively with mean physical activity ($R = 0.945$, $p < 0.001$) and negatively with BMI ($R = -0.818$, $p = 0.004$) only in patients with T2DM. Multiple linear regression analysis for the dependent variable bfATP including basal metabolic parameters (see Methods) identified basal plasma FFAs and waist-to-hip ratio as significant and independent predictors of bfATP (Table 3). In model 1, basal FFAs alone explained 28% of the variance of bfATP. In model 2, waist-to-hip ratio was included in addition to basal plasma FFAs, thereby explaining 44% of the variance of bfATP. Multiple regression analysis for the dependent variable ifATP identified insulin sensitivity (M) as a significant predictor and explained 30% of the variance of ifATP (Table 3).

Whole Body Metabolism During Hyperglycemic-Hyperinsulinemic Clamps

The effect of combined glucose and insulin stimulation on ifATP was studied in four of the T2DM volunteers during hyperglycemic–hyperinsulinemic clamps (Figure 5). Mean plasma glucose almost doubled (9.6 ± 0.8 mmol/l versus 5.5 ± 0.3 mmol/l; $p < 0.001$) resulting in 70% higher M-values compared to normoglycemic hyperinsulinemia (8.1 ± 4.4 mg/[kg body weight × min] versus 4.8 ± 2.5 mg/[kg body weight × min]; $p = 0.048$). Intramyocellular G-6-P concentrations doubled compared to normoglycemic hyperinsulinemia (240 min: 0.29 ± 0.01 mmol/l muscle versus 0.15 ± 0.05 mmol/l muscle; $p = 0.010$). Mean bfATP, ifATP and change of fATP during insulin stimulation did not differ between normoglycemic and hyperglycemic clamps (bfATP: 9.5 ± 2.2 μmol/[g muscle × min] versus 8.1 ± 1.7 μmol/[g muscle × min], $p = 0.506$; ifATP: 10.3 ± 2.4 μmol/[g muscle × min] versus 8.6 ± 3.2 μmol/[g muscle × min], $p = 0.156$, $p = 0.628$ basal versus insulin stimulation during hyperglycemic–hyperinsulinemic clamp; change of fATP during insulin stimulation: 0.9 ± 3.3 μmol/[g muscle × min] versus 0.03 ± 1.8 μmol/[g muscle × min], $p = 0.579$).

Discussion

Patients with well-controlled T2DM have impaired flux through muscle ATP synthesis occurring independently of glucose transport/phosphorylation and lipid deposition. Lipid availability primarily determines bfATP, whereas insulin sensitivity defines ifATP. Furthermore, the reduction in insulin-stimulated glucose disposal despite normal glucose

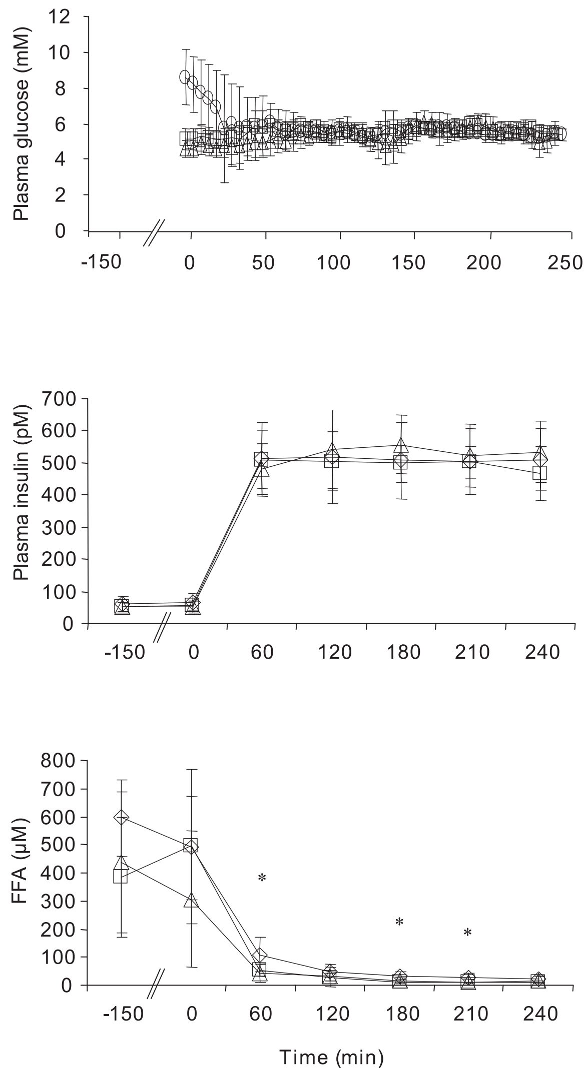

Figure 1. Plasma Glucose, Insulin and FFAs

Time course of plasma glucose (top), insulin (middle), and FFAs (bottom) concentrations during euglycemic (approximately 5.5 mM glucose) – hyperinsulinemic (approximately 500 pM insulin) clamps in patients with T2DM ($n = 10$) (diamonds), age- and BMI-matched controls (CONo; $n = 10$) (squares), and young healthy controls (CONy; $n = 11$) (triangles). All units expressed as means ± SD. * $p < 0.05$ T2DM versus controls.

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transport/phosphorylation suggests further abnormalities, mainly in glycogen synthesis of these patients with T2DM.

We studied slightly overweight patients with well-controlled T2DM featuring a lower degree of whole-body insulin resistance compared with previous studies on mitochondrial function in insulin resistant states [9,20,21]. Of note, BMI, waist-to-hip ratio, fasting lipid, and insulin concentrations were comparable to an age-matched control group. These patients with overt T2DM exhibit somewhat reduced muscular ATP synthesis as assessed by noninvasive in vivo ^{31}P MRS.

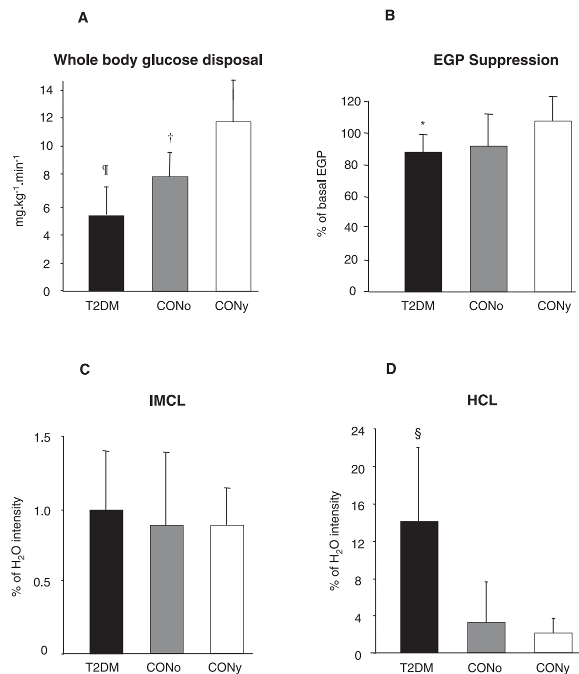

Figure 2. Glucose Metabolism and Intracellular Lipids of Skeletal Muscle and Liver

Whole-body glucose disposal (A) and ΔEGP (B) during euglycemic–hyperinsulinemic clamp ($n = 31$). IMCL in skeletal muscle ($n = 31$) (C) and liver ($n = 29$) (HCL) (D). Patients with T2DM (black columns), CONo (grey columns), and CONy (white columns). All results are means ± SD. ¶ $p < 0.001$ T2DM versus CONy; † $p < 0.01$ CONo versus T2DM and CONy; * $p < 0.05$ T2DM versus CONy; § $p < 0.001$ T2DM versus controls.

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Fasting Conditions

Both control groups featured similar bfATP, which was nearly doubled compared to a group of participants who were 15 years older, in a previous study [11]. Thus, aging-dependent alterations in mitochondrial number, morphology, and in vivo function do not seem to be present in our CONo group, but cannot be completely excluded, because we did not perform muscle biopsies in this study [5,11,22]. bfATP of the patients with T2DM was higher than in other insulin-resistant groups, but lower only compared to the CONy group [11,12,23]. This variation suggests that nondiabetic insulin-resistant groups, such as first-degree relatives of patients with T2DM, exhibit peculiar abnormalities in bfATP which can be confounded by secondary metabolic events in the overt diabetic state. Physical activity and body mass were the major determinants of bfATP only in the patients with T2DM, confirming the relationship between exercise training and mitochondrial function and further pointing to a specific susceptibility of mitochondrial function to lifestyle factors in patients with T2DM [24].

Fasting plasma FFAs and waist-to-hip ratio were identified as independent predictors of bfATP. FFAs could decrease ATP synthesis by protonophoric action on the inner mitochondrial membrane, activation of uncoupling protein-1 (UCP-1), and damage of mitochondrial proteins and DNA,

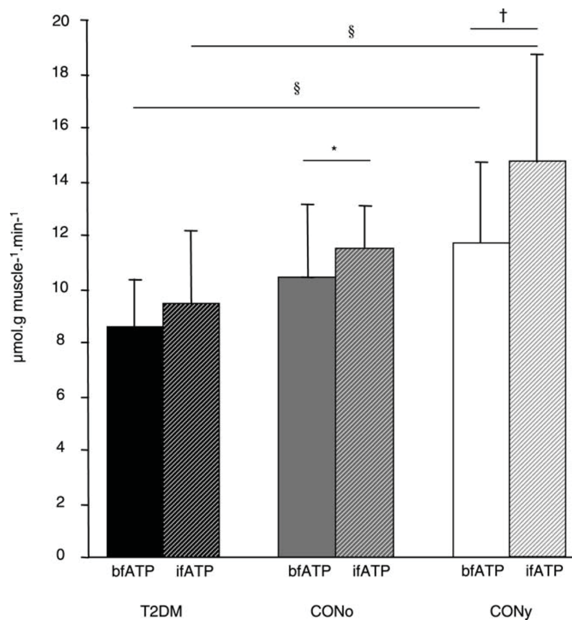

Figure 3. Rates of ATP Synthetic Flux

Rates of ATP synthetic flux (means ± SD) during fasting (bfATP, full columns) and during insulin stimulation (ifATP, hatched columns). Patients with T2DM (black columns), CONo controls (grey columns) and CONy controls (white columns) ($n = 31$). § $p < 0.05$ T2DM versus CONy; *, $p < 0.05$; †, $p < 0.01$.

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resulting from formation of reactive oxygen species [25–28]. Lipid-induced dysfunction of mitochondria might also occur via down-regulation of nuclear genes involved in genes that are involved in oxidative phosphorylation (OXPHOS) and mitochondrial biogenesis [29]. Thus, environmental factors could serve as an epigenetic trigger affecting mitochondrial biogenesis in patients with T2DM [30]. The frequently observed inverse association between lipid accumulation in skeletal muscle and insulin sensitivity is influenced by various factors such as gender, ethnicity, genes, and total body fat content, and it depends on muscle fiber composition [1]. In the present study, IMCL did not differ between groups, suggesting that FFAs and their intracellular metabolites, rather than lipid deposition, are related to mitochondrial function.

Despite their good long-term metabolic control, the patients with T2DM were hyperglycemic during bfATP measurements due to withdrawal of glucose-lowering medication. Of note, transcription of OXPHOS genes can be normalized by near-normoglycemia in patients with poorly controlled T2DM [31]. Hyperglycemia also increases reactive oxygen species formation due to an imbalance between glucose phosphorylation by mitochondria-bound hexokinase and reactions decreasing G-6-P such as glycolysis, pentose phosphate shunt, and glycogen synthesis. The resulting rise in G-6-P would cause feedback inhibition of hexokinase, decrease the mitochondrial ADP:ATP ratio, and thereby disrupt ADP recycling [32,33]. As fasting intramyocellular G-6-P was not different between individuals with and without diabetes, increased plasma glucose cannot completely account for the reduction of bfATP in patients with T2DM.

Table 2. Associations between ATP Synthesis Rates and Physiological Variables

Variable	bfATP	ifATP
HbA1c	$R = -0.417$, $p = 0.019$	$R = -0.453$, $p = 0.010$
M	$R = 0.488$, $p = 0.005$	$R = 0.587$, $p = 0.001$
Basal FFAs	$R = -0.462$, $p = 0.009$	$R = -0.482$, $p = 0.006$
HCL	$R = -0.290$, $p = 0.127$	$R = -0.434$, $p = 0.019$

Spearman correlation coefficients *(R)* for the association of bfATP and ifATP with insulin sensitivity during the clamp test (M), fasting FFAs, HCL, and HbA1c. Insulin sensitivity was calculated from whole-body glucose disposal (M) during the last 30 min (210–240 min) of a normoglycemic-hyperinsulinemic clamp.

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Insulin Stimulated Conditions

Although glucose disposal was markedly lower in patients with T2DM than in the controls during insulin stimulation, the 50% increase in G-6-P was surprisingly comparable between all three groups. Hyperinsulinemia of 515 pmol/l resulted in stimulation of glucose transport/phosphorylation in patients with T2DM similar to that in persons who do not have diabetes, and it rose even further during hyperglycemic hyperinsulinemia. The rise in G-6-P in the face of impaired glucose disposal could result from a defect of either glucose oxidation or glycogen synthesis relative to glucose transport via GLUT-4 and/or phosphorylation (hexokinase II), both of which lead to reduction of insulin-stimulated ATP synthesis.

Glucose transport was shown to be rate controlling for glucose disposal at lower plasma insulin concentrations and in obese patients with poorly controlled T2DM [34]. Reduced expression and/or flux through hexokinase II indicate that glucose phosphorylation can also be impaired in patients with T2DM [32]. One might speculate that the insulin-induced rise in G-6-P observed in our patients with T2DM could have reduced the activity of hexokinase II and lower the ADP:ATP ratio which in turn would slow down stimulation of ATP synthase flux [35]. However, both absolute concentrations and percentage increase of G-6-P were almost identical, rendering this possibility unlikely. Thus, the insulin-sensitive rise in G-6-P seems to depend critically on the degree of insulinemia and glycemia and probably also on long-term metabolic control. These mechanisms could explain why a 20-fold rise in plasma insulin concentrations during hyperglycemia completely normalizes the diminished insulin-stimulated increment in G-6-P of patients with poorly controlled T2DM [36]. On the other hand, lean insulin-resistant relatives of patients with T2DM exhibit a markedly reduced insulin-sensitive rise in G-6-P despite normoglycemia, highlighting the predominant role of inherited factors also for glucose transport/phosphorylation in this group [35].

Our patients with T2DM exhibited 27% lower rates of fasting ATP synthetic flux than the CONy participants, suggesting reduced aerobic capacity in T2DM. Reduction of oxidative capacity during insulin stimulation would diminish glucose oxidation—as reflected by impaired stimulation of ATP synthesis—and simultaneously cause reduction of glucose disposal. Plasma lactate was slightly higher during

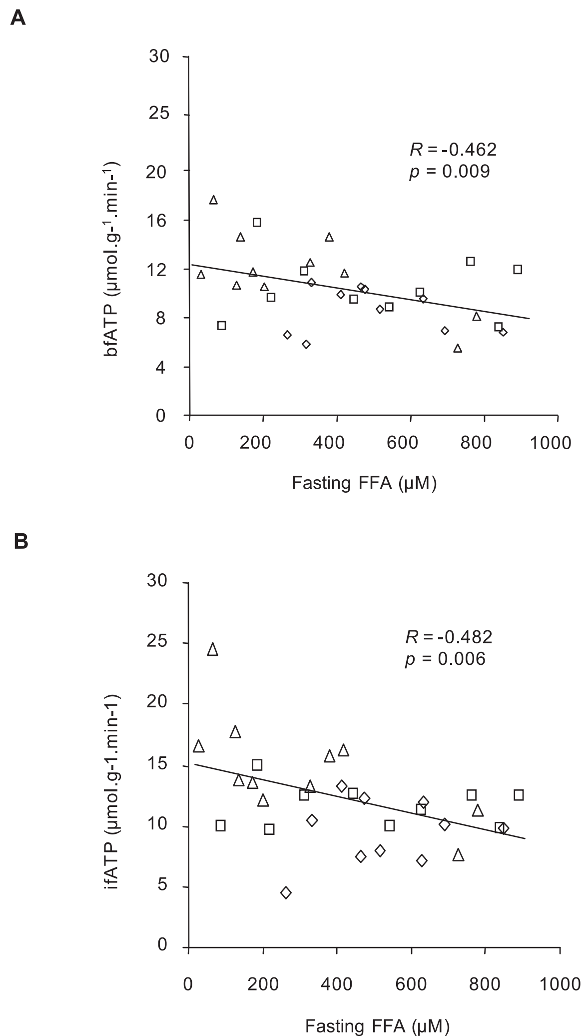

Figure 4. Associations between ATP Synthetic Flux and Metabolic Parameters

Correlation analyses of rates of ATP synthetic flux ($n = 31$).

(A) Relation between fasting plasma concentration of FFAs and bfATP across all groups.

(B) Relation between fasting plasma FFAs and ifATP across all groups. Patients with T2DM ($n = 10$) (diamonds), age- and BMI-matched controls (CONo; $n = 10$) (squares), and young healthy controls (CONy; $n = 11$) (triangles).

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fasting but comparable during insulin stimulation. Carbohydrate oxidation was therefore likely unchanged, although abnormalities cannot be ruled out, because plasma lactate levels are also determined by extramuscular sources such as adipocytes [37].

In our patients with T2DM, impaired glucose disposal despite intact glucose transport/phosphorylation could further mirror a defect in insulin-stimulated nonoxidative glucose metabolism, i.e., muscle glycogen synthesis. In insulin-resistant nonobese relatives of patients with T2DM, exercise training unmasks an independent abnormality in muscle glycogen synthesis as shown by doubling of insulin-stimulated G-6-P without normalization of nonoxidative glucose metabolism [38]. One limitation of the present study resides in the lack of data on muscle glycogen synthesis, which could not be measured due to time restrictions in this protocol. Nevertheless, the available data suggest a distinct post-transport/phosphorylation defect, particularly in glycogen synthesis, in these patients with well-controlled T2DM [39–41]. Another limitation resides in the small sample size of this study, which affects the probability of detecting associations between parameters and differences between groups. Of note, some normality approximations do not necessarily hold true for small numbers.

In young healthy humans, insulin markedly stimulated ATP synthesis, which primarily results from augmented substrate availability, mainly glucose, and stimulation of respiratory chain enzyme activities within 1–3 h of hyperinsulinemia, and from increased mitochondrial protein expression after 6 h of prolonged hyperinsulinemia [7,13,23]. ATP synthesis rose only modestly in CONo participants (11%) but not in patients with T2DM. Despite the known age-related mitochondrial abnormalities, in vivo mitochondrial function can be maintained in humans up to 70 years of age depending on physical activity and body mass [42,43]. Likewise, insulin sensitivity can be unimpaired but also reduced upon correction for body mass in the elderly [11,44–46]. In the present study, adjustment for BMI and waist-to-hip ratio did not affect the association between age and insulin sensitivity ($R = -0.634$, $p < 0.001$). Moreover, the lower increase in ifATP in our elderly participants is in line with the previously observed simultaneous occurrence of decreased basal ATP synthesis and decreased insulin sensitivity, and supports the contention that age-dependent changes in ifATP relate to those of insulin sensitivity [11].

The participants with T2DM, to which the controls were matched for age and BMI, failed to exhibit an increase in ifATP, similar to recent findings in young nonobese insulin-resistant offspring of patients with T2DM. Thus, it can be

Table 3. Multiple Regression Analysis for the Dependent Variables bfATP and ifATP

Dependent Variable	Model	Constant	R^2	Adjusted R^2	Standardized Coefficient (β)	*p*-Value
bfATP	1	Basal FFAs	0.282	0.254	−0.531	0.004
	2	Basal FFAs	0.438	0.392	−0.599	0.001
		Waist to hip ratio			−0.401	
ifATP	1	Insulin sensitivity	0.303	0.274	0.550	0.004

Coefficients of the models explaining bfATP and ifATP variations.

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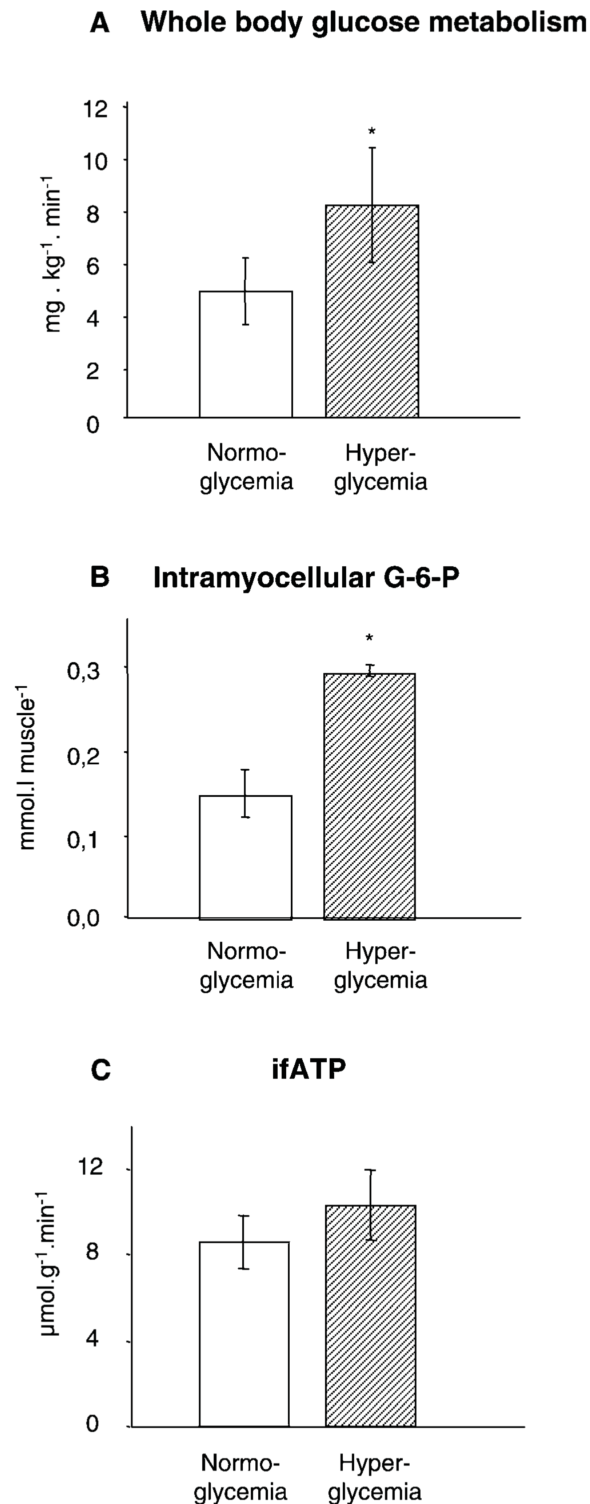

Figure 5. Glucose Metabolism and ATP Synthetic Flux during Hyperglycemic–Hyperinsulinemia in T2DM

Whole body glucose disposal (A), concentration of intramyocellular G-6-P (B), and ifATP (C) during euglycemic (approximately 5.5 mM glucose)–hyperinsulinemic (approximately 500 pM insulin) (white columns) and hyperglycemic (approximately 9.5 mM glucose)–hyperinsulinemic (approximately 500 pM insulin) clamps (hatched columns) in patients with T2DM ($n = 4$). * $p < 0.05$.

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hypothesized that insulin resistance is responsible for impaired ATP synthesis during insulin stimulation, which is supported by the multivariate analyses across all groups in the present study. The nonobese patients with T2DM featured defective ifATP despite normal G-6-P stimulation, suggesting an abnormality independent of glucose transport that does not disappear by matching glucose disposal to that of older controls and resulting in doubling of G-6-P by hyperglycemic hyperinsulinemia.

Intramuscular P_i concentrations increased by 17%, in agreement with previous studies [47]. The finding of similar basal and insulin-stimulated P_i in all groups seems to exclude abnormal P_i transport as the explanation for the ifATP variability in patients with T2DM. In contrast, lean insulin-resistant relatives of patients with T2DM exhibit markedly decreased insulin-stimulated P_i transport [23]. The difference between this group and our patients with overt T2DM could result from specific inherited mitochondrial abnormalities in the relatives or secondary metabolic changes obscuring the effect on P_i transport in the patients with T2DM. Alternatively, increased lipid availability could explain lower ifATP by interference with insulin signaling, which is in line with FFA-dependent ifATP inhibition [13]. Of note, plasma levels of FFAs during fasting was found to be tightly related to both bfATP and ifATP in the present study. Finally, ifATP correlated with HCL and elevation of HCL was identified as an early abnormality in patients with well-controlled T2DM, supporting the concept that ectopic fat in the liver rather than in muscle relates to whole body insulin sensitivity [48].

Different variants of insulin resistance can be found in humans. Insulin-resistant lean normoglycemic relatives of patients with T2DM present with IMCL accumulation along with clearly impaired insulin-stimulated P_i transport, mitochondrial ATP synthesis, and glucose transport and/or phosphorylation. In contrast, insulin-resistant nonobese near-normoglycemic patients with T2DM exhibit only slightly reduced ATP synthesis but have completely normal IMCL, P_i transport, and G-6-P increase. The difference could be explained by genes and secondary metabolic alterations such as glucolipotoxicity affecting the patients with T2DM. However, comparable fasting lipid concentrations and body mass in patients with T2DM and the age-matched control group render the latter possibility unlikely. To which extent accumulation of ectopic lipids (particularly HCL, which is seen in both offspring of and patients with overt T2DM) serve as common mediators or indicators of insulin resistance regardless of muscular abnormalities requires further investigation.

In conclusion, (i) patients with well-controlled insulin resistant T2DM have slightly lower ATP synthesis independent of glucose transport/phosphorylation and lipid deposition in muscle, (ii) lipid availability primarily determines bfATP, whereas (iii) insulin sensitivity defines ifATP, and (iv) reduction in insulin-stimulated glucose disposal suggests further abnormalities, mainly in glycogen synthesis of these patients with T2DM.

This study underlines the strong relationship between insulin sensitivity and function of mitochondria, the cells' power plants. Even a small degree of overweight and physical inactivity is associated with reduction in mitochondrial function, confirming the importance of lifestyle for development and prevention of insulin resistance and T2DM. Further research is needed, however, to delineate whether abnormalities in mitochondrial number and/or function are the cause or consequence of T2DM and to address the mitochondria as a target for novel therapeutic regimens.

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Editors' Summary

Background. Diabetes mellitus is an increasingly common chronic disease characterized by high blood sugar (glucose) levels. In normal individuals, blood sugar levels are maintained by the hormone insulin. Insulin is released by the pancreas when blood glucose levels rise after eating (glucose is produced by the digestion of food) and "instructs" insulin-responsive muscle and fat cells to take up glucose from the bloodstream. The cells then use glucose as a fuel or convert it into glycogen, a storage form of glucose. In type 2 diabetes, the commonest type of diabetes, the muscle and fat cells become nonresponsive to insulin (a condition called insulin resistance) and consequently blood glucose levels rise. Over time, this hyperglycemia increases the risk of heart attacks, kidney failure, and other life-threatening complications.

Why Was This Study Done? Insulin resistance is often an early sign of type 2 diabetes, sometimes predating its development by many years, so understanding its causes might provide clues about how to stop the global diabetes epidemic. One theory is that mitochondria—cellular structures that produce the energy (in the form of a molecule called ATP) needed to keep cells functioning—do not work properly in people with insulin resistance. Mitochondria change (metabolize) fatty acids into energy, and recent studies have revealed that fat accumulation caused by poorly regulated fatty acid metabolism blocks insulin signaling, thus causing insulin resistance. Other studies using magnetic resonance spectroscopy (MRS) to study mitochondrial function noninvasively in human muscle indicate that mitochondria are dysfunctional in people with insulin resistance by showing that ATP synthesis is impaired in such individuals. In this study, the researchers have examined both baseline and insulin-stimulated mitochondrial function in nonobese patients with well-controlled type 2 diabetes and in normal controls to discover more about the relationship between mitochondrial dysfunction and insulin resistance.

What Did the Researchers Do and Find? The researchers determined the insulin sensitivity of people with type 2 diabetes and two sets of people (the "controls") who did not have diabetes: one in which the volunteers were age-matched to the people with diabetes, and the other containing younger individuals (insulin resistance increases with age). To study insulin sensitivity in all three groups, the researchers used a "hyperinsulinemic–euglycemic clamp." For this, after an overnight fast, the participants' insulin levels were kept high with a continuous insulin infusion while blood glucose levels were kept normal using a variable glucose infusion. In this situation, the glucose infusion rate equals glucose uptake by the body and therefore measures tissue sensitivity to insulin. Before and during the clamp, the researchers used MRS to measure glucose-6-phosphate (an indicator of how effectively glucose is taken into cells and phosphorylated), ATP synthesis, and the fat content of the participants' muscle cells. Insulin sensitivity was lower in the patients with diabetes than in the controls, but muscle lipid content was comparable and hyperinsulinemia increased glucose-6-phosphate levels similarly in all the groups. Patients with diabetes and the older controls had lower fasting ATP synthesis rates than the young controls and, whereas insulin stimulation increased ATP synthesis in all the controls, it had no effect in the patients with diabetes. In addition, fasting blood fatty acid levels were inversely related to basal ATP synthesis, whereas insulin sensitivity was directly related to insulin-stimulated ATP synthesis.

What Do These Findings Mean? These findings indicate that the impairment of muscle mitochondrial ATP synthesis in fasting conditions and after insulin stimulation in people with diabetes is not due to impaired glucose transport/phosphorylation or fat deposition in the muscles. Instead, it seems to be determined by lipid availability and insulin sensitivity. These results add to the evidence suggesting that mitochondrial function is disrupted in type 2 diabetes and in insulin resistance, but also suggest that there may be abnormalities in glycogen synthesis. More work is needed to determine the exact nature of these abnormalities and to discover whether they can be modulated to prevent the development of insulin resistance and type 2 diabetes. For now, though, these findings re-emphasize the need for people with type 2 diabetes or insulin resistance to reduce their food intake to compensate for the reduced energy needs of their muscles and to exercise to increase the ATP-generating capacity of their muscles. Both lifestyle changes could improve their overall health and life expectancy.

Additional Information. Please access these Web sites via the online version of this summary at http://dx.doi.org/10.1371/journal.pmed.0040154.

- The MedlinePlus encyclopedia has pages on diabetes
- The US National Institute of Diabetes and Digestive and Kidney Diseases provides information for patients on diabetes and insulin resistance
- The US Centers for Disease Control and Prevention has information on diabetes for patients and professionals
- American Diabetes Association provides information for patients on diabetes and insulin resistance
- Diabetes UK has information for patients and professionals on diabetes

Article No. 5.

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Skeletal Muscle Phosphodiester Content Relates to Body Mass and Glycemic Control

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Abstract

Background: Aging and insulin resistance have been related to reduced mitochondrial function and oxidative stress. Muscular phosphodiesters (PDE) are comprised of metabolites of phospholipid breakdown and may reflect membrane damage. We aimed to test the hypothesis that myocellular PDE are increased in patients with type 2 diabetes (T2D) and correlate inversely with mitochondrial ATP turnover.

Methods: A Cross-sectional study in the Clinical Research Facility of an University hospital was performed. 10 nonobese middle-aged patients with T2D, 10 healthy humans matched for sex, age and physical activity index (CONm) and 18 young healthy humans (CONy) were included. Myocellular PDE and unidirectional flux through ATP synthase (fATP) were measured with ^{31}P magnetic resonance spectroscopy (MRS). Intramyocellular (IMCL) and hepatocellular lipid deposition (HCL) were quantified with ^{1}H MRS. Insulin sensitivity (Rd) was assessed from hyperinsulinemic-euglycemic clamp tests in 10 T2D, 10 CONm and 11 CONy.

Results: During fasting, T2D and CONm had 1.5 fold greater PDE than CONy (2.8±0.2, 2.5±0.2, 1.7±0.1 mmol/l, P = 0.004). Stimulation by insulin did not affect PDE in any group. PDE correlated negatively with Rd ($r = -0.552$, $p<0.005$) and fATP ($r = -0.396$, $p<0.05$) and positively with age ($r = 0.656$, $p<0.001$) and body mass ($r = 0.597$, $p<0.001$). PDE also related positively to HbA1c ($r = 0.674$, $p<0.001$) and fasting plasma glucose ($r = 0.629$, $p<0.001$) within T2D and across all participants.

Conclusions: Muscular PDE concentrations associate with age, lower resting mitochondrial activity and insulin resistance, which is determined mainly by body mass and glycemia.

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Introduction

Type 2 diabetes mellitus (T2D) represents one of the world's greatest economic and health care challenges. T2D results from an imbalance between insulin responsiveness and insulin secretion. Skeletal muscle is mainly responsible for whole-body insulin resistance and determines substrate oxidation particularly during exercise. Reduced mitochondrial oxidative capacity, activity and/or content have been related to aging, intramyocelluar lipid content (IMCL) and muscular insulin resistance [1,2,3,4].

It has been hypothesized that aging-associated insulin resistance results from cumulative free radical damage leading to lower mitochondrial function and increased IMCL [5]. Of note, preventing oxidative damage by overexpression of antioxidant defense mechanisms indeed protected rodent models from lipid-induced and age-associated insulin resistance [5,6]. However, a causal relationship between aging, mitochondria and the development of T2D has been questioned [1,7,8]. Of note, there is no evidence for disruption of the cellular integrity and contractile function in skeletal muscle of T2D, while patients with myopathies can exhibit mitochondrial abnormalities and insulin resistance [9,10,11].

Membrane phospholipids are hydrolyzed to free fatty acids (FFA) and phosphodiesters (PDE) which are regarded as cell membrane degradation products [12]. Myocellular PDE are elevated in patients with myopathies [13,14] and in elderly [15,16] and increase in response to treatment with statins [17]. PDE may reflect fiber atrophy, accumulated sarcolemmal damage [18] or structural and functional changes during loss of muscle mass possibly resulting from cumulative oxidative damage [19].

Thus, PDE may serve as marker of biomembrane integrity [18,20]. The relationship between PDE content and mitochondrial function and insulin sensitivity in healthy middle-aged humans and in patients with T2D has not yet been reported.

We hypothesized that T2D have elevated myocellular PDE which relates to age, mitochondrial activity and insulin sensitivity. Thus, we measured PDE, unidirectional flux through ATP synthase (fATP) during fasting and insulin stimulation as a marker of resting mitochondrial activity [21,22], insulin stimulated glucose-6-phosphate (ΔG6P) as a marker of insulin-stimulated glucose transport/phosphorylation, whole-body glucose disposal (Rd) and endogenous glucose production (EGP) in T2D, non-diabetic age-matched (CONm) and younger humans (CONy).

Methods

Ethics Statement

All studies were carried out in accordance with the most-recent version of the Declaration of Helsinki and approved by the local ethics committee of the Medical University of Vienna. Informed written consent has been obtained from all participants.

Subjects

Ten T2D, 10 CONm matched for sex, age and physical activity and 18 CONy were included. PDE content in all participants from one study [22] and 7 controls from another study [23] are reported. They underwent complete medical history, clinical examination and lab tests. All participants had comparable physical activity according to Baeckes questionnaire, refrained from any physical exercise for three days and fasted for 12 h before the study. T2D had neither islet cell antibodies nor signs of neurological disorders including diabetes-related neuropathy. Only sulfonylurea and/or metformin were allowed as glucose-lowering medication but withdrawn three days before the study. Four T2D and none of the healthy participants received statins, which were not withdrawn before the study. Control subjects had no family history of T2D.

Experimental Protocol

After baseline blood sampling, D-[6,6-$^2H_2$]glucose (98% enriched; Cambridge Isotope Laboratories, http://www.isotope.com/cil/index.cfm) was given as primed-continuous infusion [0 min to 5 min: 3.6 mg.(kg body weight)$^{-1}$.(fasting plasma glucose in mg/dl)/(90 mg/dl); −115 min to +240 min 0.036 mg.min^{-1}.(kg body weight)$^{-1}$] to assess Rd and EGP from +220 to +240 min. From 0 to +240 min, hyperinsulinemic-euglycemic clamp tests were performed in 10 T2DM, 10 CONm and 11 CONy, while 7 CONy were only examined at baseline. Insulin (Actrapid; Novo, Bagsvaerd, Denmark) was administered as primed-continuous infusion [40 mU.(m body surface area)$^{-2}$.min^{-1}] and plasma glucose was controlled by a variable 20% dextrose infusion, 2% enriched with D-[6,6-$^2H_2$]glucose according to the hot-glucose-infusion protocol.

IMCL, PDE and other phosphorus metabolites were measured at baseline and during insulin stimulation.

Magnetic Resonance Spectroscopy (MRS)

Measurements were performed on subjects lying supine inside a 3-Tesla MR spectrometer (Medspec S300-DBX; Bruker, Ettlingen, Germany). The right lower leg positioned on a 10-cm circular double resonant $^1H/^{31}P$ surface coil (125.6/50.8 MHz) so that the isocenter of the magnetic field was placed ~2 cm into the medial head of the gastrocnemius muscle [22]. Phosphorus compounds were measured from the ratio of the integrated peak intensities and ß-ATP resonance intensity in spectra without inversion and saturation (pulse length 150 µs/90°, 2k data, 8 averages, repetition time of 15 s) assuming constant ATP concentrations of 5.5 mmol/l muscle [22]. In human skeletal muscle, the peak between inorganic phosphate (Pi) and phosphocreatine (PCr) represents the

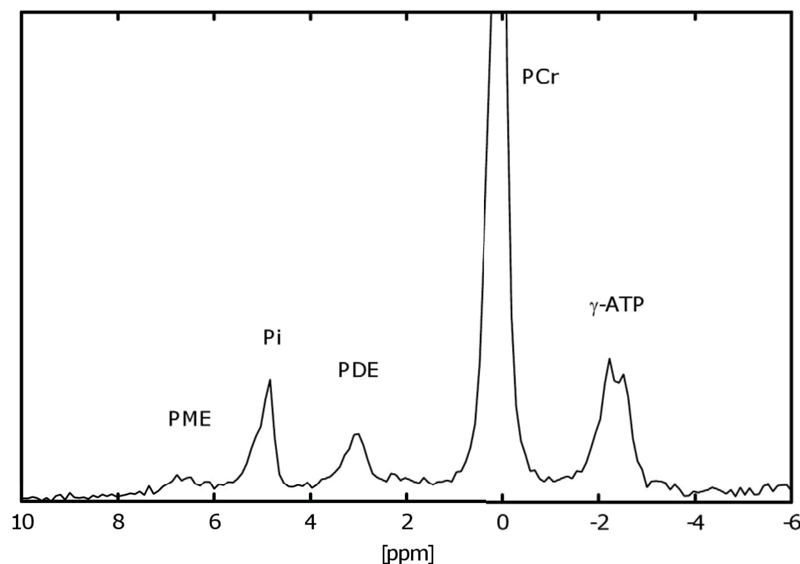

Figure 1. Representative ^{31}phosphorous magnetic resonance spectrum of skeletal muscle. Phosphorus compounds were measured from the ratio of the integrated respective peak intensities and ß-ATP resonance intensity in spectra without inversion and saturation (pulse length 150 µs/90°, 2k data, 8 averages, repetition time of 15 s) assuming a constant ATP concentration of 5.5 mmol/l muscle.
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signal arising from PDE which is mostly attributed to glycerophospho-ethanolamine/-choline, membrane-bound phospholipid metabolites (**Figure 1**) [24]. Measurement of PDE has been validated against chemical analysis employing preparations of muscular ethanol-soluble PDE and exogenous glycerol-3-phosphorylcholine, which confirmed that MRS-measured PDE represent the peak resonating at 0.13 ppm [24].

Myocellular fATP [μmol.(g muscle $^{-1}$).min^{-1}] was measured with ^{31}P MRS employing the saturation transfer experiment to the Pi/ATP exchange [22,25]. Glucose transport/-phosphorylation was assessed from the increase in G6P during the clamp (ΔG6P). IMCL in soleus muscle and liver fat content (HCL) were measured by ^{1}H MRS [22,26].

Analytical measurements

Plasma glucose was assessed by the glucose oxidase method (Beckman, Fullerton, CA), FFA microfluorimetrically (Wako Chem. USA Inc., Richmond, VA) and plasma insulin by double-antibody radioimmunoassay (Pharmacia, Uppsala, Sweden). All other measurements were assessed in the routine lab.

Calculations and statistics

At baseline, rates of glucose appearance (R_a) were calculated by dividing the tracer D-[6,6- $^{2}H_2$]glucose infusion rate times tracer enrichment by the percent of tracer enrichment in plasma and subtracting the tracer infusion rate [27]. During the clamp, R_a was calculated using Steele's non-steady state equations [28]. EGP is given as the difference between R_a and mean glucose infusion rates.

Group data are presented as means and SD (Text) or SEM (Figures) and compared with ANOVA and Tukey post-hoc testing as appropriate. Within-group differences were assessed with two-tailed t-tests. Linear correlations are Pearson product-moment correlations. Partial correlation analysis was performed to study the linear relationship between PDE and other variables after excluding the effect of age, BMI or HbA1c. Differences were considered significant at the 5-% level.

Results

All groups were matched for sex (50% female). BMI tended to be (T2D: 27±3; CONm: 26±3; CONy: 24±2 kg/m^2) and waist circumference was higher in T2D and CONm (97±9; 92±17; 80±8 cm, $p<0.05$ T2D vs. CONy). T2D and CONm were also matched for age (59±6; 57±7; 29±5 years, $p<0.001$ both vs. CONy). T2D had greater fasting plasma glucose (8.9±1.7; 5.2±0.4; 4.8±0.7 mmol/l, $p<0.001$ vs. both CON groups) and HbA1c (6.9±0.7; 5.5±0.3; 5.2±0.2 mmol/l, $p<0.001$ vs. both CON), but comparable plasma FFA (0.57±0.11; 0.44±0.22; 0.48±0.26 mmol/l) and low-density lipoprotein (LDL: 3.4±0.8; 3.8±0.9; 2.6±0.5 mmol/l).

During clamp steady-state (220–240 min), mean plasma glucose, insulin and FFA were 5.5±0.5 mmol/l, 514±96 pmol/l and 0.02±0.01 mmol/l without differences between groups. T2D had ~33% and ~54% lower Rd than CONm and CONy; CONm had ~31% lower Rd than CONy (5.7±0.5, 8.5±0.8, 12.4±1.0 mg.kg^{-1}.min^{-1}, $p<0.05$, $p<0.001$ T2D vs. CONm and CONy, $p<0.005$ CONm vs CONy). Insulin-suppressed EGP reflecting hepatic insulin resistance was greater in T2D and CONm (0.23±0.05; 0.22±0.09; −0.14±0.09 mg.kg^{-1}.min^{-1}, $p<0.05$ both vs. CONy).

Resting mitochondrial activity (fATP) was lower in T2D than in CONy but comparable to CONm [22]. Stimulation by insulin increased fATP in CONm and CONy but not in T2D and unmasked impaired mitochondrial adaptation compared to CONm [22].

In the fasted state, myocellular PDE contents were ~65% and ~47% higher in T2D and CONm compared to CONy, but not different between T2D and CONm (**Figure 2A**). IMCL and ΔG6P were comparable in all groups as reported [22] and did not relate to PDE. Insulin stimulation did not affect PDE (basal vs. insulin-stimulated: 2.8±0.8 vs. 2.9±0.7; 2.5±0.7 vs. 2.5±0.7; CONy: 1.8±0.3 vs. 1.8±0.3 mmol/l), which were ~55% and ~38% higher in T2D and CONm compared to CONy ($p<0.001$, $p<0.05$, **Figure 2B**). Changes in PDE did not relate to ΔG6P.

PDE correlated negatively with fasting fATP (**Figure 3A**) and insulin-mediated Rd (**Figure 3B**), but not with suppressed EGP or insulin-stimulated fATP. PDE related positively to BMI (**Figure 3C**), waist circumference ($r=0.668$, $p<0.001$), age ($r=0.656$, $p<0.001$), HCL ($r=0.446$, $p<0.01$), HbA1c (**Figure 3D**) and to fasting plasma glucose ($r=0.629$, $p<0.001$) across all participants and within T2D ($r=0.652$, $r=0.653$, $p<0.05$). There was no correlation between muscle PDE and HbA1c in the pooled group of non-diabetic subjects. PDE neither related to physical activity nor to plasma FFA, LDL or IMCL. Correlations of PDE with fATP and Rd were abolished after selective adjustment for either age, BMI or HbA1c.

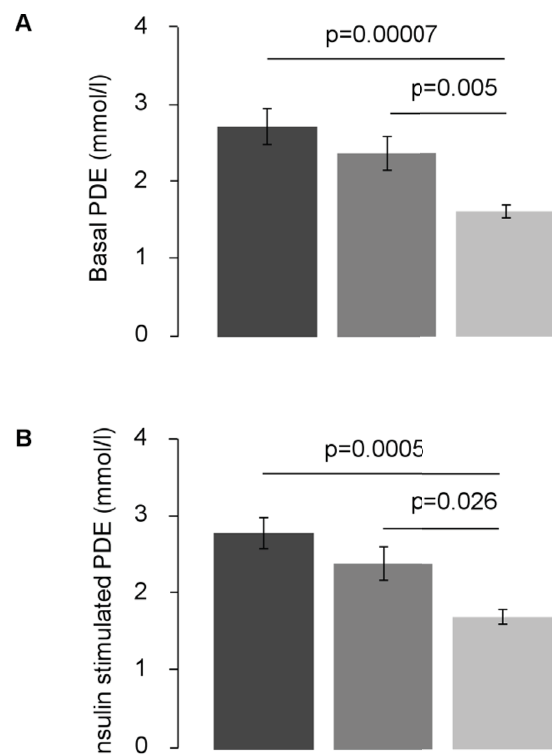

Figure 2. Phosphodiester (PDE; means ± SEM) content in 10 patients with type 2 diabetes (T2D, black columns), 10 age-matched (CONm, grey columns) and 18 lean young controls (CONy, empty columns) during fasting (A) and during insulin stimulation (B).
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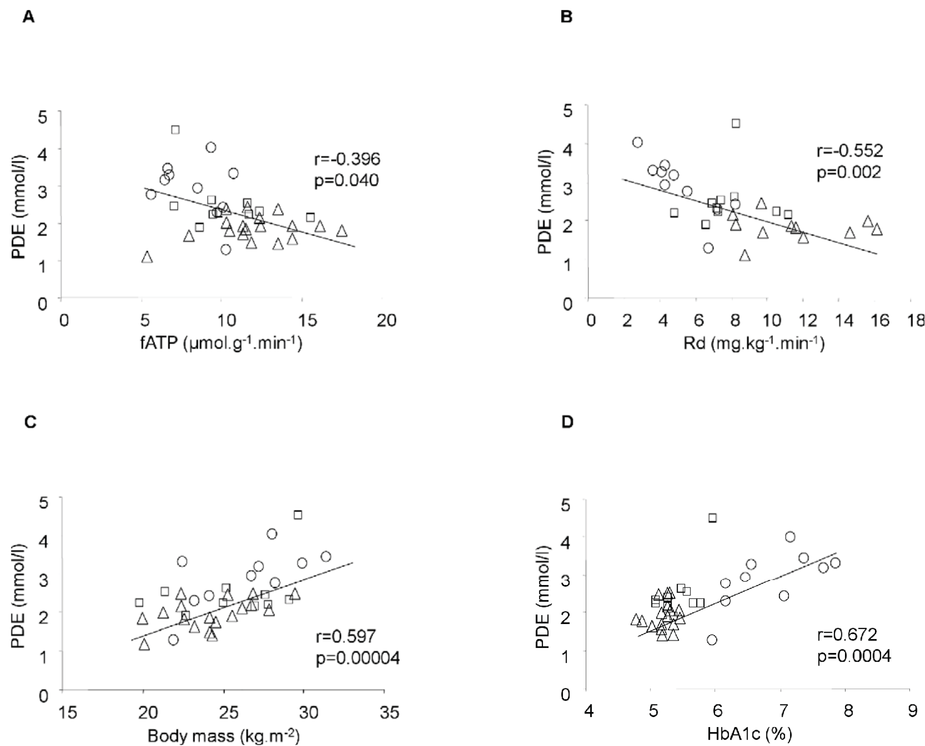

Figure 3. Association between fasting myocellular phosphodiester (PDE) contents and flux through ATP synthase (fATP) (A), whole-body glucose disposal (Rd) (B), body mass index (BMI) (C) and glycemic control (HbA1c) (D) in 10 patients with type 2 diabetes (T2D, circles), 10 age-matched (CONm, squares) and 11–18 lean young controls (CONy, triangles) during fasting.
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Discussion

Myocellular PDE levels are higher in middle-aged patients with T2D and in age-matched controls than in young non-diabetic humans. The relationships of PDE with insulin resistance and mitochondrial function seem to depend mainly on aging, body mass, but also glycemia.

The greater PDE in the middle-aged humans with or without T2D is in accordance with its positive relationship to age as reported previously [16], but confounding factors such as insulin resistance or mitochondrial function were not identified before [18]. Aging-related alterations of skeletal muscle could simply result from reduced voluntary physical activity, but PDE - in line with previous data [29] - did not relate to physical activity index. All groups of the present study were sedentary and matched for physical activity, but exercise testing was not performed. PDE has been shown to be increased in professional road cyclists [30], but to be significantly lower in sprinters than in sedentary and long-distance runners [31]. Accordingly, a large PDE signal has been reported as a sign of a larger relative content of type 1 fibers, i. e. fast-to-slow twitch muscle transformation [32]. However, this observation does not fit with increased PDE in aging or diabetic subjects, who rather have increased [33,34,35] or normal fast glycolytic fiber fractions [36,37]. Also, statins have been shown to increase muscle PDE contents [17], but only four of our patients with T2D were on statins and their PDE content was not different from the other patients. Alternatively, aging-associated cumulative oxidative damage may affect mitochondrial DNA and function and lead to muscular degenerative processes [38]. Our T2D showed lower fATP than both control groups during insulin stimulation, while resting fATP was similarly lower in T2D and CONm than in CONy [22].

In vivo and ex vivo measures reported lower oxidative capacity in exercising and resting muscle of T2D patients which result from intrinsic impairment and lower mitochondrial content [3,4,39,40,41]. fATP, reflecting demand-driven resting mitochondrial activity, is lower in insulin-resistant elderly [21,22] and non-diabetic offspring of T2D patients [42], but can be normal in T2D compared to carefully matched non-diabetic humans [22,43]. Alternatively, abnormalities in insulin signaling may be the primary events leading to impaired mitochondrial function, or both phenomena may be mutually interrelated [44]. Of note, lower mitochondrial content could be compensated for by greater mitochondrial activity suggesting that insulin sensitivity and mitochondrial function are not uniformly coupled [8,45,46,47]. Nevertheless, partial correlation analysis revealed that PDE contents relate to aging independently of BMI and physical activity, but not independently of fATP and insulin sensitivity. Thus, the impact of aging on PDE is likely mediated by insulin sensitivity and fATP.

Partial correlation analysis further identified PDE contents as an independent indicator of glucometabolic control (HbA1c and fasting plasma glucose) in T2D. Elevated PDE as observed in muscle damage [17], pain syndromes [29] and muscle dystrophies, has been previously attributed to oxidative stress [18,20,48]. One might therefore speculate that glucotoxicity-induced chronic

oxidative stress could contribute to the greater PDE as well as to the lower fATP and insulin sensitivity in our T2D. This study found a weak negative relationship between muscular PDE and fATP, which disappeared upon adjusting for age, BMI and HbA1c. While this does not exclude any role of mitochondrial damage for raising PDE levels, it makes such mechanism rather unlikely. However, fATP is only one feature of mitochondrial function and in the present study mitochondrial content and oxidative capacity were not measured. Furthermore, fATP as assessed from the ATP saturation transfer experiment with ^{31}P MRS, reflects in vivo ATP synthase flux resulting from basal energy demand and supplying processes and is therefore a measure of resting mitochondrial activity. [49].

Muscular PDE related negatively to whole-body glucose disposal but not specifically to insulin-mediated glucose transport/phosphorylation and did not change during insulin stimulation even in insulin sensitive humans. While PDE do not seem to directly reflect insulin-dependent metabolic processes, its tight relationship with BMI points to obesity-associated alterations such as fatty acid composition of membrane phospholipids which may influence insulin binding and action [50]. While lipid-induced insulin resistance generally arises from increased plasma FFA and intracellular lipids [51], this study found no association between PDE, FFA and IMCL. Nevertheless, PDE are generated by increased activities of phospholipases [16] which also yield ceramides and diacylglycerols (DAG), possible mediators of insulin resistance [52,53].

Some limitations need to be taken into account. First, no biopsies were taken so that identification of individual components of the PDE peak is not possible. However, the analysis of individual compounds contributing to the PDE signal is difficult, because ischemia during tissue handling will impact on analysis of extracted tissue [24]. Second, no indicator of oxidative stress was determined to analyse the possible role of PDE contents as a biomarker of oxidative stress. However, available markers of oxidative stress rely on various assumptions and do not offer direct measures [6,54]. Finally, our T2D cohort comprised of normal weight (n = 4), overweight (n = 4) and obese (n = 2) Caucasians yielding a mean BMI of 27 kg/m^2, which was not significantly different but tended to be higher than in controls. While T2D cohorts mostly have mean BMI exceeding 30 kg/m^2, other T2D cohorts have lower mean BMI values, e. g. 28.5 kg/m^2 in the ADVANCE study [55]. Thus, our T2D group might be suitable and representative to analyse the impact of T2D per se on PDE contents.

In conclusion, muscular PDE are higher in middle-aged patients with or without T2D than in young non-diabetic humans. Body mass and glycemia mainly determine the correlations of PDE with resting mitochondrial activity, insulin resistance and age.

Author Contributions

Conceived and designed the experiments: JS AIS WW MR. Performed the experiments: JS AIS MC MK TP AK-W PN. Analyzed the data: JS AIS MC MK TP AK-W PN. Contributed reagents/materials/analysis tools: MW WW MR. Wrote the paper: JS MR. Revised the manuscript: MK WW MW AK-W TP.

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Article No. 6.

Lower Fasting Muscle Mitochondrial Activity Relates to Hepatic Steatosis in Humans

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PATHOPHYSIOLOGY/COMPLICATIONS

OBJECTIVE

Muscle insulin resistance has been implicated in the development of steatosis and dyslipidemia by changing the partitioning of postprandial substrate fluxes. Also, insulin resistance may be due to reduced mitochondrial function. We examined the association between mitochondrial activity, insulin sensitivity, and steatosis in a larger human population.

RESEARCH DESIGN AND METHODS

We analyzed muscle mitochondrial activity from ATP synthase flux (fATP) and ectopic lipids by multinuclei magnetic resonance spectroscopy from 113 volunteers with and without diabetes. Insulin sensitivity was assessed from *M* values using euglycemic-hyperinsulinemic clamps and/or from oral glucose insulin sensitivity (OGIS) using oral glucose tolerance tests.

RESULTS

Muscle fATP correlated negatively with hepatic lipid content and HbA_{1c}. After model adjustment for study effects and other confounders, fATP showed a strong negative correlation with hepatic lipid content and a positive correlation with insulin sensitivity and fasting C-peptide. The negative correlation of muscle fATP with age, HbA_{1c}, and plasma free fatty acids was weakened after adjustment. Body mass, muscle lipid contents, plasma lipoproteins, and triglycerides did not associate with fATP.

CONCLUSIONS

The association of impaired muscle mitochondrial activity with hepatic steatosis supports the concept of a close link between altered muscle and liver energy metabolism as early abnormalities promoting insulin resistance.

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Insulin resistance associates with ectopic lipid deposition in muscle (intramyocellular lipid content [IMCL]) and liver (hepatocellular lipid content [HCL]), increased lipid availability, and cardiovascular complications (1,2). It has been suggested that muscle insulin resistance predisposes for atherogenic dyslipidemia and nonalcoholic fatty liver disease (NAFLD) by partitioning the postprandial flux of ingested carbohydrates away from muscle glycogen synthesis toward hepatic de

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novo lipogenesis (3). This would increase VLDL export and in turn raise circulating triglycerides (TGs) and LDL cholesterol (3). Some exercise studies support this concept in that reversal of muscle insulin resistance decreases HCL and dyslipidemia in humans at risk for type 2 diabetes (4,5).

However, the interaction between liver and muscle metabolism in the development of insulin resistance is not fully understood. Increased insulin-stimulated de novo hepatic lipogenesis after carbohydrate intake might represent an important sink for excess glucose, ultimately improving glucose homeostasis. On the other hand, steatosis may result from inadequate hepatic oxidation rates (6), subsequently leading to increased lipid flux to skeletal muscle. Indeed, insulin resistance frequently associates with accumulation of IMCL or lipid metabolites (3). As greater lipid availability does not necessarily increase IMCL (7,8), muscle lipid oxidation could be the link between liver and muscle metabolism (9,10). Muscle mitochondrial activity assessed from ATP synthetic flux rates (fATP) reflects ADP-stimulated oxidative phosphorylation under variable conditions of energy demand and insulinemia (11). Although fasting fATP can be lower in the state of insulin resistance (12), mitochondrial activity, capacity, and content do not necessarily correlate with insulin sensitivity in humans (9,13). These findings suggest that such dissociation between insulin sensitivity and mitochondrial function might depend on an interplay between muscle energy metabolism and hepatic fat storage. Of note, previous studies generally included small groups, thereby providing lower statistical power. To overcome this limitation, we analyzed data from a series of studies performed in well-phenotyped volunteers with and without type 2 diabetes using identical methodology for assessing fATP and HCL on the same magnetic resonance (MR) spectrometer.

RESEARCH DESIGN AND METHODS

Participants

Data were collected from six clinical experimental studies, published between 2007 and 2011, including 113 individuals who underwent assessment of muscle fATP, IMCL, and HCL by one single method applied by one research group (7,9,14–17). All participants were sedentary according to Baecke et al. (18) in all studies. Female subjects were postmenopausal or studied between days 5–9 of their menstrual cycle. For 3 days prior to every study day, participants refrained from any physical exercise, consumed an isocaloric diet, and then fasted for 12 h before the start of the experiments. The number of individuals, their glucose tolerance, and the methods for measuring glucose, insulin, and C-peptide concentrations and for assessing insulin sensitivity have been described in detail in the studies summarized in Table 1. The parameters age, sex, BMI, fATP, and either oral glucose insulin sensitivity (OGIS) or *M* value had to be available for all individuals. Individuals with type 1 diabetes and with endocrine diseases other than type 2 diabetes were excluded from this analysis. Data were obtained at baseline, i.e., in the fasted state without or before any planned interventions. The selection process of the studies is described in a flowchart (Supplementary Fig. 1). The local institutional ethics board approved all study protocols, and all subjects gave written informed consent after the nature and possible consequences of the studies had been explained to them.

Measures of Insulin Sensitivity

For the oral glucose tolerance tests (OGTTs), participants drank a solution containing 75 g of glucose dissolved in water and underwent sampling of venous blood before (zero time) and at timed intervals for 2 h. Measurements of plasma glucose and insulin at zero time, 90 min, and 120 min were used for calculating the oral glucose insulin sensitivity (OGIS) (19). OGIS is a measure of glucose clearance and represents an index of whole-body insulin sensitivity, which has been validated against the euglycemic-hyperinsulinemic clamp (20). Euglycemic (~5.5 mmol/L)-hyperinsulinemic (~500 pmol/L) clamps were performed as previously reported using a primed-continuous insulin infusion (40 mU $\cdot$ m^{-2} body surface area $\cdot$ min^{-1}) and a variable 20% dextrose infusion. Whole-body insulin sensitivity was assessed from glucose infusion rates (*M*) during the last 30 min of the clamp (9). For comparison of measures of insulin sensitivity, we checked the available files comprising data from both OGTT and euglycemic-hyperinsulinemic clamps in the same people regardless of their glucose tolerance or underlying pathology. The resulting data sets allowed the development of an equation to obtain a reconstructed *M* value (*Mr*) from OGIS. After testing several different models, a simple linear regression finally yielded the best result (R = 0.75, P = 0.0001) (Fig. 1*A*) as given by the equation *Mr* (mg $\cdot$ min^{-1} $\cdot$ kg^{-1}) = 0.023[OGIS (mL $\cdot$ min^{-1} $\cdot$ m^{2})] – 4.1. The equation was validated in several datasets from other studies, from which euglycemic-hyperinsulinemic clamps and OGTT were available (data not shown). This equation was then applied for the first time to the data of the present analysis to derive *Mr* values from OGTT data, which yielded a tight correlation (R = 0.67, $P < 0.0001$) between *Mr* and measured *M* values.

In Vivo MR Spectroscopy

All measurements were performed by MR spectroscopy (MRS) experts with

Table 1—Baseline characteristics (mean ± SD) of all participants, showing the number of participants per subgroup, sex, mean age, and glucose tolerance: normal glucose tolerance/impaired fasting glucose or impaired glucose tolerance/type 2 diabetes

n (male/female)	Age (years)	Diabetes status	Assessment of insulin sensitivity	Reference
7 (4/3)	45 ± 11	3/4/0	OGTT	17
7 (7/0)	26 ± 2	7/0/0	Clamp	7
31 (19/12)	47 ± 16	21/0/10	Clamp	9
5 (5/0)	27 ± 1	5/0/0	OGTT/clamp	14
36 (17/19)	39 ± 12	28/8/0	OGTT	15
27 (0/27)	36 ± 5	25/2/0	OGTT/clamp	16

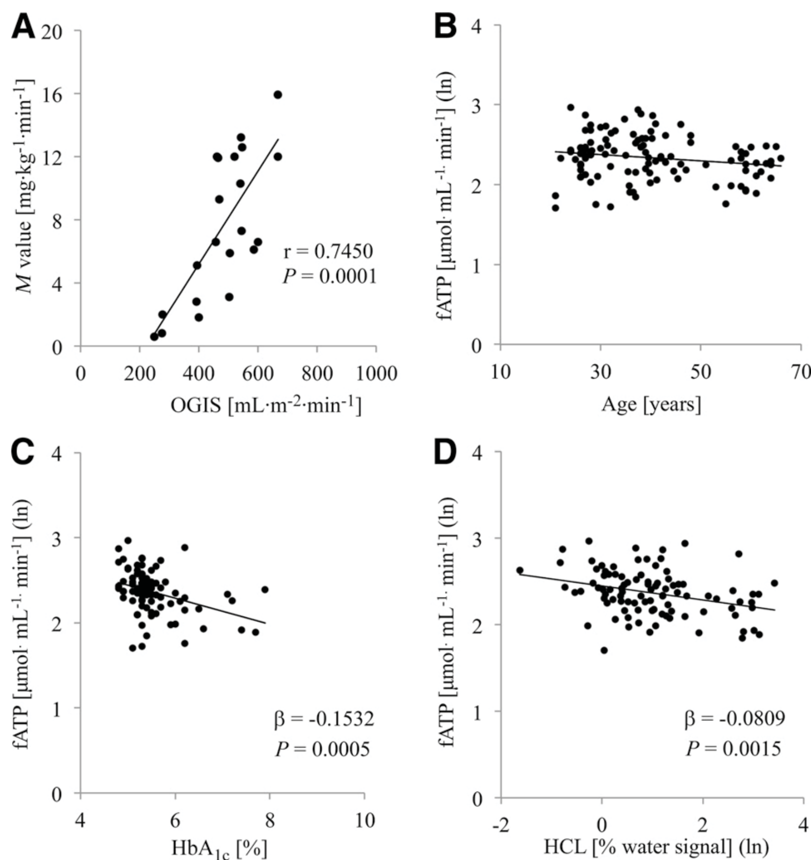

Figure 1—Relationship between OGIS and *M* value (*n* = 21 who underwent both euglycemic-hyperinsulinemic clamps and OGTT) (*A*), and associations between flux through ATP synthase during fasting (fATP [ln]) and age (*n* = 113) (*B*), HbA_{1c} (*n* = 86) (*C*), and HCL (*n* = 95) (*D*).

participants lying supine inside a 3 Tesla Medspec MR spectrometer (Bruker Biospin, Ettlingen, Germany). ^{31}P-MRS was used with a surface coil positioned ~2 cm into the medial head of the right gastrocnemius muscle and the saturation transfer experiment to measure fATP from the exchange between Pi and ATP as described earlier (7,9,12). ^{1}H-MRS was used to quantify IMCL in soleus muscle (7) and HCL as described previously (21).

Analytical Procedures

Plasma glucose was measured by the glucose oxidase method (Glucose Analyzer II; Beckman Coulter). Plasma free fatty acids (FFAs) were assayed microfluorometrically in samples containing orlistat to prevent in vitro lipolysis (Wako USA) (7). Plasma lactate was determined enzymatically (Roche) (7). Plasma insulin and C-peptide were determined by commercial double antibody RIA (7).

Statistical Analyses

Data are presented as mean ± SD. We applied univariate and multivariate linear regression analyses to evaluate the relationships between fATP and insulin sensitivity, parameters of glucose, and lipid metabolism.

Because of their skewed distributions, fATP, *M* value, HCL, fasting plasma C-peptide, a surrogate marker of β-cell function, insulin, FFA, TG, and the fasting TG-to-HDL cholesterol ratio (TG/HDL-C), which has been used as a surrogate of insulin resistance, were transformed to their natural logarithms (ln) before further analyses. In order to estimate the predictive power of individual variables, we performed regression analyses with multiple adjustments for parameters potentially confounding collinear associations with the dependent variable fATP. The multiple regression analysis for the dependent variable log-transformed flux rates through ATP synthase (fATP [ln]) included the following parameters: age, BMI, insulin sensitivity (*M* value or OGIS-derived reconstructed *Mr* value), HbA_{1c}, plasma FFAs, HCL, and IMCL (ln) as independent variables. Multiple regression with many predictor variables might serve as extension of linear regression with two predictor variables. Of note, the inclusion of a bundle of independent variables automatically induces the selection of a specific subgroup of our study population.

Thus, we performed model analyses using different models (M1–M5) for each dependent variable and controlled for a potential influence of the study group, examining investigators, and time point of investigation by adjusting for the study effect (regression models M2–M5). Model 1 (M1) was not adjusted, whereas M2 was adjusted for study effects, M3 for study effects, age, and sex, and M4 for study effects, age, sex, and BMI. The last model (M5) was further adjusted for glucose tolerance status (i.e., normal glucose tolerance, impaired fasting glucose/glucose intolerance, and type 2 diabetes). Because of the previous reports on positive associations between *M* value and muscle fATP and negative associations between *M* value and HCL, we also examined the influence of HCL on the relationship between fATP and *M* value. To this end, the analysis of fATP and *M* value in M6 was adjusted for study effects, age, sex, BMI, glucose tolerance, and, additionally, HCL.

As we aimed to compare the effect of independent variables, all of which were expressed in different units, on the dependent variable, we report standardized coefficients rather than correlation coefficients. All analyses were performed using SAS for Windows version 9.2 software (SAS Institute, Cary, NC).

RESULTS

Baseline Characteristics

The study population comprised 113 (52 male and 61 female) participants with mean age 40 ± 13 years and mean BMI 25.0 ± 3.5 kg/m^2, of whom 87 were glucose tolerant, 16 were prediabetic,

i.e., having impaired fasting glucose and/or glucose intolerance, and 10 had overt type 2 diabetes (Table 1). The individuals were rather insulin sensitive (*M* value and *Mr* value: 7.5 ± 4.6 and 7.0 ± 2.7 mg · min^{-1} · kg^{-1}) and metabolically well controlled (HbA_{1c} = 5.52 ± 0.62% [37 ± 4 mmol/mol], TG = 95 ± 49 mg/dL, FFA = 462 ± 206 μmol/L).

Fasting Muscle Mitochondrial Activity, Age, and Glucose Metabolism

Regression analyses were performed with fATP (ln) as dependent variable and the following independent variables: age, insulin sensitivity, fasting C-peptide (ln), fasting insulin (ln), fasting glucose, and HbA_{1c} (Table 2). Also, parameters potentially influencing the associations were identified and subsequently used for adjusting the regression analyses. The fATP (ln) correlated negatively with age when adjusted for study effects only, but not in M4 or M5 (Fig. 1*B*). The fATP (ln) correlated positively with insulin sensitivity (*M* value [ln] or *Mr* value [ln]) upon adjustment in M2, M3, M4, and M5. The correlation between fATP (ln) and *M* value (ln) remained upon additional adjusting for HCL (M6: P = 0.043, β = 0.225, n = 95). Fasting plasma C-peptide levels (ln) related negatively to fATP (ln) in M2, M3, M4, and M5. Whereas fasting plasma glucose and insulin did not associate with fATP (ln), HbA_{1c} correlated negatively with fATP in M1, M2, and M3 (Fig. 1*C*), but not in M4 and M5.

Fasting Muscle Mitochondrial Activity and Fat Distribution

Regression analyses were performed with fATP (ln) as dependent variable and BMI, HCL (ln), and IMCL (ln) as independent variables (Table 3). BMI as well as IMCL (ln) did not associate with fATP (ln). fATP (ln) correlated negatively with HCL in all tested models (Fig. 1*D*). In a multiple stepwise regression model, HCL was also confirmed as the only independent predictor of fATP (standardized coefficient, B: −0.339, P = 0.003). Of note, IMCL did not relate to fATP (ln) in any of the models.

Fasting Muscle Mitochondrial Activity and Lipid Metabolism

Further regression analyses were performed with fATP (ln) as dependent variable and HDL-C, TG, TG/HDL-C, and plasma FFA as independent variables (Table 3). Only fasting plasma FFA (ln) negatively related to fATP (ln), when adjusted for study effects in M2. This association disappeared either without adjustments (M1) or in M3, M4, and M5.

Table 2—Associations between fasting muscle mitochondrial activity, age, and glucose metabolism

Independent variable	*n*		Model M1	M2	M3	M4	M5
Age	113	β	−0.0039	**−0.0052**	**−0.0052**	−0.0045	−0.0033
		P value	0.0522	**0.0200**	**0.0245**	0.543	0.1869
Insulin sensitivity (ln)	113	β	0.0672	**0.2264**	**0.1905**	**0.1814**	**0.1984**
		P value	0.2434	**0.0015**	**0.0141**	**0.0331**	**0.0430**
Plasma glucose	113	β	−0.002	−0.0016	0.0029	−0.0003	0.0038
		P value	0.0719	0.1821	0.2184	0.8121	0.1182
HbA_{1c}	86	β	**−0.1532**	**−0.1382**	**−0.1088**	−0.0986	−0.0791
		P value	**0.0005**	**0.0037**	**0.0489**	0.0687	0.3196
Plasma C-peptide (ln)	84	β	−0.0793	**−0.3937**	**−0.3629**	**−0.3271**	**−0.3085**
		P value	0.1375	**0.0001**	**0.0003**	**0.0051**	**0.0167**
Plasma insulin (ln)	113	β	−0.0513	−0.00003	−0.0116	0.0190	0.0157
		P value	0.2579	0.9996	0.8439	0.7660	0.8060

Linear regression analyses with log-transformed flux rates through ATP synthase (fATP [ln]) as dependent variable and insulin sensitivity (*M* value or OGIS-derived reconstructed *Mr* value), fasting concentrations of plasma glucose, C-peptide (ln), insulin (ln), and HbA_{1c} as independent variables. All statistically significant results are represented in boldface. M1, not adjusted; M2, adjusted for study effects; M3, adjusted for study effects, age, and sex; M4, adjusted for study effects, age, sex, and BMI; M5, adjusted for study effects, age, sex, BMI, and glucose tolerance.

CONCLUSIONS

The main finding of this study is the strong and independent correlation between fasting muscle mitochondrial activity and ectopic lipid deposition in the liver. Second, this analysis, derived from a larger study population, supports previous reports on a positive relationship of fATP with age and insulin sensitivity in sedentary humans. Third, fATP relates positively to glycemia as assessed from HbA_{1c} and negatively with fasting plasma FFA. Finally, in this study population of adults with different glucose tolerance status, fATP did not relate to any other parameter of lipid metabolism or intramyocellular lipid storage.

The observed tight and significant association between muscle fATP and HCL was not detected previously. This is likely due to the lower statistical power of the individual studies, although some of the included studies at least suggested that individuals with high HCL may have lower fATP (9,16,17). Whereas steatosis and NAFLD are known to relate to insulin resistance, and even predict type 2 diabetes (6,21,22), the interaction between HCL and muscle energy metabolism is less clear. According to one theory, decreased nonoxidative storage of ingested carbohydrates in skeletal muscle represents a primary abnormality in insulin-resistant states. This would redirect glucose to the liver to serve as substrate for hepatic de novo lipogenesis and subsequently cause hyperinsulinemia, dyslipidemia, and steatosis (4). In the present analysis, muscle fATP was measured during fasting, suggesting that muscle energy metabolism may also interfere with liver energy storage in the postabsorptive state. In this context, a recent study reported higher muscle complex I activities but lower mitochondrial content in morbidly obese NAFLD patients undergoing bariatric surgery (23). This suggests that increased activities of electron transport chain components reflect adaptation of muscle mitochondria to fat overload and mitochondrial damage. Indeed, muscle complex I activity gradually increased with progressing steatosis and fibrosis in that study. Another study

Table 3—Associations between fasting muscle mitochondrial activity, fat distribution, and lipid metabolism

Independent variable	n		Model M1	M2	M3	M4	M5
BMI	113	β	−0.0119	−0.0131	−0.0099		−0.0087
		P value	0.1120	0.0968	0.2189		0.2769
HCL (ln)	95	β	**−0.0809**	**−0.0805**	**−0.0662**	**−0.0684**	**−0.0759**
		P value	**0.0015**	**0.0025**	**0.0185**	**0.0264**	**0.0232**
IMCL (ln)	112	β	0.0270	0.0614	0.0691	0.0752	0.0728
		P value	0.5591	0.2321	0.1731	0.1371	0.1542
Plasma TG (ln)	105	β	−0.0350	−0.0425	−0.0192	−0.0047	−0.0067
		P value	0.5323	0.4502	0.7427	0.9389	0.9135
Plasma HDL-C	98	β	0.0759	0.0911	0.1504	0.1430	0.1372
		P value	0.3872	0.3008	0.1199	0.1641	0.1836
TG/HDL-C (ln)	91	β	−0.0604	−0.0674	−0.0587	−0.0628	−0.0629
		P value	0.2375	0.1950	0.2904	0.3016	0.3060
Plasma FFA (ln)	104	β	−0.0981	**−1.2135**	−0.0930	−0.0989	−0.0831
		P value	0.0759	**0.0379**	0.1291	0.1090	0.1883

Linear regression with fATP (ln) as dependent variable and BMI, HCL (ln), and IMCL (ln) as independent variables. Further linear regression analyses include parameters of systemic lipid profile, namely, fasting plasma concentrations of TG (ln), HDL-C, TG/HDL-C, and plasma FFAs as independent variables. All statistically significant results are represented in boldface. M1, not adjusted; M2, adjusted for study effects; M3, adjusted for study effects, age, and sex; M4, adjusted for study effects, age, sex, and BMI; M5, adjusted for study effects, age, sex, BMI, and glucose tolerance.

found that lifestyle intervention resulting in greater muscle glucose disposal also improved steatosis and NAFLD (5). Although muscle mitochondrial function was not measured in that study, muscle mitochondrial adaptation could be one mechanism to modulate hepatic fat loading during the development of type 2 diabetes.

On the other hand, liver mitochondria could exhibit a similar abnormality as muscle mitochondria of people at risk for type 2 diabetes, which would reduce hepatic fat oxidation and thereby favor TG deposition. In support of this contention, lower liver ATP levels (21), fATP (6), and ATP recovery upon fructose challenge (24) provide evidence for lower hepatic mitochondrial function, at least in insulin-resistant groups such as severe obesity or type 2 diabetes. Furthermore, impaired hepatic energy metabolism could subsequently raise plasma FFA via lipolysis of VLDL (3) and in turn induce lipid-mediated muscle insulin resistance (25). Our observation of an independent correlation between HCL, fasting plasma FFA, insulin resistance, and lower muscle fATP is in line with this concept. Nevertheless, these cross-sectional studies do not allow definite conclusions, and, of course, potentially coincident but significant relationships do not imply any causality.

Furthermore, the positive correlation between whole-body insulin sensitivity and muscle fATP is in agreement with data of one of our smaller group studies, reporting a correlation of *M* value with fasting fATP in patients with type 2 diabetes (9). Lower fATP in insulin-resistant compared with insulin-sensitive healthy humans has also been observed in other studies during fasting (9,12,16,17), during hyperinsulinemia (9,26), after exercise (15), and during acute lipid-induced insulin resistance (7), all without reporting an association of fATP with insulin sensitivity. In vivo mitochondrial oxidative capacity as assessed from postexercise muscle phosphocreatine repletion was also reduced in patients with type 2 diabetes but did not correlate with insulin sensitivity (27). Likewise, ex vivo measures of mitochondrial function, such as mitochondrial content (28), oxidative enzyme capacity (29,30), and mitochondrial morphology (31), were lower in insulin-resistant humans, but again without association with insulin sensitivity. However, only a few cross-sectional studies found a correlation between insulin sensitivity and markers of mitochondrial function in untrained, metabolically well-controlled humans with and without type 2 diabetes (9,32,33). On the other hand, some lifestyle intervention studies found associations between improvement of insulin sensitivity and various parameters of mitochondrial function and suggest that raising glucose and lipid oxidation rates could underlie reduction of insulin resistance (34). However, aerobic exercise training can consistently improve muscle mitochondrial response in all age-groups, whereas only younger participants also improved their insulin sensitivity (35). In this context, the present analyses reported a weak negative correlation of fATP with age in sedentary humans, which underlines the predominant role of physical activity as the major determinant of mitochondrial biogenesis and function (35). These previous findings may be explained by the small size of the studied groups; however, dissociation between mitochondrial function and insulin sensitivity was also suggested. Although the individuals participating in the present combined analysis were rather insulin sensitive, lean, or overweight and mostly glucose tolerant and metabolically well controlled, fATP correlated with insulin sensitivity also upon adjustment for age, sex, BMI, and glucose tolerance. Of note, even HCL did not disrupt the relationship between fATP and insulin sensitivity. Thus, analyzing this much larger study population revealed that reduced mitochondrial activity might indeed be an early abnormality occurring during the development of insulin resistance.

Nevertheless, impairment of mitochondrial function can result from chronic lipid- and glucose-mediated increases in oxidative substrate flux rates causing oxidative stress and thereby damaging mitochondrial proteins (11). Of note, the present analysis identified HbA_{1c} as a strong predictor of fATP and fasting plasma FFA as the only lipid parameter correlating (negatively) with fATP. Increase of mitochondrial oxidative capacity conferred by lipid-induced stimulation

of the peroxisome proliferator–activated receptor γ coactivator 1-α (10,36) might coincide with lipid-induced insulin resistance in skeletal muscle. This might explain why others did not find a correlation of mitochondrial function and insulin sensitivity. On the other hand, hyperinsulinemia increases ATP synthesis (9,26), whereas this effect is blunted in insulin-resistant humans (9,26) and during lipid exposure (7). Accordingly, C-peptide levels, as surrogate of insulin secretion, related negatively to fATP. Thus, insulin deficiency and insulin resistance at the level of mitochondria and poor metabolic control, comprising impaired adaptation to prevalent metabolic conditions rather than insufficient oxidative capacity, might limit oxidation rates (11). In accordance, some (1,29), but not all, studies found higher IMCL in patients with type 2 diabetes and no correlation with insulin resistance or fasting fATP (27), which is in line with our finding.

This analysis of a number of studies, consistently acquiring data on the same MR scanner, benefits from the application of one single methodology for quantification of muscle ATP production as a measure of mitochondrial activity to a metabolically well-characterized larger study population. The applied saturation transfer method for assessing fATP by means of ^{31}P-MRS has been validated in a variety of in vitro systems and in human muscle biopsy samples, as reviewed recently (37). The limitations of this approach are that this method provides a measure of ATP synthesis/hydrolysis cycle at rest driven by energy demands rather than maximal oxidative capacity and that differences in individual mitochondrial content are not taken into account. Nevertheless, fATP associates with changes in mitochondrial content, coupling, and oxygen consumption (38) and tightly correlates with postexercise muscle phosphocreatine (39), indicating that skeletal muscle with higher maximal oxidative ATP synthetic rates is also metabolically more active at rest. An increased prevalence of insulin resistance in Asian-Indian men was associated with a twofold increase in HCL compared with Caucasian men. These data demonstrate important ethnic differences in the pathogenesis of insulin resistance and steatosis (40). One further limitation of the study might be that only Caucasian participants of identical ethnic background have been included.

In conclusion, the association of impaired muscle mitochondrial activity with hepatic steatosis supports the concept of a close link between altered muscle and liver metabolism as early abnormalities promoting insulin resistance.

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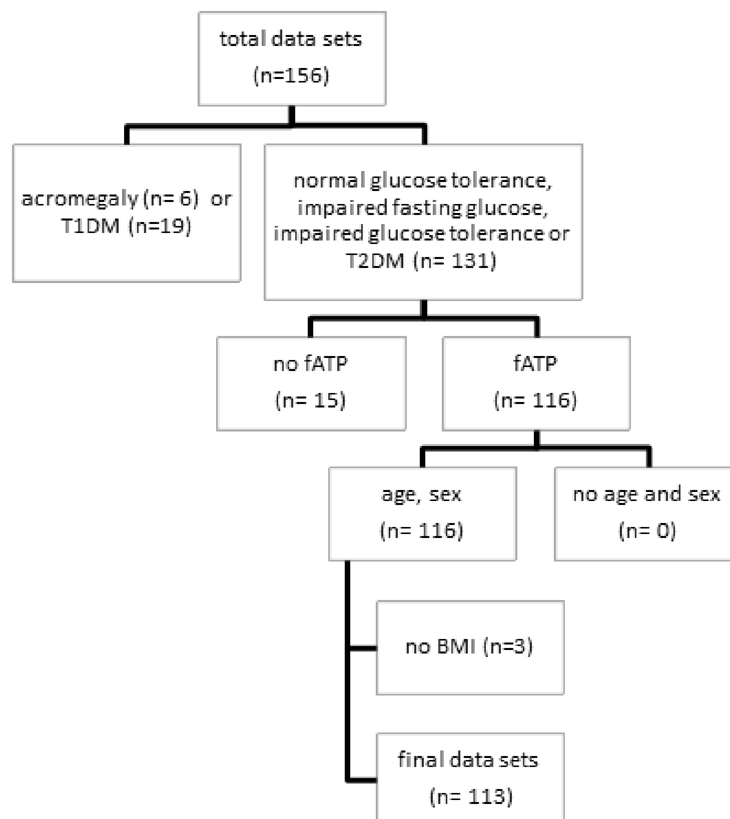
SUPPLEMENTARY DATA

Supplementary Figure 1. Flow chart of inclusion criteria: presence of flux through ATP synthase (fATP), age, body mass index (BMI), M-value, or Mr-value. Only those data sets that satisfied the depicted criteria were included into analysis. Type 1 diabetes (T1DM), type 2 diabetes (T2DM).



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Article No. 7.

Impaired Mitochondrial Function and Insulin Resistance of Skeletal Muscle in Mitochondrial Diabetes

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OBJECTIVE — Impaired muscular mitochondrial function is related to common insulin resistance in type 2 diabetes. Mitochondrial diseases frequently lead to diabetes, which is mostly attributed to defective β-cell mitochondria and secretion.

RESEARCH DESIGN AND METHODS — We assessed muscular mitochondrial function and lipid deposition in liver (hepatocellular lipids [HCLs]) and muscle (intramyocellular lipids [IMCLs]) using $^{31}P/^{1}H$ magnetic resonance spectroscopy and insulin sensitivity and endogenous glucose production (EGP) using hyperinsulinemic-euglycemic clamps combined with isotopic tracer dilution in one female patient suffering from MELAS (myopathy, encephalopathy, lactic acidosis, and stroke-like episodes) syndrome and in six control subjects.

RESULTS — The MELAS patient showed impaired insulin sensitivity (4.3 vs. 8.6 ± 0.5 mg · $kg^{-1} \cdot min^{-1}$) and suppression of EGP (69 vs. 94 ± 1%), and her baseline and insulin-stimulated ATP synthesis were reduced (7.3 and 8.9 vs. 10.6 ± 1.0 and 12.8 ± 1.3 $\mu mol \cdot l^{-1} \cdot min^{-1}$) compared with those of the control subjects. HCLs and IMCLs were comparable between the MELAS patient and control subjects.

CONCLUSIONS — Impairment of muscle mitochondrial fitness promotes insulin resistance and could thereby contribute to the development of diabetes in some patients with the MELAS syndrome.

Diabetes Care 32:677–679, 2009

The MELAS (myopathy, encephalopathy, lactic acidosis, and stroke-like episodes) syndrome is caused by a maternally inherited mtDNA mutation, resulting in defective cellular respiration. MELAS-associated diabetes has been primarily attributed to insufficient insulin secretion due to mitochondrial dysfunction of pancreatic β-cells. Insulin-resistant populations show reduced mitochondrial function and increased soleus muscle lipids (intramyocellular lipids [IMCLs]) and liver lipids (hepatocellular lipids [HCLs]) (1). The sequence of events, particularly the possibility of muscle mitochondria leading to insulin resistance, is unclear.

RESEARCH DESIGN AND METHODS

One female patient (age 37 years, BMI 24 kg/m^2, A1C 7.4%) suffering from MELAS (mtDNA mutation A3243G, ~60% heteroplasmy in leukocytes, confirmed by PCR amplification) was compared with six nondiabetic female control subjects (age 45 ± 4 years, BMI 24 ± 1 kg/m^2, A1C 5.2 ± 0.1%). The protocol was approved by an institutional ethics board, and written informed consent was obtained. The patient developed bilateral labyrinthine hypacusis and insulin-dependent diabetes at 26 years of age, underwent surgery for ptosis, and showed vascular lesions in her periventricular white matter.

Whole-body insulin sensitivity (insulin-mediated glucose disposal, *M*) and endogenous glucose production (EGP) were assessed during a normoglycemic-hyperinsulinemic clamp with infusion of insulin (40 mU · m body surface $area^{-1} \cdot min^{-1}$) (Actrapid; Novo Nordisk, Copenhagen, Denmark) and 20% dextrose containing 2%-enriched D-[6,6-$^{2}H_2$]glucose (2).

Plasma glucose was measured by the glucose oxidase method (Glucose analyzer II; Beckman Coulter, Brea, CA). Free fatty acid (FFA) was measured microfluorimetrically (Wako Chemicals, Richmond, VA). Insulin was measured by radioimmunoassay (2).

Open-air spirometry (MasterScreen CPX; Jaeger Viasys Healthcare, Hoechberg, Germany) was combined with continuous heart rate recording (SporttesterPE4000; Polar Electro, Oulu, Finland). Resting energy expenditure (REE) was assessed using the Weir equation: $REE = [3.9(V_{O_2}) + 1.1(V_{CO_2})]1.44$. Exercise testing was performed on an electronically braked cycle ergometer (Lode-Excalibur Sport, Groningen, the Netherlands).

$^{31}P/^{1}H$ magnetic resonance spectroscopy (MRS) was performed at baseline and repeated between 120 and 240 min of the clamp to assess flux through ATP synthase (fATPase) in gastrocnemius muscle with saturation transfer experiment (3-T spectrometer; Bruker, Ettlingen, Germany) (2). Glucose-6-phosphate (G-6-P) and inorganic phosphate (P_i) were measured from the ratio of integrated respective peak intensities and β-ATP resonance intensity (2). Localized ^{31}P-MRS was performed to assess PCr recovery using STEAM (the stimulated echo acquisition

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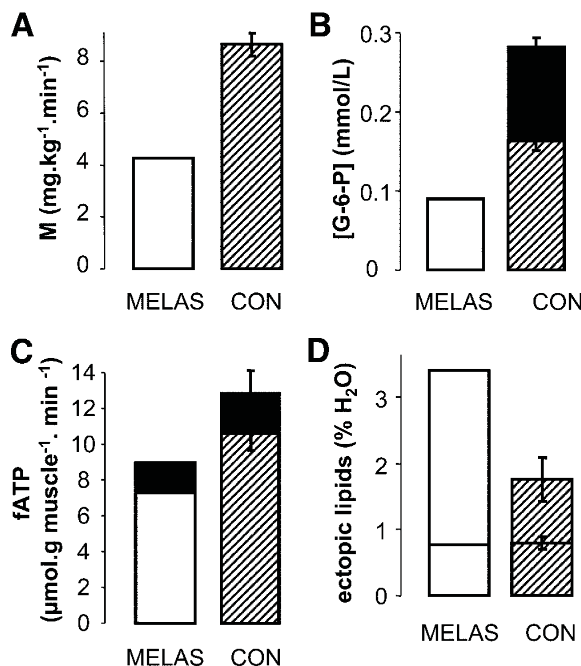

Figure 1—*Whole body insulin sensitivity* (M *value*) (A)*, glucose phosphorylation/transport (IMCL G-6-P)* (B)*, and IMCL ATP synthesis as well as ectopic lipids in skeletal muscle (IMCL, lower portion of the columns)* (C) *and liver (HCL, total columns)* (D) *in a female patient with MELAS syndrome* (□) *compared with matched control subjects* (*CON*, ▨, n = 6). ■, *Insulin-stimulated increases in G-6-P and ATP synthesis.*

mode) and the time domain fit routine AMARES (advanced method for accurate, robust, and efficient spectral fitting of MRS data). Aerobic plantar flexion exercise was performed at 50% maximal contraction until fatigue. PCr amplitudes were fitted to a mono-exponential curve using nonlinear least squares. Ectopic lipids were measured with ^{1}H-MRS (2). The patient's PCr recovery was compared with that of healthy male subjects from a previous study of ours (3), and REE was compared with the value predicted by the Harris-Benedict equation for women: $(665.1 + 9.6 \times \text{weight}) + (1.8 \times \text{height}) - (4.7 \times \text{age})$. All other tests were performed on the patient and six control subjects, and data are shown as means ± SEM.

RESULTS

Glucose and FFA metabolism

The MELAS patient had normal fasting EGP (1.7 vs. 1.7 ± 0.1 mg · kg^{-1} · min^{-1}), plasma FFA (385 μmol/l), and insulin suppression of lipolysis (95%) in comparison with the control subjects. EGP suppression (69 vs. 94 ± 1%) and *M* (4.3 vs. 8.6 ± 0.5 mg · kg^{-1} · min^{-1}) were markedly lower in the patient than in the control subjects (Fig. 1*A*).

Energy expenditure

The patient's respiratory quotient was 0.88, indicating reliance on glucose oxidation during fasting. REE was lower than predicted (1,108 vs. 1,355 kcal/24 h). Maximal power (88 W) and $V_{O_{2max}}$ (19.4 mg · kg^{-1} · min^{-1}) were reduced.

Intracellular metabolites and ATP synthesis

G-6-P did not increase after insulin stimulation, proving impaired muscle glucose transport/phosphorylation (control: +75% vs. baseline, $P < 0.001$) (Fig. 1*B*). Fasting and insulin-stimulated fATPase were lower in the MELAS patient (7.3 and 8.9 μmol · l^{-1} · min^{-1}) than in the control subjects (10.6 ± 1.0 vs. 12.8 ± 1.3 μmol · l^{-1} · min^{-1}, $P < 0.05$) (Fig. 1*C*). The patient performed plantar flexion for 6.4 min, resulting in ~60% PCr depletion, demonstrating exhaustive exercising. Postexercise PCr recovery was twice (66 ± 17 s) that of healthy volunteers (3), indicating compromised mitochondrial fitness (4). Resting ATP concentration (4.7 mmol/l) and PCr-to-P$_i$ ratio (5.0) were reduced by ~40% compared with those in healthy volunteers studied under identical conditions (5). HCLs, albeit within the normal range (<5%) (6), were higher in the MELAS patient than in the control subjects, whereas IMCLs were similar (Fig. 1*D*).

CONCLUSIONS— The MELAS patient exhibited severe reductions in ATP concentrations and synthesis at baseline, after exercise, and during insulin stimulation. This was paralleled by muscular and hepatic insulin resistance. While the cause of insulin resistance could likely be explained by primary defects of mitochondrial number and fitness, both abnormalities could also result from lipotoxicity. Lipotoxicity can cause not only β-cell dysfunction but also muscular insulin resistance via inflammatory pathways, decreasing fat oxidation and raising IMCLs (7). Normal fasting and insulin suppression of FFA as well as low IMCLs would argue against the operation of lipotoxicity in our patient. However, her severe hepatic insulin resistance and higher HCLs are in line with the contention that hepatic signals, e.g., release of lipids (6), contributed to muscular insulin resistance and mitochondrial dysfunction. Finally, despite good long-term metabolic control, chronic hyperglycemia could have aggravated insulin resistance and mitochondrial dysfunction via glucotoxicity.

Similar to most MELAS patients, our patient had normal body mass, manifested diabetes in early adulthood, and required insulin treatment (8). Although some studies found that patients with mitochondrial diabetes are insulin resistant (8), others reported normal insulin sensitivity, suggesting that the A3243G mutation in skeletal muscle does not play a causative role in diabetes development (9). As the majority of studies show that MELAS patients present with impaired glucose-stimulated insulin secretion, occurrence of diabetes has been attributed to impaired β-cell function, which strongly depends on intact mitochondrial metabolism (7). Our patient presented with severe insulin resistance, impaired insulin-stimulated glucose transport/ phosphorylation, reduction of myocellular concentrations, and synthetic flux of ATP. These alterations are similar to findings in insulin-resistant populations (elderly, obese nondiabetic humans, and first-degree relatives of patients with type 2 diabetes) who are at increased risk of diabetes (10,11). Previous reports found

increased prevalence of A3243G in patients with type 2 diabetes (12). Thus, insulin resistance along with lower muscular mitochondrial fitness could contribute to the manifestation of diabetes in MELAS patients.

In conclusion, impairment of muscle mitochondrial fitness contributes to insulin resistance in our patient with MELAS and diabetes, which resembles the mechanism described for first-degree relatives of type 2 diabetic patients. However, given the broad spectrum of MELAS defects and heterogeneity of insulin sensitivity in these patients, the present report does not permit the conclusion that MELAS is a typical model for the pathogenesis of type 2 diabetes.

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No potential conflicts of interest relevant to this article were reported.

Parts of this study were presented in abstract form at the 68th Scientific Sessions of the American Diabetes Association, San Francisco, California, 6–10 June 2008.

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Article No. 8.

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Reduced Basal ATP Synthetic Flux of Skeletal Muscle in Patients with Previous Acromegaly

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Abstract

Background: Impaired mitochondrial function and ectopic lipid deposition in skeletal muscle and liver have been linked to decreased insulin sensitivity. As growth hormone (GH) excess can reduce insulin sensitivity, we examined the impact of previous acromegaly (AM) on glucose metabolism, lipid storage and muscular ATP turnover.

Participants and Methods: Seven AM (4f/3 m, age: 46±4 years, BMI: 28±1 kg/m^2) and healthy volunteers (CON: 3f/4 m, 43±4 years, 26±2 kg/m^2) matched for age and body mass underwent oral glucose testing for assessment of insulin sensitivity (OGIS) and ß-cell function (adaptation index, ADAP). Whole body oxidative capacity was measured with indirect calorimetry and spiroergometry. Unidirectional ATP synthetic flux (fATP) was assessed from ^{31}P magnetic resonance spectroscopy (MRS) of calf muscle. Lipid contents of tibialis anterior (IMCLt) and soleus muscles (IMCLs) and liver (HCL) were measured with ^{1}H MRS.

Results: Despite comparable GH, insulin-like growth factor-1 (IGF-I) and insulin sensitivity, AM had ~85% lower ADAP ($p<0.01$) and ~21% reduced VO_2max ($p<0.05$). fATP was similarly ~25% lower in AM ($p<0.05$) and related positively to ADAP ($r=0.744$, $p<0.01$), but negatively to BMI ($r=-0.582$, $p<0.05$). AM had ~3fold higher HCL ($p<0.05$) while IMCLt and IMCLs did not differ between the groups.

Conclusions: Humans with a history of acromegaly exhibit reduced insulin secretion, muscular ATP synthesis and oxidative capacity but elevated liver fat content. This suggests that alterations in ß-cell function and myocellular ATP production may persist despite normalization of GH secretion after successful treatment of acromegaly.

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Introduction

Acromegaly generally results from slowly growing monoclonal pituitary adenomas secreting growth hormone (GH). As a result of its slow and often insidious onset, it frequently remains unrecognized for an extended time, which may give rise to sustained metabolic alterations [1]. Although improving, the overall standardized mortality of patients with acromegaly is about 1.5fold higher compared with the general population [2,3]. Among other factors, the higher prevalence of glucose intolerance and overt diabetes mellitus could contribute to the increased mortality [4].

GH stimulates protein anabolism and at the same time augments lipolysis and reduces insulin-dependent glucose disposal [5]. Patients with active acromegaly frequently exhibit mild hepatic [6] and more pronounced muscular insulin resistance [7,8] which is similar to the metabolic alterations during aging and states of obesity, inherited risk of or overt type 2 diabetes (T2DM). Noninvasive magnetic resonance spectroscopy (MRS) made it possible to identify impaired muscle glucose transport/phosphorylation from monitoring basal and insulin stimulated glucose-6-phosphate (G6P) concentrations as well as reduced hepatic glycogen synthesis as key mechanisms underlying reduced glucose disposal in human insulin resistance [9]. According to the current paradigm, inherited and environmental factors (elevated glucose or free fatty acids, FFA) lead to reduced Krebs cycle flux and ATP synthetic flux (fATP) suggesting impaired mitochondrial oxida-

tion/phosphorylation which in turn increases the ectopic deposition of lipids in hepatocytes (hepatocellular lipids, HCL) and myocytes (intramyocellular lipids, IMCL) [10]. Mitochondrial function is impaired in insulin resistant states including obese nondiabetic humans, relatives of patients with T2DM and overt T2DM. Electron microscopic examination revealed abnormalities of the morphology of muscle mitochondria in a patient with acromegaly which resolved upon surgical treatment [11]. Recently, evidence was provided that an acute 4-fold increase in plasma GH not only increased plasma insulin-like growth factor-1 (IGF-I), insulin, glucose and FFA but also shifted fuel selection into the direction of fat oxidation and stimulated muscle ATP production rate and citrate synthase activity [12]. However, to our knowledge, no data are currently available on muscle ATP synthesis in patients with acromegaly.

In addition, patients with active acromegaly may also feature altered ß cell function with hyperinsulinemia either resulting from direct effects of GH/IGF-I or from adaptation to changes in glucose and FFA availability [13,14]. Glucose intolerance secondary to acromegaly generally improves following treatment of the underlying disease, whereas no data are available on ATP synthesis and ectopic lipid deposition under these conditions.

We tested the hypothesis that the alterations associated with active acromegaly are completely reversed by normalizing the endocrine and metabolic environment implicating normal fATP. To this end, we measured insulin sensitivity and ß cell function as well as whole body energy expenditure and physical fitness. Employing noninvasive multinuclear magnetic resonance spectroscopy (MRS), we further assessed in vivo fATP, flux through creatine kinase (fCK) and glucose metabolites (G6P) in skeletal muscle as well as ectopic lipid deposition (IMCL, HCL) in patients with prior acromegaly.

Methods

Volunteers

We included seven patients with prior acromegaly (AM) and seven healthy subjects (CON) matched for age, BMI and physical activity [15]. The mean duration between assumed clinical onset based on typical symptoms and diagnosis of acromegaly was 10±4 years. The participants had no first-degree relatives with T2DM. The mean interval between successful treatment until inclusion into the study was 14±3 years, but at least 7 years. All but one patient underwent transsphenoidal surgery for GH-secreting pituitary adenomas, one patient also received adjuvant radiotherapy and gamma knife treatment. Two of them required replacement of hormones (thyroid hormone, hydrocortisone and in one case additionally estrogen and gestagens) due to postoperative hypophyseal insufficiency. Three patients had been on pre- and postsurgical treatment with somatostatin analogues and bromocriptin, but only one patient required pharmacological treatment of acromegaly during the last two years. This patient was on octreotide and pegvisomant, had normal GH and IGF-I concentrations and similar insulin secretion compared to the rest of the group, but was glucose intolerant based on the 2-h plasma glucose concentration during the oGTT. Fasting plasma concentrations of IGF-I and GH and suppression during OGTT were within the normal range at repeated measurements during the last years indicating successful treatment in patients with acromegaly.

Experimental protocol

The protocol was approved by the local human ethical board (Ethics Committee of the Medical University of Vienna), and written informed consent was obtained from each volunteer. All participants were instructed to ingest a mixed diet (25–30 kcal per kg bodyweight per day consisting of 60% carbohydrates, 25% fat, 15% proteins) for three days prior to the studies. They refrained from any physical exercise for three days and fasted for 12 hours and did not take any medications before the experiments. A 75-gram oral glucose tolerance test (OGTT) was performed to assess glucose tolerance, insulin sensitivity and secretion. Magnetic resonance spectroscopy (MRS) and respiratory gas exchange measurements were carried out on separate days with intervals of at least one week each at identical daytimes in the morning.

MRS

All measurements were performed during resting conditions in participants lying supine inside a 3-T spectrometer (Bruker, Germany) using a 10-cm circular double resonant surface coil for $^{1}H/^{31}P$ measurements [16]. ^{31}P MRS allowed determination of fATP from the exchange between inorganic phosphate (Pi) and ATP (Pi→ATP) applying saturation transfer to calf muscle as described [16,17]. In analogous fashion, the exchange between phosphocreatine (PCr) and ATP (PCr→ATP) was used to calculate total (cytosolic and mitochondrial) flux through creatine kinases (fCK) [18,19]. Intramyocellular concentrations of G6P, Pi, PCr and phosphodiesters (PDE) were measured from the ratio of the respective integrated peak intensities and ß-ATP resonance intensity in spectra without inversion and saturation assuming constant ATP concentrations of 5.5 mmol/l muscle [20]. ^{1}H MRS allowed to measure IMCL in soleus and tibialis muscles [21] and HCL as described [22].

OGTT

The volunteers drank a solution containing 75 grams of glucose and venous blood samples were collected before and in 30-min intervals during 150 min for measurements of plasma glucose, insulin, C-peptide. Plasma concentrations of insulin, C-peptide and IGF-I were determined by double antibody radio immunoassay [22]. Further, dynamic insulin sensitivity were assessed with the OGIS [23], a measure of glucose clearance which has been validated against insulin sensitivity obtained from the euglycemic-hyperinsulinemic clamp [24]. Insulin secretion in relation to ambient insulin sensitivity was assessed with the insulinogenic index (ISEC), an indicator of first phase insulin secretion which is calculated as the ratio between the supra-basal increments at 30 min of insulin and glucose concentration [24]. The adaptation index (ADAP) is based on the areas under the concentration curves of plasma glucose and C-peptide during the OGTT and is a marker of responsive ß cell function [25].

Respiratory gas exchange measurement

After a 30-min resting period, measurements were performed on subjects in supine position under a canopy using an open-air spirometry system (Jaeger/Viasys MasterScreen CPX. Wuerzburg. Germany) combined with continuous heart rate recording (Sporttester PE4000, Polar Electro, Kempele, Finland). Resting energy expenditure (REE, kcal/24 h) was assessed from two 30-min sets which were separated by 15-min breaks in a quiet air conditioned laboratory (21°C) using the equation: 3.91 VO_2 −1.10 VCO_2 – 1.93 N [26,27]. Thereafter, the volunteers performed an incremental exercise test on an electronically-braked cycle ergometer (Lode Excalibur Sport, Groningen, Netherlands). The exercise test started at an initial level of 20 W followed by 7, 10, 15 or 20 W increments every min until exhaustion. The respiratory compensation point (RCP) was determined from incremental testing as described previously [28].

Statistical analysis

Data are presented as means±SEM. Statistical comparisons between study groups were performed using two-tailed Student's *t* tests or the Mann-Whitney-Wilcoxon test for parameters not having a normal distribution (ADAP, ISEC, GH, IGF-I, HCL, IMCLs) as assessed from the Levene test. Linear correlations are Pearson product-moment correlations. Differences were considered significant at the 5% level.

Results

Anthropometric and laboratory data are summarized in **Table 1**. In AM, fasting plasma concentrations of IGF-I and GH did not differ from CON and were within the normal range indicating successful treatment of the patients with previous acromegaly. Fasting plasma glucose was ~17% higher in AM ($p<0.05$) and four patients had impaired fasting glucose (defined as fasting plasma glucose levels of 5.6 to 6.9 mmol/l), whereas plasma insulin and C-peptide were not different. All but one participant had normal glucose tolerance (as defined by the 2-h plasma glucose concentration less than 7.8 mmol/l the during OGTT) during active acromegaly and at follow-up visits following successful treatment. Fasting plasma insulin and C-peptide related positively to IGF-I levels ($r=0.552$ and $r=0.646$, $p<0.05$). Insulin secretion indices (ADAP, ISEC) were ~85% lower in AM than in CON ($p<0.05$). Insulin sensitivity (OGIS) was comparable between groups (**Figure 1**) and correlated negatively with fasting plasma FFA ($r=-0.744$, $p<0.05$). FFA further related positively to GH levels ($r=0.782$, $p<0.01$).

Parameters of whole body oxidative capacity during resting and exercise are summarized in **Table 2**. REE, resting O_2 consumption (AM: 0.25±0.02, CON: 0.28±0.02 $l.min^{-1}$) and CO_2 production (AM: 0.20±0.01, CON: 0.23±0.02 $l.min^{-1}$), the respiratory quotient (RQ), as well as the derived oxidation rates for glucose (GOX) lipids (LOX) and proteins (POX) were comparable. GOX related negatively to FFA ($r=-0.718$, $p<0.05$) and GH ($r=-0.729$, $p<0.05$) and LOX related positively to skeletal muscle fATP ($r=0.637$, $p<0.05$).

AM had ~21% lower maximal oxygen uptake (VO_2max) and oxygen uptake at the RCP (VO_2rcp) ($p<0.05$, **Figure 1**), the respective power output measures tended to be lower in AM but did not reach significance.

Table 1. Anthropometric and laboratory data (means±SEM) in subjects with prior acromegaly (AM) and controls (CON).

	AM	CON
N (f/m)	4/3	3/4
Age (years)	46±4	43±4
BMI (kg/m²)	27.8±1.3	25.6±1.5
Waist-to-hip ratio	0.86±0.02	0.86±0.04
HbA1c (%)	5.4±0.2	5.3±0.1
Insulin-like growth factor-1 (IGF-I, ng/ml)	227±59	141±18
Growth hormone (GH, U/ml)	2.4±0.6	3.3±1.5
Fasting glucose (mmol/l)	5.5±0.2	4.7±0.1 *
2-hour glucose (mmol/l)	6.2±0.9	5.2±0.4
Fasting insulin (pmol/l)	43.4±8.2	37.2±5.7
Fasting C-peptide (pmol/ml)	2.4±0.3	1.9±0.3
Fasting plasma FFA (μmol/l)	368±96	285±29
ISEC (×10⁻⁶)	0.8±0.3	6.5±1.5 §
ADAP (l.min⁻¹.m⁻².10⁻³)	0.4±0.2	2.7±0.6 §

*$P<0.01$.
§$P<0.05$ vs. AM.
doi:10.1371/journal.pone.0003958.t001

Intramyocellular G-6-P (both groups: 0.09±0.01 mmol/L) and pH (7.05±0.0) were comparable in both groups. Skeletal muscle fATP was ~25% lower in AM ($p<0.05$, **Figure 1**) and related positively to ADAP ($r=0.744$, $p<0.01$) and ISEC ($r=0.687$, $p<0.01$) and negatively to BMI ($r=-0.582$, $p<0.05$). The rate constant of ATP synthesis (*k*1) was ~25% lower in AM (AM: 0.06±0.01, CON: 0.08±0.01 s^{-1}, $p<0.01$), PCr/Pi ratios (AM: 7.7±0.6, CON: 7.3±0.2) and fCK (AM: 317±23, CON: 350±13 $\mu mol.l^{-1}.min^{-1}$) were comparable.

HCL was three-fold higher in AM than in CON (**Figure 2**). IMCLs and IMCLt did not differ between the groups. IMCLs, but not IMCLt, related negatively to insulin sensitivity ($r=-0.799$, $p<0.001$). IMCLt, but not IMCLs, related positively to plasma IGF-I ($r=0.726$, $p<0.01$) (**Figure 3**).

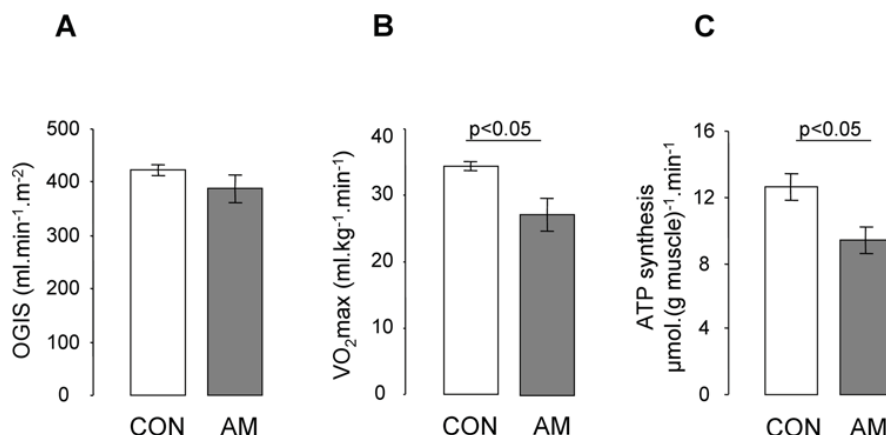

Figure 1. Whole-body insulin sensitivity, maximal oxygen consumption and muscle mitochondrial ATP production (means±SEM): (A) OGIS (B) VO_2max ($p<0.05$) and (C) flux through ATP synthesis (fATP) ($p<0.05$) in 7 subjects with previous acromegaly (AM, full bars) and 7 age- and body mass index-matched controls (CON, empty bars).
doi:10.1371/journal.pone.0003958.g001

Table 2. Expired gas analysis during resting (indirect calorimetry) and exercise (spiroergometry).

	AM	CON
Resting energy expenditure (kcal/24 h)	1678±118	1889±167
Glucose oxidation ($mg.kg^{-1}.min^{-1}$)	1.5±0.2	1.4±0.3
Lipid oxidation ($mg.kg^{-1}.min^{-1}$)	0.6±0.1	0.7±0.1
Protein oxidation ($mg.kg^{-1}.min^{-1}$)	0.7±0.1	0.7±0.2
Respiratory quotient	0.84±0.04	0.83±0.04
VO_2max ($ml.kg^{-1}.min^{-1}$)	27±2	34±1 §
Maximal power output (W)	180±26	229±17
VO_2rcp ($ml.kg^{-1}.min^{-1}$)	22±2	28±1 §
Power output at RCP (W)	130±16	179±12

Fasting substrate oxidation and parameters of physical fitness (means±SEM) in subjects with prior acromegaly (AM) and controls (CON).
§P<0.05 vs. AM.
doi:10.1371/journal.pone.0003958.t002

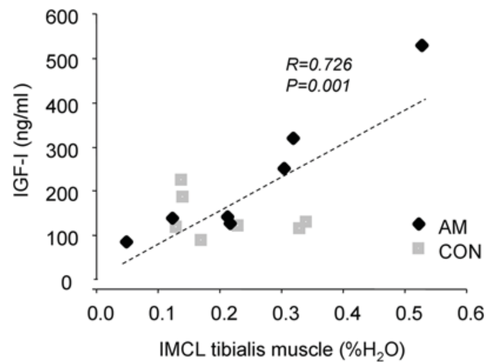

Figure 3. Relationship between ectopic lipid content of tibialis muscle (IMCLt) and plasma concentrations of insulin-like growth factor-1 (IGF-I) in 7 subjects with previous acromegaly (AM, black squares) and 7 age- and body mass index-matched controls (CON, grey squares) (r = 0.726, p<0.05).
doi:10.1371/journal.pone.0003958.g003

Discussion

Humans with a history of acromegaly exhibit normal whole body insulin sensitivity despite impaired ß cell function. Nonetheless, noninvasive measurements unmasked abnormalities in energy metabolism such as impaired muscular ATP synthesis during resting conditions, reduced maximal oxygen consumption and increased hepatic triglyceride contents years after successful treatment of acromegaly. This is similar to the reduced mitochondrial function observed in elderly or type 2 diabetic patients [17,29,30].

This study is consistent with previous reports showing that whole-body insulin sensitivity is normal after cure of acromegaly [31,32]. It is well known that reduced insulin-sensitive muscular glucose uptake in untreated acromegalic patients is mediated by prolonged increases in GH and IGF-I [8,33]. The cellular mechanisms of acromegaly-associated insulin resistance involve direct GH-mediated expression of suppressors of cytokine signaling (SOCS-1/-6) [34] or indirect activation of nutrient-sensing pathways [35] due to excessive lipolysis [20] and proteolysis [36,37]. Dietary proteins and amino acids promote insulin resistance via the mammalian target of rapamycin, a nutrient sensor that activates a negative feedback loop toward insulin receptor substrate-1 signalling [37]. But for the most part, increased plasma FFA can cause serine phosphorylation of insulin-receptors substrate-1 and thereby inhibit proximal steps of insulin signaling [38]. Accordingly, in the present study, FFA related positively to plasma levels of GH and negatively to insulin sensitivity. In addition, therapeutic interventions may also affect insulin action and secretion: first, transsphenoidal surgery can either be not sufficient to normalize GH and IGF-I secretion or alternatively lead to deficiency of other hypophyseal hormones involved in metabolism [39] and second, somatostatin analogues and GH receptor analogues can specifically affect insulin secretion or glucose metabolism. In the present study, only one patient was on acromegaly-specific medication, but all patients had plasma concentrations of GH, IGF-I, other hypophyseal hormones as well as circulating FFA which were not different from the matched control group. Thus, it is not surprising that whole-body insulin sensitivity was not impaired in these patients with a history of acromegaly.

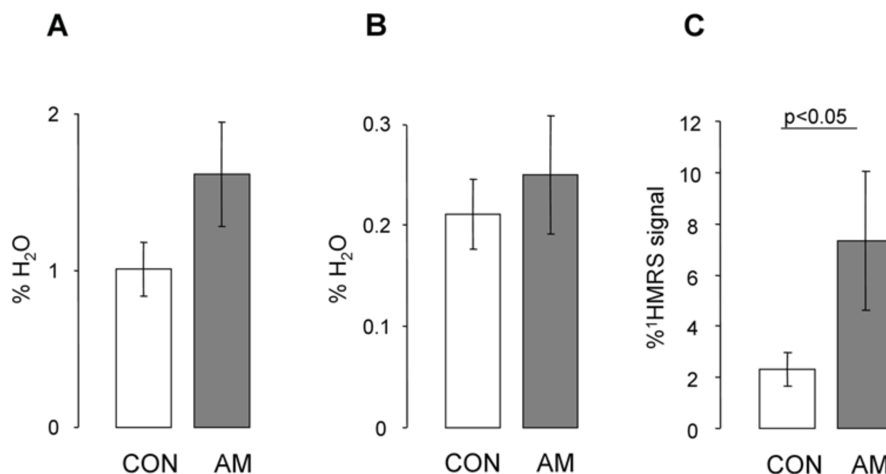

Figure 2. Ectopic lipid deposition: (A) in M.soleus (IMCLs), (B) M.tibialis ant. (IMCLt) and (C) in the liver (HCL) (p<0.05).
doi:10.1371/journal.pone.0003958.g002

On the other hand, the participants with previous acromegaly exhibited severe impairment of dynamic insulin secretion. Both insulin and IGF-I are important regulators of ß cell development, ß cell mass and function [40,41]. Chronic GH/IGF-I excess during active acromegaly induces hyperinsulinemia which is likely mediated by elevation of plasma glucose as well as by direct ß cytotrophic effects [13,41]. Accordingly, IGF-I levels related positively to fasting plasma concentrations of insulin and C-peptide in the present study. During early stages of T2DM, exhaustive stimulation of ß cells to counterbalance insulin resistance precedes the progressive ß cell failure involving impaired function and loss of ß cell mass [42]. The pathophysiological mechanisms might involve excess of nutrient availability, particularly of FFA [43], hyperglycemia, adipocytokine-mediated endoplasmic reticulum stress (release of reactive oxygen species) and cellular inflammation [44]. These mechanisms could also apply for the present study, assuming that the participants acquired their ß cell defect during active acromegaly. Ultimately, insulin secretory responses rely on intact mitochondrial function to provide for sufficient ATP and - as recently demonstrated - GTP for glucose-stimulated insulin secretion [45]. Thus, mitochondrial impairment of ß cells could also underly reduced adaptive insulin secretion.

During active acromegaly, there is evidence that GH acutely reduces glucose oxidation and impairs glucose disposal directing glucose fluxes into the non-oxidative pathway. In line with this contention, rates of lipid oxidation and non-oxidative glucose metabolism were found to be increased while oxidative glucose metabolism was decreased [31,46]. However, this seems to hold true for high plasma FFA concentrations because no reduction in oxidative metabolism was detected in the presence of normal plasma FFA [8,47]. Accordingly, in the present study GOX related negatively to FFA and GH. However, resting energy expenditure, as well as substrate specific oxidation rates did not differ between the study groups. On the other hand, despite comparable physical activity oxygen uptake during maximal exhaustion and at the RCP were reduced in the group of patients with previous acromegaly. The RCP marks the onset of hyperventilation during incremental exercise mainly driven by the onset of lactic acidosis [48] and thus is a good marker of oxidative capacity.

To our knowledge, no previous data are available on in vivo ATP production in humans with prior acromegaly. Here, such humans showed reduction of ATP synthetic flux rates (fATP) to a similar extent as reported in other states of impaired oxidative phosphorylation [30,49]. This is also reflected by the positive relationship between fasting whole body lipid oxidation and skeletal muscle fATP. It has been shown that in combination with endurance training, GH injections can enhance the positive effects of physical activity on muscle mitochondrial enzyme activities [50]. A recent report also investigated the sole effects of continuous GH infusion for 14 h and found that GH not only induced several mitochondrial genes but also promoted an increase in mitochondrial capacity in particular for fat oxidation and a shift in whole body fuel utilization toward enhanced fat utilisation [12]. Augmented lipid availability leads to accumulation of lipid metabolites such as long-chain fatty acyl CoA, diacylglycerols and ceramides which are known to impair insulin signalling and damage mitochondria [10]. In the present study, insulin sensitivity related negatively to IMCL of soleus muscle, although IMCL were comparable in both groups. Nevertheless, the relationship between IMCL of tibialis muscle and IGF-I suggests that active acromegaly could favour prolonged lipid accumulation with long-term deleterious effects on mitochondria. On the other hand, patients with prior acromegaly had markedly increased HCL. In the absence of alcohol intake or other hepatotoxic agents, HCL accumulation, i.e. steatosis or non-alcoholic fatty liver [51], could be a key factor in the development of insulin resistance and T2DM [52]. These data are in line with a previous study reporting 4–7fold higher HCL, but unchanged IMCL contents in T2DM with impaired fATP [30]. Thus, excessive hepatic lipid storage relates to insulin resistance, hepatocellular mitochondrial dysmorphology, depletion of mtDNA and decreased activity of ETC and could be interpreted as mitochondrial impairment of hepatocytes [53].

Of note, one limitation resides in the fact that the study design does not allow discrimination whether these abnormalities directly result from the previous GH/IGF-I excess or develop as a long-term consequence of metabolic alterations due to glucose, lipids and adipocytokines, because no patients with active acromegaly were included in this study. It is also impossible to sort out whether dysfunction of ß cells or of myocytes occurs first and to which extent these alterations are interrelated. Furthermore, the observed reduction in basal ATP synthesis flux does not equal mitochondrial dysfunction but could reflect a decreased ATP demand, a decreased mitochondrial content, and/or an impaired intrinsic mitochondrial function [54].

Long-term prospective studies in patients before and after successful acromegaly treatment are required to address these issues. Regardless of the cause and sequence of abnormalities in humans with prior acromegaly, the sustained disruption of ATP synthesis along with severely impaired ß cell function could contribute to an increased risk of this population for developing diabetes despite successful acromegaly therapy.

Author Contributions

Conceived and designed the experiments: AIS MR. Performed the experiments: JS EZ AIS MC GS PN. Analyzed the data: JS AIS MC GP GS OW MR. Contributed reagents/materials/analysis tools: GK PN OW CS GS KK. Wrote the paper: JS MR.

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Article No. 9.

Abnormal Hepatic Energy Homeostasis in Type 2 Diabetes

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Increased hepatocellular lipids relate to insulin resistance and are typical for individuals with type 2 diabetes mellitus (T2DM). Steatosis and T2DM have been further associated with impaired muscular adenosine triphosphate (ATP) turnover indicating reduced mitochondrial fitness. Thus, we tested the hypothesis that hepatic energy metabolism could be impaired even in metabolically well-controlled T2DM. We measured hepatic lipid volume fraction (HLVF) and absolute concentrations of γATP, inorganic phosphate (Pi), phosphomonoesters and phosphodiesters using noninvasive $^{1}H/^{31}P$ magnetic resonance spectroscopy in individuals with T2DM (58 ± 6 years, 27 ± 3 kg/m²), and age-matched and body mass index–matched (mCON; 61 ± 4 years, 26 ± 4 kg/m²) and young lean humans (yCON; 25 ± 3 years, 22 ± 2 kg/m², $P < 0.005$, $P < 0.05$ versus T2DM and mCON). Insulin-mediated whole-body glucose disposal (M) and endogenous glucose production (iEGP) were assessed during euglycemic-hyperinsulinemic clamps. Individuals with T2DM had 26% and 23% lower γATP (1.68 ± 0.11; 2.26 ± 0.20; 2.20 ± 0.09 mmol/L; $P < 0.05$) than mCON and yCON individuals, respectively. Further, they had 28% and 31% lower Pi than did individuals from the mCON and yCON groups (0.96 ± 0.06; 1.33 ± 0.13; 1.41 ± 0.07 mmol/L; $P < 0.05$). Phosphomonoesters, phosphodiesters, and liver aminotransferases did not differ between groups. HLVF was not different between those from the T2DM and mCON groups, but higher ($P = 0.002$) than in those from the yCON group. T2DM had 13-fold higher iEGP than mCON ($P < 0.05$). Even after adjustment for HLVF, hepatic ATP and Pi related negatively to hepatic insulin sensitivity (iEGP) ($r = -0.665$, $P = 0.010$, $r = -0.680$, $P = 0.007$) but not to whole-body insulin sensitivity. *Conclusion:* These data suggest that impaired hepatic energy metabolism and insulin resistance could precede the development of steatosis in individuals with T2DM. (HEPATOLOGY 2009;50:1079-1086.)

Approximately 70% of patients who are obese or who have type 2 diabetes mellitus (T2DM) have nonalcoholic fatty liver (NAFL)[1-5] of whom 20% show progression to nonalcoholic steatohepatitis (NASH).[6] Nonalcoholic fatty liver disease (NAFLD) refers to hepatocellular lipid contents of >5.5% with varying degrees of inflammation in individuals consuming less than 20 g of alcohol per day.[7-10]

Liver fat content relates negatively to hepatic insulin sensitivity as assessed from impaired insulin-suppressed endogenous glucose production (iEGP).[7,11] Increased liver fat may precede and even predict the onset of T2DM[12,13] and cardiovascular disease.[14] Those with T2DM typically not only feature steatosis and hepatic insulin resistance, but also whole-body insulin resistance, which in turn frequently associates with increased intramyocellular lipid contents (IMCL).[15] Skeletal muscle of individuals with T2DM and their nondiabetic first-degree relatives shows reduced mitochondrial activity, which has been related to insulin resistance and may result from impaired function and/or density of mitochondria.[16] The role of ectopic lipid deposition in skeletal muscle as cause or consequence of reduced oxidative capacity is yet unclear.[17,18]

Interestingly, liver biopsies of patients with steatohepatitis also reveal mitochondrial dysmorphologies and re-

Abbreviations: EGP, endogenous glucose production; HCL, hepatocellular lipids; HLVF, hepatic lipid volume fraction; iEGP, insulin-suppressed endogenous glucose production; IMCL, intramyocellular lipid contents; jMRUI, Java-based Magnetic Resonance User Interface; mCON, age-matched and BMI-matched controls; M, insulin-mediated whole-body glucose disposal; MRSI, magnetic resonance spectroscopic imaging; NAFL, nonalcoholic fatty liver; NAFLD, nonalcoholic fatty liver disease; [P], concentrations of phosphorus metabolites; PCr, phosphocreatine; Pi, inorganic phosphate; Pi_a, γATP_a, Pi and γATP concentrations adapted for HLVF; PME, PDE phosphomonoesters and phosphodiesters; R_a, rates of glucose appearance; r, Pearson correlation coefficients; R, Spearman correlation coefficients; T2DM, type 2 diabetes mellitus; yCON, young lean human controls.

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duced activities of respiratory chain enzymes.[19,20] The possible role of mitochondrial function in the development of liver steatosis and insulin resistance could be as follows[15]: increased free fatty acid availability increases hepatocellular acyl-coenzyme A molecules which impair insulin signaling thereby enhancing EGP and stimulating both storage of triglycerides and lipid oxidation. Increased lipid oxidation and citrate cycle activity will give rise to reactive oxygen species and diminish mitochondrial function. As a result, hepatocellular adenosine triphosphate (ATP) concentrations would decrease. Combined lipotoxicity, oxidative stress, and proinflammatory mediators, also key factors in the development of muscular insulin resistance,[1] would further damage hepatic mitochondria leading to mitochondrial dysmorphologies and promoting the transition of steatosis to NASH.[2,11] In this context, hyperlipidemia was found to decrease both insulin sensitivity and mitochondrial function at the level of skeletal muscle.[21] However, no data on the association between ectopic fat content, insulin sensitivity, and mitochondrial function are currently available at the level of the liver.

Thus, we hypothesized that humans with hepatic insulin resistance have decreased hepatocellular ATP concentrations and that the reduction of hepatic phosphorus compounds relates to hepatic insulin resistance and to lipid contents. To this end, we studied patients with T2DM, who present with hepatic insulin resistance, and tested the hypotheses that (1) patients with T2DM have decreased hepatic inorganic phosphate (Pi) and γATP and that these phosphorus compounds relate negatively to (2) hepatic lipid contents and (3) insulin sensitivity.

In order to address these hypotheses, we applied a recently developed technique using noninvasive phosphorus magnetic resonance spectroscopy (^{31}P MRS). This method allows measurement of phosphorus metabolites in various regions of the liver using three-dimensional MRS imaging with an external reference standard for the absolute quantification of hepatocellular γATP, Pi, and phosphodiesters and phosphomonoesters (PDE, PME) in humans.[22] Due to its low intraindividual variability, this technique makes it possible to trace subtle changes of hepatocellular phosphorus metabolites.

Table 1. Clinical Characteristics and Absolute Concentrations of Hepatic Phosphorous Metabolites (Means ± SD) of Patients with Type 2 Diabetes (T2DM), Matched Controls (mCON), and Young Controls (yCON)

Characteristic	T2DM	mCON	yCON
Sex (female/male)	3/6	4/5	3/6
Age (years)	58 ± 6	61 ± 4	25 ± 3*
BMI (kg/m^2)	26.9 ± 3.4	25.5 ± 3.6	21.9 ± 1.7†
HbA1c (%)	7.1 ± 1.0‡	5.5 ± 0.3	5.1 ± 0.2
ALT (U/L)	27 ± 11	22 ± 6	19 ± 6
Pi_a (mmol/L)	1.08 ± 0.25	1.50 ± 0.39§	1.43 ± 0.23§
γATP_a (mmol/L)	1.88 ± 0.38	2.50 ± 0.61§	2.26 ± 0.29§
PME (mmol/L)	1.81 ± 0.58	2.43 ± 0.77	2.29 ± 0.29
PDE (mmol/L)	10.36 ± 2.46	12.24 ± 2.93	11.9 ± 2.72
Pi/γATP	0.58 ± 0.02	0.59 ± 0.02	0.64 ± 0.02
PME/γATP	1.06 ± 0.24	1.10 ± 0.31	1.06 ± 0.17
PDE/ γATP	6.20 ± 1.20	5.53 ± 1.05	5.40 ± 0.90

Glycosylated hemoglobin A1c (HbA1c), alanine aminotransferase (ALT), inorganic phosphate (Pi), and γATP adapted for HLVF (Pi_a and γATP_a), phosphomonoesters (PME) and phosphodiesters (PDE).

*$P < 10^{-6}$ yCON versus T2DM and mCON.

†$P < 0.05$ yCON versus T2DM and mCON.

‡$P < 10^{-5}$ T2DM versus CONm and yCON.

§$P < 0.05$ versus T2DM.

Patients and Methods

Human Subjects. Twenty-seven volunteers consisting of individuals with T2DM and age-matched and body mass index (BMI)-matched (mCON) and young nondiabetic human (yCON) controls were included in this study (Table 1). All participants were recruited by means of public advertisement and underwent complete medical history, examination, and laboratory tests to exclude viral hepatitis and other major diseases. Self-reported alcohol consumption was below 20 g/day. Patients presenting with islet cell antibodies and diabetes-related complications were excluded. The control groups had neither any family history of diabetes nor any medication use. None of the participants were trained or performed intense exercise. The study protocol conformed with the ethical guidelines of the 1975 Declaration of Helsinki as reflected by a priori approval by the appropriate institutional review committee. Informed consent was obtained from each volunteer after explanation of the purpose, nature, and potential complications of the study.

Experimental Protocol. All participants refrained from any physical exercise and were on an isocaloric diet

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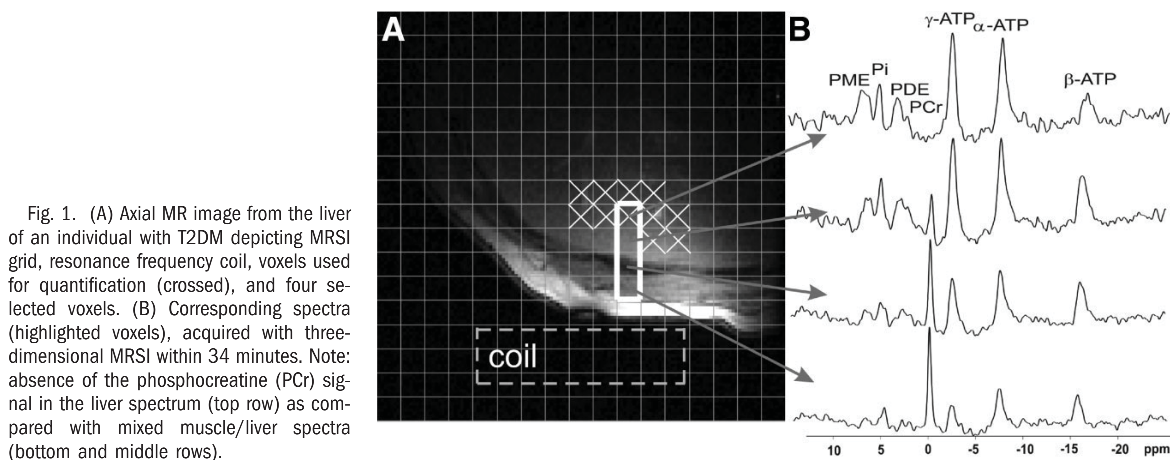

Fig. 1. (A) Axial MR image from the liver of an individual with T2DM depicting MRSI grid, resonance frequency coil, voxels used for quantification (crossed), and four selected voxels. (B) Corresponding spectra (highlighted voxels), acquired with three-dimensional MRSI within 34 minutes. Note: absence of the phosphocreatine (PCr) signal in the liver spectrum (top row) as compared with mixed muscle/liver spectra (bottom and middle rows).

for 3 days and fasted for 12 hours before the start of the experiments. According to previous studies, sulfonylurea were withdrawn at one, metformin and alpha-glucosidase inhibitors at 3 days before the clamps.[7,9] Patients treated with insulin (glitazones), incretins, or lipid-lowering drugs were not included in the study. In the morning of the study day, hepatic lipids and phosphorus compounds were measured with $^{1}H/^{31}P$ MRS (n = 27). On a separate day, whole-body glucose disposal (M) and endogenous glucose production (EGP) were measured in 16 participants (T2DM: three female, four male; mCON: four female, three male; yCON: one female, one male) using clamp studies as described.[7] Briefly, a primed-continuous infusion of D-[6,6-$^{2}H_2$]glucose (98% enriched; Cambridge Isotope Laboratories, Andover, MA) was commenced at 6:30 AM. The euglycemic-hyperinsulinemic clamp was started at 10:00 AM with a primed-continuous insulin infusion (Actrapid; Novo, Bagsvaerd, Denmark; 40 mU/m^2 body surface area/minute) lasting for 240 minutes. A 20% dextrose infusion labeled with D-[6,6-$^{2}H_2$]glucose (2% enriched) was periodically adjusted to maintain euglycemia.[7]

Analytical Procedures. Plasma glucose was measured by the glucose oxidase method (Glucose analyzer II; Beckman Instruments Inc., Fullerton, CA). Plasma triglycerides were assayed colorimetrically (Roche, Vienna, Austria) and insulin was quantified radioimmunometrically.[7]

$^{31}P/^{1}H$ MRS. Volunteers were scanned in the prone position with the 10-cm-diameter linearly polarized surface coil positioned under the lateral aspect of the liver in a 3-T whole-body scanner (Medspec S30/80; Bruker Biospin, Ettlingen, Germany). The ^{31}P three-dimensional *k*-space–weighted MRS imaging (MRSI) localization technique (13 × 13 × 13 matrix; field of view: 20 cm × 20 cm × 20 cm; repetition time: 1 second) with adiabatic excitation pulse was used to acquire ^{31}P spectra (Fig. 1). The protocol including setup required 45 minutes. Quantification was performed as described[22] using an MRSI software tool developed in our laboratory[23] and quantified in jMRUI (Java-based Magnetic Resonance User Interface)[24] using AMARES (advanced method for accurate, robust and efficient spectral fitting of MRS data)[25] on the basis of prior knowledge.[26] Complete quantification of one MRSI data set lasted 20-30 minutes.

Reproducibility of the assessment of hepatic lipids was determined in humans in different segments of the liver and resulted in variations of lower than 10%, indicating good reproducibility.[27] Reproducibility of the measurement of phosphorous metabolites has been tested in vitro using phantoms and in vivo in humans. The standard error of the mean (SEM) was 0.85% of the mean of the reference concentration in a phantom containing K_3PO_4.[22] SEMs were 1.55% and 7.88% of the means of ATP and Pi, respectively, from three measurements in one volunteer, each done after complete repositioning of the volunteer.[22] Furthermore, SEMs were 0.40% and 2.16% of the means of the ATP and Pi concentrations, respectively, from measurement by three independent operators.

Localized ^{1}H MRS was performed to measure hepatic lipid contents.[7] Data were converted into absolute concentrations expressed as percent fat per volume,[28,29] using the equations by Longo et al.[29] Hepatic lipid volume fraction (HLVF), calculated as HCL hepatocellular lipids/ (1.138 − 0.339 HCL) is tightly correlated with biopsy-measured liver fat concentrations.[29] Measured concentrations of phosphorus [P] metabolites were corrected for the volume captured by lipid droplets within hepatocytes using the equation $[P_a] = [P]*(1 - HLVF)^{-1}$ to yield [P] adapted for liver fat infiltration.

Calculations and Statistics. M values were assessed from steady-state glucose infusion rates during the last 30 minutes of the clamp. Rates of glucose appearance (R_a)

were calculated by dividing the tracer infusion rate times tracer enrichment by percent of tracer enrichment in plasma and subtracting tracer infusion rate.[7] Clamp-R_a was calculated using Steele's non–steady state equations.[7] EGP was calculated from the difference between R_a and mean glucose infusion rates. Insulin-suppressed EGP (iEGP) represents EGP during the last 30 minutes of the clamp.

Statistical analyses were performed using SPSS 6.0 software (SPSS Inc., Chicago, IL). Data are given as means ± standard deviation in text and tables and means ± SEM in figures. Group comparisons were done using analysis of variance with Tukey post hoc testing or Kruskal-Wallis test for parameters with skewed distribution (HLVF, [P_a], glycosylated hemoglobin [$HbA1_C$]). Spearman correlations were used for HLVF and $HbA1_C$, and Pearson product-moment correlations were used for all other variables. Differences and correlations were considered significant at $P < 0.05$ for phosphorus metabolites and EGP, and at $P < 0.01$ for other parameters to correct for interrelated comparison. Multiple linear regression analysis was performed for the dependent variables Pi and γATP, including the following parameters: age, BMI, M, iEGP, $HbA1_C$, HLVF, plasma concentrations of aminotransferases, triglyceride, and low-density lipoprotein cholesterol/high-density lipoprotein cholesterol.

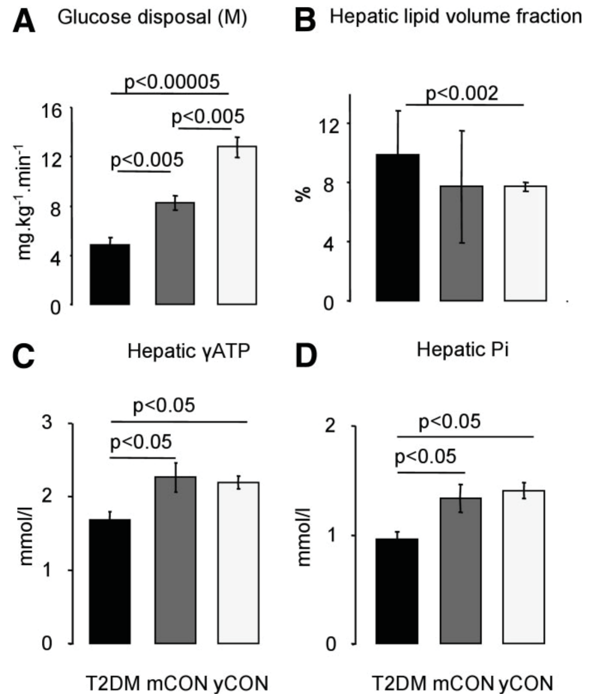

Fig. 2. (A) Insulin-stimulated glucose disposal (M value), (B) hepatic lipid volume fraction, and hepatic concentrations of (C) γATP and (D) inorganic phosphate (Pi) in patients with type 2 diabetes (T2DM), matched controls (mCON), and young controls (yCON). Data are means ± SEM.

Results

Clinical Characteristics. Individuals in the T2DM and mCON groups were older and more obese than those in the yCON group. They were metabolically well-controlled based on analysis of $HbA1_C$. All other parameters were comparable between the groups (Table 1). Only one male T2DM had an alanine aminotransferase (ALT) level of 46 U/L, exceeding the upper limit of ALT for males of <41 U/L.

Insulin Sensitivity and Liver Fat. The T2DM group had 42% and 63% lower M values than the mCON and yCON groups, respectively (Fig. 2A). T2DM featured 13-fold higher iEGP than mCON (0.48 ± 0.12 versus −0.04 ± 0.15 mg/kg body weight/minute; $P < 0.05$) indicating hepatic insulin resistance. HLVF was not different between the T2DM and mCON groups, but was higher ($P = 0.002$) than in the yCON group (Fig. 2B). Steatosis, as defined by HLVF of >5.5%, was present in six individuals with T2DM, three from the mCON group, and none of those in the yCON group.

Hepatic Phosphorus Metabolites. Also, γATP in T2DM was 26% and 23% lower compared with the mCON and yCON controls, respectively (Fig. 2C). Absolute Pi concentrations were 28% and 31% lower in T2DM than in mCON and yCON, respectively (Fig. 2D). Likewise, PI_a and γATP_a showed similar alterations, whereas PME, PDE, and derived ratios were comparable (Table 1).

Hepatic Pi and γATP were not associated with M values, but related negatively to hepatic insulin resistance (iEGP) (Fig. 3), $HbA1_C$, BMI, and HLVF (Table 2). Hepatic Pi correlated positively with plasma ALT levels. Adjustment for HLVF abolished these correlations except for that between Pi and γATP and iEGP (r = −0.680, $P = 0.007$; r = −0.665, $P = 0.010$). In parallel, Pi_a and γATP_a related negatively to iEGP (R = −0.650, $P = 0.009$; R = −0.639, $P = 0.010$) only.

Multiple linear regression analysis for the dependent variable Pi and γATP including clinical characteristics (see Patients and Methods section) and HLVF, identified iEGP as the single significant independent predictor explaining 57% of Pi and γATP variances ($P = 0.001$) (Table 3).

Discussion

This study demonstrates that individuals with metabolically well-controlled T2DM have markedly lower hepatocellular concentrations of Pi and γATP than both

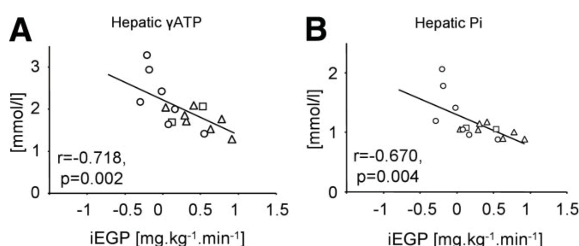

Fig. 3. Correlation between hepatic inorganic phosphate (Pi) and γATP versus insulin-mediated endogenous glucose production (iEGP) as a measure of hepatic insulin resistance in patients with type 2 diabetes (T2DM: triangles, n = 7), matched controls (mCON: circles, n = 7), and young controls (yCON: squares, n = 2).

age-matched and BMI-matched and young lean nondiabetic humans. We employed a recently developed method for absolute quantification of hepatic phosphorus compounds and for assessment of their relationships to insulin resistance and ectopic lipid deposition.[22] Before this method, various tracer dilution methods were applied to assess surrogate parameters of human hepatic mitochondrial function.[30] However, these methods rely on a range of assumptions, involve whole-body protein turnover, and do not assess hepatic energy metabolism directly. The time course of ATP resynthesis upon fructose infusion has been used as a more direct marker of hepatic mitochondrial function but apparently becomes less efficient with increasing BMI.[31]

Previous liver ^{31}P MRS studies were limited by the inability to estimate absolute concentrations, making it necessary to express the intensities of phosphorus signals as ratios to that of another metabolite and/or to the assumed concentration of a metabolite. Consequently, qualitative MRS would fail to detect these alterations in the presences of parallel increases or decreases of metabolites as shown in alcoholic liver disease.[32] Therefore, we conclude that it is highly recommended to use absolute quantification based on external references rather than peak ratios.

The measurement on a 3-T scanner provides for greater signal-to-noise ratio and thereby improved accuracy compared to MRS studies performed on 1.5-T to 2-T MR scanners,[33,34] which is necessary for an accurate measurement of metabolite concentration. Further advantages include improved spatial resolution, speed-up of the measurement due to *k*-space–weighted acquisition, B_1 homogeneity–insensitive excitation, the possibility to quantify a very high number (on average 55±5) of spectra per experiment, and high reproducibility of the metabolite concentrations.

Overall, the absolute concentrations measured here are in agreement with that of 1.4-2.8 mmol/L for Pi and 1.6-3.8 mmol/L for γATP.[32-37] In addition to MRS-dependent differences, the great interindividual variability within and across these studies could be due to the nutritional status and variations of age and BMI. To overcome such influences, we controlled for dietary intake and matched the controls for age and BMI. Of note, the high intraindividual and interobserver reproducibilities of γATP and Pi made it possible to investigate more subtle changes in phosphorus metabolites across a range of hepatic insulin resistance and fat contents.[22]

The decrease in absolute levels of γATP in viral and alcoholic hepatitis[32,37] and obesity[38] has been commonly interpreted as “energy deficit” or impaired “ATP homeostasis”. Decreased γATP could result from hepatocellular γATP depletion due to increased ATP utilization by energy-demanding processes such as Na^+/K^+ adenosine triphosphatases, lipogenesis, or gluconeogenesis. However, stimulation of catabolism would imply increase of Pi levels and/or Pi/γATP ratios, which reflect the bioenergetic state of the cell and have been used as a marker for reduced phosphorylation status[39] in livers of humans suffering from obesity,[38] viral cirrhosis,[40] or malignancies.[41] In contrast, individuals from our T2DM group showed comparable reductions of Pi and γATP, indicating balanced anabolic/catabolic rates. However, one cannot rule out that they had decreased mitochondrial ATP synthesis arising from impaired glycolysis, fat oxidation, or oxygen consumption.

Alternatively, parallel reduction of γATP by 25%-36% and of Pi by 37%-50% in alcoholic hepatitis with ALT elevation has been explained by loss of functional hepatocytes due to necrosis and replacement with

Table 2. Association Between Absolute Phosphorus Metabolites and Other Parameters

	iEGP (n = 16)	$HbA1_c$ (n = 27)	BMI (n = 27)	HLVF (n = 27)	ALT (n = 27)
[γATP]	r = −0.718 *P* = 0.002	R = −0.496 *P* = 0.010	r = −0.429 *P* = 0.025	R = −0.551 *P* = 0.004	n.s.
[Pi]	r = −0.670 *P* = 0.004	R = −0.567 *P* = 0.003	r = −0.535 *P* = 0.004	R = −0.577 *P* = 0.003	r = −0.497 *P* = 0.010

Pearson correlation coefficients (r) and Spearman correlation coefficients (R) for the association of inorganic phosphate (Pi) and γATP with insulin-suppressed endogenous glucose production (iEGP), HbA1c, body mass index (BMI), hepatic lipid volume fraction (HLVF), and alanine aminotransferase (ALT).

Table 3. Multiple Regression Analysis for the Dependent Variables Inorganic Phosphate (Pi) and γATP with Physiologic Parameters

Dependent Variable	Model	Independent Variable	R^2	Adjusted R^2	Standardized Coefficient (β)	*P* Value
γATP	1	iEGP	0.567	0.534	−0.753	0.001
Pi	1	iEGP	0.569	0.536	−0.754	0.001

Multiple linear regression analysis was performed including the following parameters: hepatic (iEGP, Insulin-Suppressed Endogenous Glucose Production) and Whole-Body Insulin Sensitivity (M, Insulin-Mediated Whole-Body Glucose Disposal), Body Mass Index, age, liver fat content (HLVF, hepatic lipid volume traction) plasma concentration of aminotransferases, triglycerides, and low-density lipoprotein cholesterol/high-density lipoprotein cholesterol.

fat and collagen.[32] In our T2DM group, similar reductions of γATP and Pi were observed without any clinical or laboratory evidence of liver damage, which does not rule out excessive hepatocellular fat deposition as a cause of lower phosphorus metabolites. However, even after correcting the absolute γATP and Pi concentrations for hepatocellular lipid volume, γATP and Pi remained lower in the T2DM group, indicating reduced energy metabolism independent of liver fat content.

Reduction of Pi and γATP could also be a feature of hepatic insulin resistance preceding hepatocellular lipid accumulation. Increased circulating lipids derived from dietary intake and impaired inhibition of lipolysis stimulate lipid oxidation and production of reactive oxygen species, giving rise to lipid peroxidation and damage of mitochondrial proteins and DNA, reduction of oxidative capacity, and subsequent lipid accumulation.[42,43] In support of this contention, patients with NASH exhibit paracrystalline inclusions in megamitochondria,[11,44] increased mitochondrial DNA mutations,[45] and lower respiratory complex activities in the liver.[20] But even obese subjects already may have lower liver ATP resynthesis upon fructose administration.[31] In our study, HLVF was similar in patients with T2DM and mCON with only five individuals from all groups with excessive steatosis at HLVF of $>10\%$ and only one man with T2DM with slightly elevated ALT. This indicates that our patients had steatosis and were unlikely to be suffering from NASH. Of note, HLVF related inversely to Pi and γATP, which strongly relied on the negative association of hepatic insulin resistance with Pi and γATP, which was identified as an independent predictor of the concentrations of these metabolites.

A recent study reported higher PME/Pi and PME/ATP ratios along with lower insulin sensitivity in overweight patients with NAFLD, as diagnosed with ultrasound, compared to lean subjects.[46] Because PME not only comprises phosphocholine, phosphoethanolamine and adenosine monophosphate but also glucose-6-phosphate and 3-phosphoglycerate,[47] the higher PME signal was interpreted as a surrogate of increased gluconeogenesis. More likely, the increase in PME/Pi or PME/ATP simply resulted from reduction of the absolute concentrations of Pi or γATP. Of note, increased PME ratios have been repeatedly found in patients with severe liver diseases including hepatitis, liver cirrhosis, or neoplasia and therefore rather reflect extensive membrane remodeling.[48-50] In this study, PME concentrations and ratios did not differ between the groups, which further supports the absence of liver damage in our T2DM individuals.

Up to 60% of the general population shows defects of the hepatic respiratory chain despite normal liver function.[51] Because the majority of this group is older than 50 years, aging-induced mitochondrial damage could contribute to the observed alterations of phosphorus metabolites in our T2DM individuals. Reduced energy metabolism could be due to loss of functional hepatocytes or reduction of mitochondrial contents and/or fitness.[15] However, reduction of absolute Pi and γATP concentrations were found in T2DM but not in age-matched and BMI-matched healthy humans. In the face of age-dependent decline of whole-body insulin sensitivity across the groups, these data suggest a diabetes-related or even diabetes-specific effect causing the reduction of hepatic Pi and γATP. This is corroborated by a negative relationship between Pi and γATP and glucometabolic control as assessed from $HbA1_C$ and iEGP, which not only reflects hepatic insulin sensitivity but also defines plasma glucose concentrations.

Some limitations need to be taken into account. First, no biopsies were done, and thus exact information on inflammatory status is lacking. This was due to the fact that biopsies are not considered ethical in subjects without any evidence of liver disease. Second, not all participants were available for the clamp test. Nevertheless, the overall number of participants allowed detection of associations with clinically relevant parameters. Finally, our T2DM cohort comprised normal weight ($n = 3$), overweight ($n = 3$), and obese ($n = 3$) patients resulting in a mean BMI of ~ 27 kg/m^2. Because T2DM cohorts mostly have higher BMI values, the results obtained in our cohort cannot be necessarily extrapolated to all patients with T2DM. In Europe, however, Caucasian co-

horts with T2DM and/or NAFLD may present with mean BMI values below 30 kg/m^2.[52-54] Likewise, HLVF values did not cover a broad range from normal fat content to excessive steatosis. However, we detected alterations in phosphorus metabolites even within the normal range.

In conclusion, patients with metabolically well-controlled T2DM already show reduced hepatic ATP concentrations which relates to hepatic insulin resistance independent of hepatic lipid contents. These data further suggest that impaired hepatic energy metabolism and hepatic insulin resistance could precede the development of steatosis in patients with T2DM.

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Article No. 10.

Liver ATP Synthesis Is Lower and Relates to Insulin Sensitivity in Patients With Type 2 Diabetes

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OBJECTIVE—Steatosis associates with insulin resistance and may even predict type 2 diabetes and cardiovascular complications. Because muscular insulin resistance relates to myocellular fat deposition and disturbed energy metabolism, we hypothesized that reduced hepatic ATP turnover (fATP) underlies insulin resistance and elevated hepatocellular lipid (HCL) contents.

RESEARCH DESIGN AND METHODS—We measured hepatic fATP using ^{31}P magnetic resonance spectroscopy in patients with type 2 diabetes and age- and body mass–matched controls. Peripheral (M and M/I) and hepatic (suppression of endogenous glucose production) insulin sensitivity were assessed with euglycemic-hyperinsulinemic clamps.

RESULTS—Diabetic individuals had 29% and 28% lower peripheral and hepatic insulin sensitivity as well as 42% reduced fATP than controls. After adjusting for HCL, fATP correlated positively with peripheral and hepatic insulin sensitivity but negatively with waist circumference, BMI, and fasting plasma glucose. Multiple regression analysis identified waist circumference as an independent predictor of fATP and inorganic phosphate (P_I) concentrations, explaining 65% (P = 0.001) and 56% (P = 0.003) of the variations. Hepatocellular P_I primarily determined the alterations in fATP.

CONCLUSIONS—In patients with type 2 diabetes, insulin resistance relates to perturbed hepatic energy metabolism, which is at least partly accounted for by fat depots.

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Alterations in liver function are frequently encountered in patients with type 2 diabetes (1,2). Nonalcoholic fatty liver (steatosis) and hepatic insulin resistance are typical for type 2 diabetes and may even precede or contribute to its development (2,3). It also relates to the development of cardiovascular diseases (4), besides the progression into steatohepatitis and cirrhosis (5). Steatosis likely results from excessive lipid overflow originating from augmented lipid stores in peripheral or visceral adipocytes, lipogenesis, or high dietary intake (2).

Magnetic resonance spectroscopy (MRS) allows noninvasive direct measurements of metabolite concentrations and tracing of metabolic fluxes in situ. Quantification of intramyocellular lipids and hepatocellular lipids (HCL) by ^{1}H MRS revealed that both are linked to whole-body (peripheral) insulin resistance, abnormal endogenous glucose production (EGP), and liver glycogen storage in line with hepatic insulin resistance (2). According to one current paradigm, excessive ectopic lipid deposition precedes the development of type 2 diabetes (2). Yet, the underlying metabolic processes leading to lipid accumulation and insulin resistance in skeletal muscle and liver are not fully understood.

In skeletal muscle, insulin resistance relates not only to elevated lipids but also to impaired rates of phosphorylation (flux through ATP synthesis, fATP), as measured by ^{31}P MRS in elderly (6) type 2 diabetic patients (7), their insulin-resistant first-degree relatives (8), and nondiabetic individuals with elevated plasma free fatty acids (FFAs) (9).

Although it has been suggested that nonalcoholic steatohepatitis is associated not only with insulin resistance but also with mitochondrial abnormalities (10), no data on the quantification of fATP in patients with type 2 diabetes have been reported so far in the human liver. Our previous studies showed that type 2 diabetic individuals have lower hepatic concentrations of P_I and ATP (11). After developing a saturation transfer experiment for assessment of hepatocellular fATP (12), we tested the hypothesis that type 2 diabetic patients have lower hepatocellular fATP than nondiabetic controls, which should relate independently of HCL to hepatic insulin resistance.

RESEARCH DESIGN AND METHODS

The study protocol conformed with the Declaration of Helsinki (2008) and was approved by the appropriate institutional ethical board. Informed consent was obtained from each study participant after explanation of the purpose, nature, and potential complications of the study.

Volunteers

After recruitment by means of public advertisement, all participants underwent complete medical history, clinical examination, and laboratory tests to exclude cardiovascular, renal, and thyroid diseases, and viral hepatitis. All volunteers were white and had already participated in our previous study on absolute quantification of liver phosphorus metabolites (11). The study excluded patients taking insulin or thiazolidinediones, or presenting with islet cell antibodies and

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diabetes-related complications. Controls had similar body mass and age, but normal glucose tolerance during a 75-g oral glucose tolerance test and had neither a family history of diabetes nor regular drug treatment. None of the participants was physically trained or performed regular intense exercise. They had no evidence for liver diseases and consumed <20 g alcohol daily.

Of 24 individuals recruited, 3 did not complete the experiment because they refused to continue the MRS session. Data sets of three participants could not be used due to low quality resulting from a poor local homogeneity of the static magnetic field. Data from one individual could not be used because of a technical problem with the scanner. Thus, complete MRS datasets for 17 individuals were available for analyses (Table 1).

Type 2 diabetic patients showed acceptable glycemic (A1C: 7.06 ± 0.98% vs. 5.53 ± 0.27% in controls; $P = 0.001$) and lipid control on treatment with diet only ($n = 1$), sulfonylurea only ($n = 3$), or combined with metformin ($n = 5$) and statins ($n = 7$). Medications were withdrawn at least 3 days before the measurements. Measurements of fATP and insulin sensitivity were performed on different days under identical conditions of overnight fasting and withdrawal of medications.

Anthropometric measurements

Waist circumference was measured with a cloth tape with participants upright. Waist was defined as the midpoint between the highest point of the iliac crest and the lowest part of the costal margin in the midaxillary line (13).

Analytic procedures

Plasma glucose was measured by the glucose oxidase method (Glucose Analyzer II, Beckman Instruments Inc., Fullerton, CA) and plasma insulin by double antibody radio immunoassay (11). All other parameters were measured in the clinical chemistry laboratory with standardized quality-controlled assays.

Peripheral and hepatic insulin sensitivity

Whole-body glucose disposal (M, or M related to plasma insulin [M/I]) and EGP were measured in seven diabetic subjects (three women/four men) and in six controls (four women/two men) as described previously (7), who exhibited identical demographics as the whole group. Studies started at 6:30 A.M. with primed-continuous infusion (5 min: 3.6 mg · kg body wt^{-1} · fasting glucose [mg/dL]/90 [mg/dL]; 355 min: 0.036 mg · min^{-1} · kg body wt^{-1}) of D-[6,6-$^2H_2$]glucose (98% enriched; CIL, Andover, MA). The euglycemic (~5.5 mmol/L)-hyperinsulinemic (~70 mU/L) clamp test was started at 10:00 A.M., with a primed continuous infusion of human regular insulin (Actrapid; Novo Nordisk, Bagsvaerd, Denmark; 40 mU · [m body surface area]$^{-2}$ · min^{-1}) for 240 min. A 20% dextrose infusion labeled with D-[6,6-$^2H_2$]glucose (2% enriched) according to the hot-glucose infusion protocol was periodically adjusted to maintain euglycemia (7).

MRS

After overnight fasting, volunteers underwent a MRS protocol consisting of two consecutive sessions with a break of ~30 min in a 3-T MR scanner (Bruker Biospin, Ettlingen, Germany) using a 10-cm linear polarized surface coil tuned to ^{1}H and ^{31}P frequencies.

In session 1, subjects were supine in the scanner with the coil positioned over the lateral aspect of the liver. Axial and oblique gradient echo images were acquired during one breath-hold each. HCL was measured using localized ^{1}H MRS (STEAM, 20 × 20 × 20 mm; 5 scans: echo time, 15, 20, 30, 50, 70 ms; repetition time, 2.5 s) (Fig. 1*A*). Fat and water peaks were integrated after phasing and baseline adjustment using XWIN-NMR software (Bruker Biospin, Ettlingen, Germany). A 30-mm slab within the liver was selected using 1D-ISIS encoding for ^{31}P acquisitions (Fig. 1*A*). After a baseline scan, alternated spectra with saturation of the γ-ATP resonance frequency and the frequency mirrored relative to the P_I resonance were acquired (Fig. 1*B*). In addition, a combined saturation transfer inversion recovery experiment was performed in each individual to determine the effective longitudinal relaxation time (T_1*). Spectra were phased and baseline corrected. The peak amplitude was used for quantification because only peak ratios between consecutive scans were used as described (12).

In session 2, lasting ~50 min, the subjects were prone in the scanner on top of the surface coil with the lateral aspect of the liver over the coil center. After shimming, gradient echo-based images were acquired during one breath-hold. A 3-dimensional k-space weighted chemical shift imaging sequence (field of view: 20 × 20 × 20 cm, repetition time: 1 s, bandwidth: 5 kHz, 2,048 acquisitions in 34 min) was applied, reconstructed, and quantified offline using custom-made software in combination with jMRUI/AMARES, as described (12,14).

Calculations and analyses

Water and lipid signals were nonlinearly fitted as a single exponential for T_2 correction. HCL were calculated as lipids/(water + lipids) × 100. The chemical exchange constant, k of the saturation transfer experiment is assessed from the P_I signal before (M_0) and after saturation of γ-ATP (M_s). Then, k is calculated as $k = 1/T_1^* \times (1 - M_s/M_0)$, and fATP as fATP = $k \times P_I$.

Rates of glucose appearance (Ra) were calculated by dividing the tracer (D-[6,6-$^2H_2$]glucose) infusion rate multiplied by tracer enrichment by the percent of

Table 1—*Anthropometric and laboratory parameters*

Variable	Type 2 diabetes	Control
N (female)	9 (3)	8 (4)
Age (years)	57.7 ± 6.1	60.1 ± 4.4
Height (cm)	174 ± 11	174 ± 10
Body weight (kg)	82 ± 14	78 ± 17
BMI (kg/m^2)	26.9 ± 3.4	25.1 ± 3.9
A1C (%)	7.06 ± 0.98*	5.57 ± 0.27
Waist circumference (cm)	100 ± 10	88 ± 19
Fasting plasma glucose (mmol/L)	8.8 ± 1.9*	5.3 ± 0.6
Fasting plasma insulin (mU/L)	12.1 ± 3.9	8.5 ± 2.0
Serum LDL cholesterol (mg/dL)	119 ± 22	146 ± 33
Serum HDL cholesterol (mg/dL)	62 ± 9	59 ± 10
Plasma triglyceride (mg/dL)	163 ± 80	128 ± 79
GGT (units/L)	34 ± 17	27 ± 9
AST (units/L)	22 ± 5	24 ± 5

Data are expressed as mean ± SD, unless indicated otherwise. *$P < 0.001$, significant differences between groups.

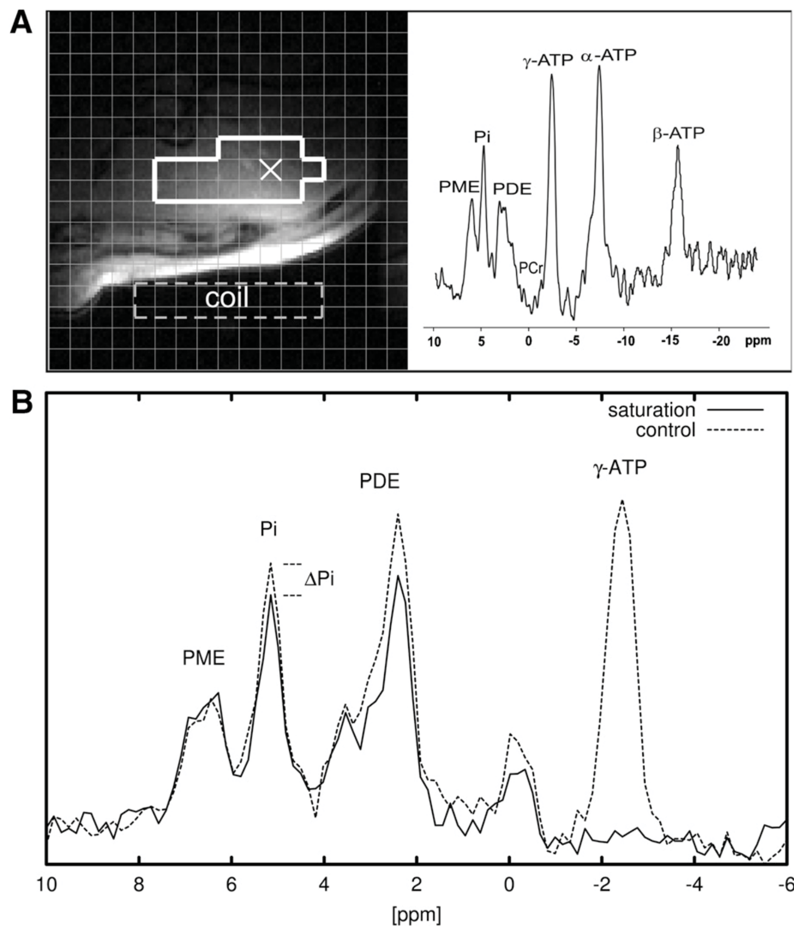

Figure 1—*Liver MR data of one patient with type 2 diabetes.* A: *Axial slice through the liver (gradient echo, prone position). The grid corresponds to the chemical shift imaging matrix. The voxels surrounded by the bold white line were selected for quantification. The MR spectrum corresponding to the "×" in the image is shown on the right panel.* B: *MR spectra from the saturation transfer experiment (solid line). The ATP resonance is completely suppressed, and the P_I signal is lower than in the baseline control experiment (dashed line).*

tracer enrichment in plasma and subtracting the tracer infusion rate. During the clamp, Ra was calculated using Steele's nonsteady state equations. EGP was calculated from the difference between Ra and mean glucose infusion rates. Insulin-mediated EGP represents EGP during the last 30 min of the clamp. EGP suppression was calculated as 100 × [(1 − EGP insulin-mediated)/(EGP baseline)].

Data are presented as means ± SD and compared between the groups using the two-tailed Student *t* test. Relationships between variables were analyzed with Pearson correlations for normally distributed data and with Spearman correlations for the scattered HCL values. Regression analyses for the dependent variables fATP and P_I were done using the independent variables of age, BMI, waist circumference, HCL, alanine aminotransferase, A1C, EGP, and FFA suppression, M/I, serum triglycerides, and low-density lipoprotein.

RESULTS—Diabetic participants had 67% higher fasting plasma glucose concentrations, 27% higher A1C, and similar body weight, but tended to have greater waist circumference (P = 0.167) than controls (Table 1). Insulin sensitivity was 29% (M) and 45% (M/I) lower in diabetic volunteers than in controls, confirming peripheral insulin resistance (Table 2).

Mean HCL content was not different between groups (Table 2). The distribution of the individual HCL values covered a broad range from <1 to 33% across the groups, with values >5.5% in six diabetic and five nondiabetic participants. Fasting EGP was similar in both groups, whereas EGP was completely suppressed during insulin stimulation only in controls but not in diabetic participants, indicating hepatic insulin resistance. Fasting plasma FFAs were comparable, but FFA suppression was lower in diabetic patients. Hepatocellular fATP was 42% lower and *k*, the chemical exchange rate constant, tended to be lower in patients compared with controls (Table 2). Previous medical treatment with or without metformin did not affect fATP (16.5 ± 6.0 vs. 16.0 ± 5.0 mmol/L/min).

fATP positively correlated with both peripheral (M: r = 0.67, P = 0.01; M/I: r = 0.61, P = 0.03) and hepatic insulin resistance (suppression of EGP: r = 0.77, P = 0.002), even after adjustment for plasma insulin during the clamp (r = 0.84, P = 0.001) across all participants (Fig. 2*A*). Also, *k* values related to M (r = 0.67, P = 0.01) but not to M/I (r = 0.47, P = 0.11). Furthermore, fATP correlated negatively with waist circumference (r = −0.78 P = 0.001; Fig. 2*B*), fasting plasma glucose (r = −0.58, P = 0.01), BMI (r = −0.55, P = 0.02), HCL (r = −0.51, P = 0.04; Fig. 2*C*), and A1C (r = −0.50, P = 0.04). Hepatocellular P_I (r = 0.77, P = 0.0003) related positively to fATP (Fig. 2*D*). After adjusting for HCL, the relationships of fATP with waist circumference (r = −0.81 P = 0.001), M (r = 0.66, P = 0.02), M/I (r = 0.64, P = 0.03), EGP suppression (r = 0.72, P = 0.009), BMI (−0.54, P = 0.03), and fasting plasma glucose (r = −0.52, P = 0.04) remained significant. fATP did not correlate significantly with fasting FFA (r = 0.203, P = 0.507) or FFA suppression (r = 0.51, P = 0.07).

Multiple regression analysis for the dependent variable fATP identified waist circumference as the independent predictor of fATP (r^2 = 0.65, adjusted r^2 = 0.62, P = 0.001). Likewise, multiple regression analysis for the dependent variable P_I identified waist circumference as the independent predictor of P_I (r^2 = 0.56, adjusted r^2 = 0.52, P = 0.003).

CONCLUSIONS—This study found that patients with type 2 diabetes have markedly lower hepatic ATP production than nondiabetic individuals of comparable age and body mass. Of note, this alteration of fATP occurred without evidence for clinical liver disease, as indicated by the

Table 2—*Liver fat, energy metabolism, and hepatic and peripheral insulin sensitivity*

Variable	Type 2 diabetes	Control
HCL (% signal)	10.6 ± 9.3	8.2 ± 10.9
Absolute P_I (mmol/L)	0.96 ± 0.19*	1.38 ± 0.38
k (s^{-1})	0.28 ± 0.07	0.33 ± 0.12
fATP ($mmol \cdot L^{-1} \cdot min^{-1}$)	16.2 ± 5.2†	28.0 ± 13.0
EGP baseline ($mg \cdot kg^{-1} \cdot min^{-1}$)	1.6 ± 0.3	1.8 ± 0.4
EGP suppression (%)	72 ± 15‡	100 ± 17
FFA baseline (μmol/L)	569 ± 122	430 ± 260
FFA suppression (%)	95 ± 2*	97 ± 2
M ($mg \cdot kg^{-1} \cdot min^{-1}$)	5.3 ± 1.3§	7.5 ± 1.9
M/I ($mg \cdot kg^{-1} \cdot min^{-1} \cdot mU^{-1} \cdot L$)	0.06 ± 0.03‖	0.11 ± 0.02

Data are shown as mean ± SD. Significant differences between groups: *P = 0.011; †P = 0.025; ‡P = 0.009; §P = 0.003; ‖P = 0.005.

lack of a history of hepatitis, alcohol consumption, or significant elevation of liver enzymes (11).

Across all subjects, hepatocellular fATP correlated negatively with HCL, as recently reported for the relationships between hepatic absolute P_I and ATP concentrations and HCL (11). However, we detected a clear difference in fATP between diabetic and nondiabetic participants, despite comparable HCL values and equal distribution of steatosis (HCL >5.5%) in both groups (67% of diabetic and 63% of nondiabetic participants). Thus, other factors than HCL more likely explain the lower fATP in patients. After adjustment for HCL, hepatic and whole-body insulin sensitivity remained tightly and negatively related to fATP.

Absolute hepatic concentrations of P_I and ATP are also associated with hepatic but not with peripheral insulin sensitivity (11). Under similar conditions of overnight fasting, myocellular fATP correlates negatively to peripheral insulin sensitivity, which is mostly accounted for by skeletal muscle, but not to hepatic insulin sensitivity (7). This suggests that hepatocellular fATP could serve as a better indicator of overall insulin resistance than myocellular fATP.

In type 2 diabetes, insulin resistance develops as a result of inherited factors as well as from acquired factors that result in glucolipotoxicity due to chronic elevation of FFAs, triglycerides, or glucose (2,3). In rats, a high-fat diet for 7 weeks induced obesity, insulin resistance, and hepatic steatosis and also reduced respiratory capacity and increased oxidative stress in liver mitochondria (15). Further, mitochondria from fatty rat liver have lower amounts of ATP synthase protein (16). A short-term high-fat diet also leads to insulin resistance in liver, but not in skeletal muscle (17), whereas a hypocaloric diet decreases both hepatic fat content and insulin resistance within 48 h (18). In conclusion, impaired energy metabolism in the liver could be an early defect in the pathogenesis of type 2 diabetes.

The lack of a correlation of fATP with circulating plasma triglycerides or FFAs suggests that increased lipid availability cannot explain the abnormality of hepatic fATP. However, hepatocellular fATP related negatively to BMI and even more tightly to waist circumference, which explained most of the variation of fATP. Of note, waist circumference is the best anthropometric marker of abdominal visceral fat (19), which suggests, but does not prove, a role of visceral fat for hepatic energy metabolism.

Hepatocellular fATP further related to short- and long-term glycemia, as measured by fasting plasma glucose and A1C, so that defective hepatic energy metabolism could also reflect deterioration of glucose metabolism in the progression of type 2 diabetes. Abnormalities in fATP have been found in skeletal muscle not only of patients with overt type 2 diabetes (7,20) but also of their nondiabetic first-degree relatives, who are at increased risk for type 2 diabetes (8). Impairment of ATP synthesis in skeletal muscle has been further related to insulin resistance, myocellular lipid deposition, and genetic susceptibility to the onset of type 2 diabetes (21). To our knowledge, however, no data on a similar association between lipid contents, insulin sensitivity, and ATP production, which also includes ATP generation from glycolytic flux, have been reported in the livers of patients with type 2 diabetes. Although our results suggest that abnormal phosphorus metabolism relates to hepatic insulin resistance and HCL, we cannot rule out that reduced ATP turnover is rather a consequence of reduced energy demand or altered energy homeostasis that may relate to increased lipid availability and hyperglycemia. In this case, impaired fATP would result from chronic hyperglycemia and/or insulin resistance due to glucose toxicity. Of note, there is evidence that insulin stimulates uptake of P_I by way of a sodium-dependent transporter, at least in cultured rat hepatocytes, so that desensitization of this system could lead to lower hepatocellular P_I levels (22).

Our MRS protocol is time-consuming, which also explains the dropout rate in this study. Alternatively, the tight relationship between hepatocellular fATP and P_I, which is a direct consequence of a comparable rate constant, k, suggests that quantification of the absolute P_I concentration could be sufficient for assessing hepatic energy metabolism. Altered tissue morphology could affect the MR relaxation rates, which in turn could affect the absolute quantification of metabolites and fATP. This simplification should, therefore, only be considered when there is strong evidence against alterations in MR relaxation times. The tight correlation between ATP and P_I (11) renders errors due to altered MR relaxation times highly unlikely. Participants in the current study did not show clinical signs of cholestasis or impaired liver function, as expressed by normal results on liver enzyme tests and function parameters, although we cannot be completely certain because we had no histologic confirmation. Thus, absolute quantification of hepatic ^{31}P metabolites (11,12), together with k from this study, appears to be a clinically applicable measure of hepatic energy metabolism. Even more, it appears that waist circumference could also serve as a viable predictor of hepatic ATP production.

Nevertheless, some limitations of the current study need to be considered. We studied elderly volunteers who had a mean age of ~60 years, which might result in lower fATP analogous to data on myocellular fATP (8). The ages of diabetic and nondiabetic participants in this study were similar, however, and fATP in these elderly controls was almost identical to that previously reported for young, lean, healthy volunteers (12).

Finally, the saturation transfer experiment provides a measure of total ATP turnover, which includes cycling at the

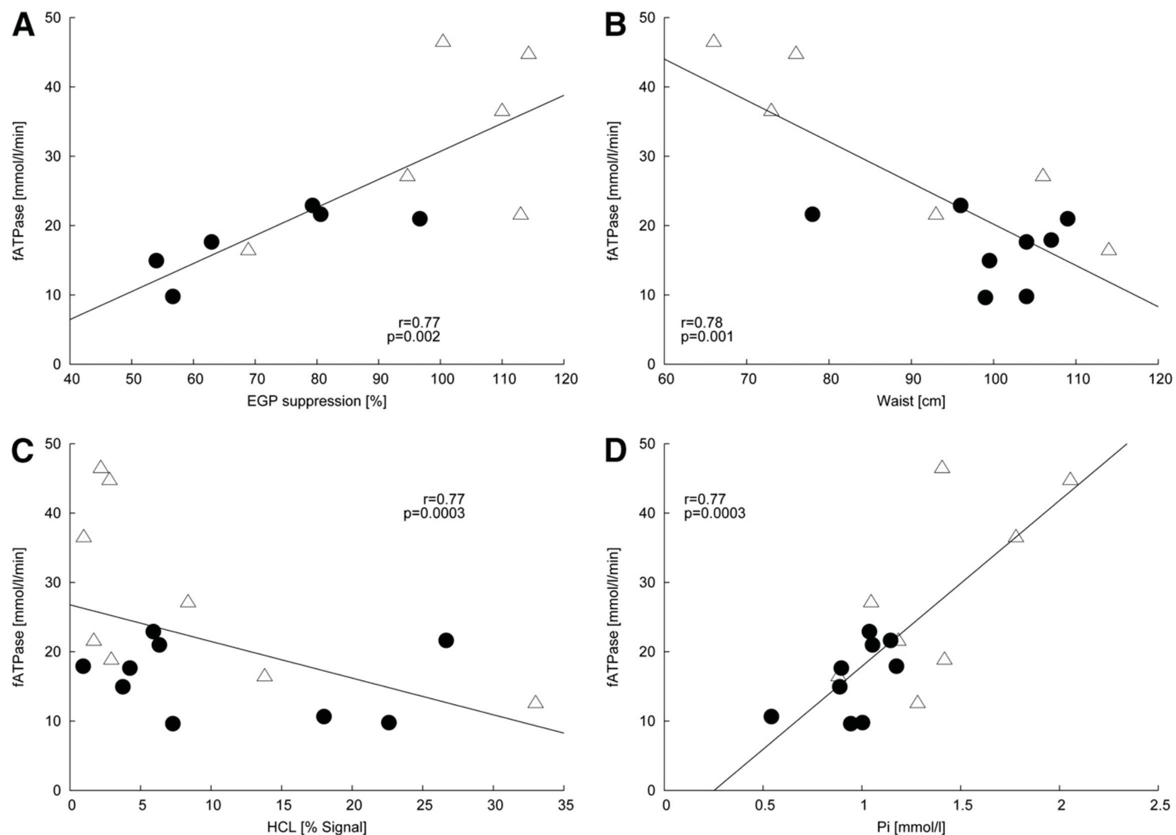

Figure 2—*Correlations with hepatocellular ATP production (fATP) with* (A) *hepatic insulin resistance (EGP suppression; all:* r = *0.77,* P = *0.002; type 2 diabetic subjects:* r = *0.79,* P = *0.01; controls:* r = *0.54,* P = *0.17),* (B) *waist circumference (all:* r = −*0.78,* P = *0.001; type 2 diabetic subjects:* r = *0.57,* P = *0.11; controls:* r = *0.68,* P = *0.064),* (C) *HCL contents (all:* r = −*0.51,* P = *0.038; type 2 diabetic subjects* r = −*0.20,* P = *0.61; controls:* r = *0.62,* P = *0.10),* (D) *hepatic concentrations of* P_I *(all:* r = *0.77,* P = *0.0003; type 2 diabetic subjects:* r = *0.57,* P = *0.11; controls:* r = *0.68,* P = *0.064) across all participants (type 2 diabetic subjects: ●, controls: △).*

levels of glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase of the glycolytic and gluconeogenic pathways (23). Because fasting EGP was comparable between both groups, despite different degrees of glycemia, a variable contribution of cycling is rather unlikely. Finally, some spectra of the saturation transfer experiment contained residual phosphocreatine resulting from a minor compromise between optimizing localization and signal-to-noise ratio. Of note, the phosphocreatine is dominating skeletal muscle spectra, implying the contamination in P_I, which determines fATP, is lower by an order of magnitude. Moreover, chemical shift displacement shifts the P_I signal further inward into the liver. The phosphodiester signals appear affected by the saturation pulse (Fig. 1*A*); this could, besides off-resonance effects, indicate chemical exchange such as ATP used in phosphodiesterase formation (24).

In conclusion, patients with type 2 diabetes without clinical liver disease exhibit lower ATP production than nondiabetic otherwise comparable individuals. The reduction in flux through ATP synthesis depends primarily on the lower hepatic P_I and ATP concentrations. Insulin resistance relates to the perturbed hepatic energy metabolism, which can be at least be partly explained by fat mass as assessed from waist circumference.

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A.I.S., J.S., and M.C. acquired and analyzed data. A.I.S. and M.R. wrote the manuscript. M.K., E.M., and J.S. contributed to the discussion and reviewed the manuscript.

Parts of the data were presented as abstracts at the following meetings: 69th Scientific Sessions of the American Diabetes Association, New Orleans, Louisiana, 2009; 45th EASD Annual Meeting, Vienna, Austria, 2009; 17th European Congress of Obesity, Amsterdam, 2009; 24th Federation of International Danube Symposia on Diabetes Mellitus, Salzburg, Austria, 2009; Keystone Symposium, Banff, Alberta, Canada, 2009; and the 11th Meeting of the Austrian Diabetes Society, Linz, Austria, 2006.

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