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Distribution of lipid metabolites in muscle biopsies from healthy volunteers after fat induced insulin resistance

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Für Marc

Zusammenfassung

Weltweit ist ein großer kontinuierlicher Anstieg der Diabetesrate zu verzeichnen. Insulinresistenz ist ein Hauptmerkmal von Diabetes mellitus Typ 2, der einen großen Teil der Diabetes-Erkrankungen darstellt. Ziel der Arbeit war es, einen Beitrag zur Klärung des Entstehungsmechanismus von Insulinresistenz zu leisten. Hierzu wurde der Mechanismus der lipidinduzierten akuten muskulären Insulinresistenz bei normalgewichtigen jungen gesunden Probanden untersucht.

Hierfür wurde jeweils zu Beginn des Versuchs und nach 4 h Lipidinfusion mit einem hyperinsulinämisch-euglykämischer Clamp Muskelbiopsien entnommen. Ab dem neunten Versuch wurde eine zusätzliche Muskelbiopsie nach 2,5 h Lipidinfusion durchgeführt. Anschließend wurden die intramuskulären Konzentrationen von Diacylglycerol (DAG) und Ceramiden sowie die Expression/Aktivität von DAG-sensiblen Protein-Kinase C-Isoformen (ß, δ, θ) analysiert.

In dieser Arbeit zeigte sich nach 4 Stunden lipid-induzierter Insulinresistenz mit durch die Lipidinfusionen bedingten hohen Plasmaspiegel von freien Fettsäuren eine erhöhte Aktivität der Proteinkinase C isoform θ (PKS θ). Darüber hinaus ist ein vorangegangener Anstieg von DAG membran- und zytosol Fraktionen zu verzeichnen.

Die erhobenen Befunde unterstützen die Hypothese, dass lipid-induzierte Insulinresistenz im Skelettmuskel mit einem Anstieg von DAG membran- und zytosol Fraktionen nach 2,5 h und damit verbundenen folgenden Aktivität der PKC θ nach 4 h in Zusammenhang steht.

Summary

Worldwide a huge continuous increase of diabetes is observed. Insulin resistance is a key feature of diabetes type 2, the most common type of diabetes. The aim of the present study was to contribute to clarify the mechanism of development of insulin resistance. For this purpose, the mechanism of lipid induced acute muscle insulin resistance was examined in lean young and healthy humans.

The muscle biopsies were sampled at the beginning and after 4 h Lipid infusion and hyperinsulinemic–euglycemic clamp. From the ninth experiment, an additional muscle biopsy was performed after 2.5 h lipid infusion. Afterwards the intramuscular concentrations of diacylglycerol (DAG) and ceramides and expression/activation of DAG sensible protein kinase isoforms (ß, δ, θ) were analyzed.

The results show that 4 hours high free fatty acid (FFA) plasma levels induced by lipid infusion with a conditional state of insulin resistance are related with an activation of protein kinase isoform (PKC) θ. Moreover, DAG membrane and cytosol fractions are preceded increased.

These observations support the assumption that lipid induced insulin resistance in skeletal muscle is associated with an early elevation of DAG membrane and cytosol fraction at 2.5 h and thereby following activation of PKC θ at 4h.

Abbreviations

ALT:	Alanine Transaminase
AS:	arachidonate/stearate
ASO:	antisense oligonucleotide
AST:	Asparate Transaminase
APE:	atom excess percent
ASO:	antisense oligonucleotide
BMI:	Body Mass Index
BSA:	Body surface area
C16:	N-palmitoyl-D-erythro-sphingosine
C17:	N-heptadecanoyl-D-erythro-sphingosine
C18:	N-stearoyl-D-erythro-sphingosine
C20:	N-arachidoyl-D-erythro-sphingosine
C22:	N-behenoyl-D-erythro-sphingosine
C24:1:	N-nervonoyl-D-erythro-sphingosine
C24:	N-lignoceroyl-D-erythro-sphingosine
CerS:	Ceramide synthases
DAG:	diacylglycerol
DGAT:	diacylglycerol O-acyltransferase
EGP:	Endogenous Glucose Production
FATP-1:	fatty acid transport protein-1
FFA:	Free fatty acidy
FFAIR:	Free Fatty Acid induced insulin resistance
GDM:	gestational diabetes
GLUT:	glucose transporter
GLUT4:	glucose transporter type 4
GS:	glycogen synthase
Hb:	haemoglobin
HbA1c:	glycosylated haemoglobin A 1c
HCL:	hepatocellular lipids
HK:	hexokinase II

HOMA:	Homeostasis model assessment Homeostasis model assessment
IC:	indirect calorimetry
IL-1RA:	interleukin-1 receptor antagonist
IMCL:	Intramyocellular content of lipids
IR:	Insulin resistance
IRS:	insulin receptor substrate
KO:	knockout
LCAD:	long-chain acyl-CoA dehydrogenase
LL:	Linoleate
LPL:	lipoproteinlipase
LS:	Linoleate/stearate
MAPK :	mitogen-activated protein kinase
mtGPAT:	mitochondrial glycerol-3-phosphate acyltransferase
mTOR:	mammalian target of Rapamycin
MUBI:	muscle biopsy
Mv:	mean value
NAFL:	Nonalcoholic fatty liver
NAFLD:	Nonalcoholic fatty liver disease
NEFA:	non-esterified fatty acids
NIDDM:	non insulin dependant diabetes mellitus
NMR:	nuclear magnetic resonance
OL:	Oleate/linoleate
OO:	Oleate
OS:	oleate/ stearate
PA:	Palmitate/arachidonate
PDH:	Pyruvate dehydrogenase
PFK:	Phosphofructokinase
PI3-K:	Phosphatidylinositol 3-kinase
PIP2:	Phosphatidylinositol-4,5-bisphosphat
PKC:	Protein kinase C
PKCθ:	Protein kinase C θ
PL:	Physical Activity during leisure excluding sport

PL:	Palmitate/linoleate
PO:	Palmitate/oleate
PP:	Palmitate
PS:	Sport during Leisure Time
PW:	Physical Activity at Work
Ra:	Rate-of-appearance
Ras:	Rat sarcoma
REE:	Resting energy expenditure
RQ:	Respiratory quotient
Sd:	standard deviation
Sem:	standard error of the mean
SPT:	serine plamotyltransferase
SP:	Stearate/palmitate
SS:	Stearate
TAG:	Triacylglycerol
Tg:	transgenic
TG:	Triglycerides
TSH:	Thyroid Stimulating hormone
TTR:	Tracer-to-tracee-ratio
UCP:	uncoupling protein-3

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1. Introduction

Insulin resistance is an important characteristic for type 2 diabetes and can precede diabetes for several years. It appears 10-20 years before diabetes type 2 (DeFronzo, 1988), (Petersen and Shulman, 2002). According to World Health Organization (WHO) the number of people with diabetes has nearly quadrupled from 108 million in the year 1980 to 422 million in the year 2014 worldwide. Type 2 diabetes is responsible for around 90% of all diabetes worldwide. Type 2 diabetes is one of the most chronically disease with a continuous rising incidence. The mortally rate is increased as well as numerous complications are developing. Thus, the disease does not only indicate a limitation of the life span but also the life quality (Paulweber et al., 2010).

According to Narayan et al. (2003) the lifetime risk of developing diabetes for persons in the United States born in the year 2000 is 32.8% for males and 38.5% for females. Patients with type 2 diabetes diagnosed at age 40 years, men will lose 11.6 life-years and 18.6 quality-adjusted life-years and women will lose 14.3 life-years and 22.0 quality-adjusted life-years (Narayan et al., 2003). For the year 2030 in Europe one in ten Europeans aged 20-79 are forecasted to develop diabetes (Lindstrom et al., 2010).

1.1. Insulin resistance

Insulin resistance is defined as the lack of adequate response to circulating insulin in several tissues, such as liver, muscle, and adipose tissue. The insulin resistant state refers to a reduction of insulin sensitivity with decreased insulin stimulated glucose uptake from the blood into insulin responsive tissues, as well as glycogen synthesis. Additionally, insulin mediated inhibition of glucose production in the liver is impaired in this state (DeFronzo, 1988), (Boden, 2001), (Abdul-Ghani and DeFronzo, 2010). Defective insulin action finally leads to fasting and postprandial hyperglycemia (Holland et al., 2007).

Beta cells, which represent 80% of the pancreatic cells, produce insulin, the only glucose lowering hormone. The insulin secretion is under physiological conditions proportional to the blood sugar level. Furthermore, insulin induces anabolic metabolic processes (e.g. glycogen synthesis, lipid synthesis, protein synthesis) and inhibits catabolic processes (e.g. glycogenolyis, lipolysis and proteolysis). Via uptake of glucose into the target cell blood sugar is reduced and maintained in a stable range. Insulin binds to insulin receptors with tyrosinkinase activity and activates a cascade, the signaling pathway. In skeletal

muscle cells and adipocytes insulin dependent glucose transporters (GLUT4) translocate from intracellular vesicle storage sites to the plasma membrane. GLUT 1 and 3 in cerebral tissue and erythrocytes and GLUT 2 in pancreatic beta cells and hepatocytes are insulin independent. They operate like an alleviated diffusion along a concentration decline (Löffler, 2005), (Petersen and Shulman, 2002).

Causes of insulin resistance

Previous studies could show a correlation between insulin resistance (IR) and aging with a reduction in the capacity of the glucose uptake in elderly subjects. In this regard post receptor defect in target tissue insulin action causes supposedly peripheral insulin resistance. As well as the number of glucose transport and metabolic units with a normally function is abated (Fink et al., 1983), (Fink et al., 1986), (Fink et al., 1986). The aging process means also a state of chronic inflammation, the so-called inflammaging. And a chronic low-grade (sterile) inflammation induces IR. So reduced insulin sensitivity is related with local and systemic elevated pro-inflammatory molecules such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, interferon (IFN)-γ, inflammatory adipokines, chemokines, and FFAs (Frasca et al., 2017), (Hotamisligil, 2017). However, studies indicate it is probably more related with age-related effects acquired during life span and less with age. It points towards a diminished energy metabolism or lifestyle-related abnormities (Phielix et al., 2011).

Sedentary lifestyle with low physical activity is often linked with obesity and insulin resistance. Mitochondrial content can increase with exercise training in diabetic type 2 patients. So oxidative capacity ameliorates parallel to insulin sensitivity. Genetic and nutrition factors including „western“, fat rich, high caloric diet are also involved in the development of insulin resistance (Bruce et al., 2006), (Reznick and Shulman, 2006), (Toledo et al., 2007), (Szendroedi and Roden, 2008), (Kacerovsky-Bielesz et al., 2009), (Szendroedi et al., 2009), (Phielix et al., 2010), (Meex et al., 2010).

Previous studies suggested the existence of a defect in mitochondrial oxidative phosphorylation in skeletal muscle in insulin resistance states (Schrauwen 2004), (Lowell and Shulman, 2005), (Morino et al., 2005), (Roden 2005), (Abdul-Ghani and DeFronzo, 2008). Such as elderly decline in mitochondrial function (Petersen et al., 2003), (Short et al., 2005).

Smoking has probably an effect on insulin action. However, the mechanism is not exactly known. The insulin sensitivity is lower in chronic cigarette smokers compared with nonsmokers. After 1 to 2 weeks of smoking cessation, the insulin sensitivity ameliorates, but do not normalize. Probable reasons are the greater saturation of skeletal muscle lipids or normalization of the increased IRS-1ser636 phosphorylation after smoking cessation (Bergman et al., 2009), (Bergman et al., 2012).

Less cellular insulin receptors are developed in different tissues and the receptors become non-sensitive as a response of prevalent high insulin secretion. Similarly, the beta cells of the pancreas react with an increased compensatory production and secretion of insulin as a result of the partially IR like vicious cycle of metabolic decompensation (Amatruda et al., 1975), (Kolterman et al., 1980), (Le Marchand-Brustel et al., 1999), (Fuentes et al., 2010).

Some studies manifest a significant link between obesity and insulin resistance (Banerji et al., 1997). The excessive body fat in obesity may lead to quantitative and functional alterations of specific adipose tissue compartments.

Lipid overload is also associated with a general low-grade chronic inflammatory state. The macrophage activation and inflammatory cytokine production are increased. Big overloaded adipocytes are distinguishable from small healthy ones. The secretory profile and inflammation degree are different (Krebs and Roden 2005), (Fuentes et al., 2010).

Generally chronic elevation of non-esterified fatty acids (NEFA) due increased fat intake and impaired inhibition of lipolysis by insulin is observed in obesity and can lead to ectopic fat deposition in non-adipose tissue (e.g. liver, skeletal muscle) (Reaven et al., 1988), (Boden, 1997), (Ferrannini, 1998), (Kelley et al., 1999), (Boden, 2001), (Bruce et al., 2006), (Vistisen et al., 2008), (DeFronzo, 2010).

Nonalcoholic fatty liver disease (NAFLD) is recognized as a significant public health problem. The prevalence of NAFLD is 20-30% of the general population of Western countries (Pappachan et al., 2017). Nonalcoholic fatty liver disease or hepatic steatosis is strongly linked with hepatic insulin resistance (Szendroedi et al., 2009), (Roden, 2006).

It is fact, that adipose tissue belongs to the endocrine system and is an active endocrine and paracrine organ. Visceral and subcutaneous fat depots release adipokines, cytokines and hormones (Ghadge et al., 2018). Adipocytes secrete different hormones, the adipokines like leptin, adiponectin or resistin amongst others in proportion to adipocyte

tissue mass (Pittas et al., 2004). The adipokines are polypeptides and coordinate multiple physiological functions such as energy balance, insulin sensitization, appetite regulation, and inflammatory response (Srinivasan et al., 2018).

Increased intramyocellular content of lipids (IMCL) is strongly assumed for the pathogenesis of IR (Perseghin et al., 2002), (Schrauwen-Hinderling et al., 2006), (Szendroedi and Roden, 2009).

Lipid-rich infusions and thereupon increased plasma free fatty acid levels induce a dose-dependent reduction of insulin-stimulated glucose disposal in insulin-sensitive humans (Roden et al., 1996). And this reversible insulin-resistance is caused by direct interaction of lipid metabolite with the insulin signal pathway. In the insulin resistant state of overt T2DM, it seems that lipid overload from energy-rich diet, increased lipolysis rates and low physical activity lead to elevation of intramycellular lipids, which together with impaired lipid oxidation due to mitochondrial dysfunction interact like a vicious cycle that fortifies insulin resistance (Shulman, 1999), (Schmitz-Peiffer, 2000), (Roden, 2004), (Belfort et al., 2005), (Brehm et al., 2006), (Szendroedi and Roden, 2009).

Measurement of insulin resistance

The hyperinsulinemic-euglycemic clamp test, first described by DeFronzo et al. is the gold standard to measure insulin resistance (DeFronzo et al., 1979), (Donga et al., 2015). In fasting condition, a continuous i.v. infusion is given to raise plasma insulin concentration. Via variable i.v. glucose solution (GI, 20%) the plasma glucose is maintained between 4.5 and 5.5 mmol/l. Therefore, the blood glucose is measured every five- ten minutes intervals. Under steady-state condition in the last 60 minutes between 80 to 120 min the whole insulin sensitivity can be calculated (Hills et al., 2004). The infused glucose rate to maintain euglycemia correlates with the tissue sensitivity to exogenous insulin. About 80 % of the insulin-stimulated glucose disposal during the clamp test are caused by skeletal muscle (Szendroedi and Roden, 2008).

1.2. Cellular mechanism of insulin resistance

1.2.1. Insulin signaling pathway

In order to determine the mechanisms underlying insulin resistance it is important to understand the mechanism of insulin signaling pathway. An overview of the insulin signaling pathway is described below.

Insulin binds to insulin membrane receptors of target tissues. The insulin receptor is a heterotetramere glycoprotein with tyrosine kinase activity. Two extracellular alpha subunits and two intracellular beta subunits are linked by disulfide bonds (Lee and Pilch, 1994).

Upon binding the insulin, the receptor changes its conformation. Thereby the intracellular part (beta subunit) of the receptor with specific several tyrosyl domains, phosphorylate each other in an ATP-dependant way (autophosphorylation) (Kasuga et al., 1982).

This tyrosine phosphorylation induces a cascade also via phosphorylation of specific tyrosyl domains of intracellular substrates including the insulin receptor substrates (IRS), Src homology collagen (Shc), and adapter protein with PH and SH2 domain (APS) binding to the receptor of SH2-domains (Myers and White, 1993), (Taha and Klip, 1999). 6 suptypes of IRS are discovered with IRS-1 and IRS-2 being the best characterized subtypes (Cai et al., 2003).

Several signaling pathways are activated.

MAPK (mitogen-activated protein kinase) pathway mediated by Ras leads to an activation of certain transcription factors (Skolnik et al., 1993) and controls gene expression and cell differentiation and growth.

The phosphatidylinositol 3-kinase (PI 3-K) pathway mediates the metabolic effects of protein synthesis, glucose transport and glycogen synthesis. Via the key enzyme protein kinase B, also called Akt, the glycogensynthase-kinase-3 (GSK3) is phosphorylated and simultaneously inhibited-thus, glycogen synthesis increases (Cross et al., 1997). Furthermore, activated Akt controls the glucose transport via translocation of GLUT 4 transporter to the plasma membrane, protein synthesis, cell growth and differentiation. PI3 3-K regulate the protein synthesis via mTOR: mammalian target of Rapamycin (White, 1998), (Czech and Corvera, 1999), (Lee and White, 2004).

Alternative pathways that are also involved on the insulin glucose uptake like protein kinase C are discussed (White, 1998), (Withers and White, 2000).

1.2.2. Lipid induced insulin resistance

In 1963 Randle presented with a so-called glucose-fatty-acid cycle, suggesting that metabolism of glucose and free fatty acids directly impact each other in muscle and adipose tissue. In an animal study with isolated heart and diaphragm preparations, a

competition between free fatty acidy (FFA) and glucose as substrate for oxidation could be detected. If one of both is metabolized, the use of the other would be inhibited. Thus, glucose oxidation was inhibited by increased FFA uptake from augmented plasma concentrations of FFA. Thereby it was concluded that this mechanism might be important for the development of lipid-induced insulin resistance (Randle et al., 1963), (Randle et al., 1964), (Hue and Taegtmeyer, 2009).

Several studies investigated the effect of administration of lipid-rich infusions in lean insulin-sensitive humans, which induced a reversible reduction of the whole body glucose disposal within a few hours. However, these studies could not confirm an inhibition of plasma glucose oxidation by high plasma FFA concentration but rather suggested direct inhibition of glucose uptake and/or phosphorylation under hyperinsulinemic-clamp condition by intracellular lipid metabolites (Wolfe et al., 1988), (Roden et al., 1996), (Shulman, 1999), (Roden, 2004).

In contrast to the mechanism described by Randle et al. in humans decreased intramyocellular levels of glucose-6-phosphate were found at elevated plasma fatty acid concentrations. This indicates that insulin resistance is caused by direct inhibition of insulin-mediated glucose transport activity, and not as proposed in the Randle cycle by inhibition of pyruvate dehydrogenase activity and glucose oxidation (Roden et al., 1996). A few authors showed that the administration of lipid-rich infusions during hyperinsulinemic-euglycemic clamp is detected to cause a reversible insulin resistance in skeletal muscle and the liver with a reduction of insulin-stimulated mitochondrial activity in healthy humans (Roden, 2004), (Brehm et al., 2006).

Recent studies indicate that lipid metabolites such as diacylglycerols (DAG), ceramides and long chain FFA (acyl CoA) are the mediators of lipid-induced insulin resistance directly inhibiting the insulin signaling pathway (Shulman, 1999), (Summers, 2006), (Holland et al., 2007). Animal studies suggest, that DAG activate sensitive protein kinase C isoforms (PKC θ, PKC ε) (Yu et al., 2002) resulting in inhibitory serine-phosphorylation of IRS-1 and in turn reduced phosphatidylinositol 3-kinase activity (Dresner et al., 1999).

Studies with transgenetic and knock out mice with reduced activity of acetyl-CoA carboxylase (Acc2), the key enzyme for the formation of Malonyl-CoA, the precursor for lipogenesis and potent inhibitor for lipid uptake into mitochondria for oxidation, showed

that increased lipid oxidation rates did not impair glucose oxidation and uptake. Contrarily these mice were protected from insulin resistance induced by high fat diet and had reduced PKC theta activation (Choi et al., 2007). The investigators proposed Acc2 inhibition as a possible target for the therapy of T2DM.

Human studies support this concept even though other PKC isoforms, namely PKC-βII and –δ were activated (Itani et al., 2002), (Erion and Shulman, 2010). Other studies accent the role of ceramide metabolites such as glucosylceramides in for the development of insulin resistance (Schmitz-Peiffer, 2010). In a further study diet-induced as well as genetically (leptin receptor deficient) obese mice were treated with myriocin, an inhibitor of serine palmitoyl transferase-1 (SPT1), which reduced de novo ceramide synthesis and intramyocellular ceramide contents. This therapy resulted in amelioration of the insulin resistance despite accumulation of intramyocellular DAG, triglycerides and acyl-CoA. The investigator suggests a therapy for insulin resistance via inhibition of SPT1 (Ussher et al., 2010).

The following figures (figure 1) present an overview of different molecular mechanism of lipid-induced insulin resistance.

Diacylglycerol induced insulin resistance:

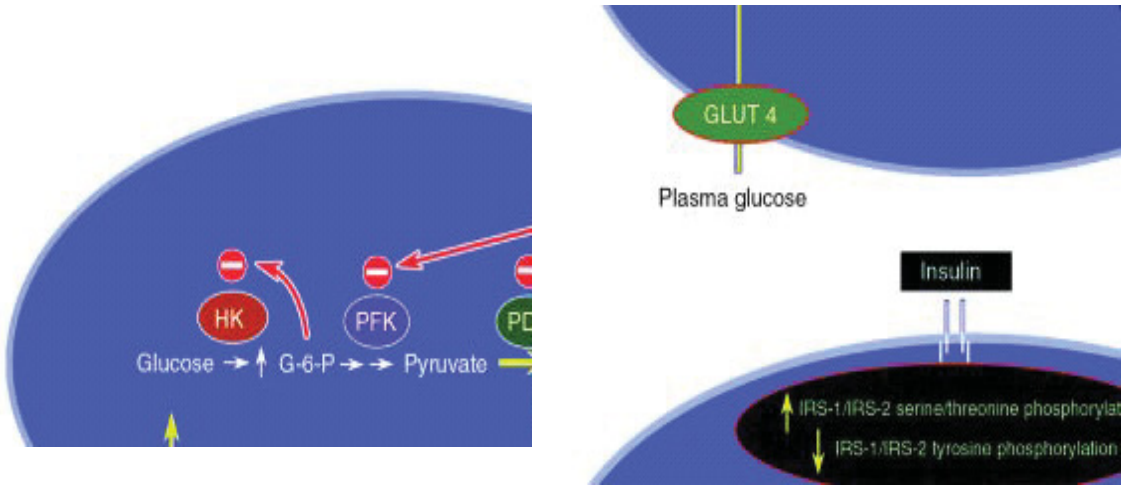

Fig. 1: Molecular cellular insulin resistance mechanism in comparison Randle cycle and updated mechanism (Shulman, 2000)

Adaption of Randle free fatty acids-glucose cycle in skeletal muscle. (Fig. 1: Molecular cellular insulin resistance mechanism in comparison Randle cycle and updated mechanism (Shulman, 2000)-left):

Increased free fatty acids concentration cause increased intramitochondrial acetyl CoA/CoA and NADH/NAD^+ ratios. This leads to an inhibition of pyruvate dehydrogenase. Thus, citrate concentrations increase and Phosphofructokinase is therefore inactivated. Consequently the intracellular glucose-6-phosphate concentration rise and would inhibit hexokinase II activity. The subsequently rise of intracellular glucose concentration and would decline muscle glucose uptake.

From Shulman et al. (2000) alternative mechanism for fatty acid–induced insulin resistance in human skeletal muscle mediated via lipid metabolites (DAG, ceramide, fatty acyl CoA) (Fig. 1: Molecular cellular insulin resistance mechanism in comparison Randle cycle
and updated mechanism (Shulman, 2000)-right):

Elevated fatty acids concentrations in muscle or decreased metabolism of fatty acids intracellular cause an increase of intracellular fatty acid metabolites such as diacylglycerol, fatty acyl CoA, and ceramides. Out of it, a serine/threonine kinase cascade (possibly by PKCθ) is initiated. First step is a phosphorylation of serine/threonine sites on insulin receptor substrates (IRS-1 and IRS-2). This results in a reduced activation of PI-3 kinase via insulin receptor. Thus, the glucose uptake is reduced and the insulin receptor signaling pathway is declined.

HK: hexokinase II; PFK: phosphofructokinase; PDH: pyruvate dehydrogenase; PKCθ: protein kinase Cθ (Shulman, 2000).

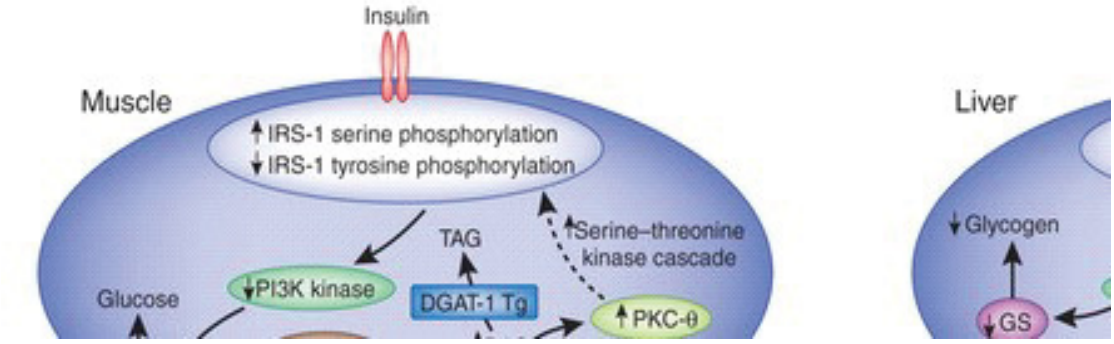

Fig. 2: Molecular cellular insulin resistance in muscle and liver in comparison

Elevated DAG levels induce an activation of PKC-θ and PKC-ε in skeletal muscle and liver. Thus, in turn, insulin-stimulated IRS-1/IRS-2 tyrosine phosphorylation is reduced. This leads also to decreased PI3K activation and downstream insulin signaling.

Skeletal muscle (Fig. 2: Molecular cellular insulin resistance in muscle and liver in comparison- left):

Muscle glycogen synthesis is diminished. This is associated with a reduced insulin-stimulated GLUT4 translocation to the plasma membrane.

Liver (Fig. 2: Molecular cellular insulin resistance in muscle and liver in comparison- right):

Hepatic glycogen synthesis is diminished resulting in reduced glycogen synthase activation and so increased hepatic gluconeogenesis.

Studies with transgenic and knockout mice, as well as antisense oligonucleotides to knock down specific proteins, leads to the supposition that DAG cause mediated insulin resistance via impaired insulin signalling pathway

KO: knockout; GLUT4: glucose transporter type 4; TAG: triacylglycerol; LPL: lipoproteinlipase; Tg: transgenic; FATP-1: fatty acid transport protein-1; DGAT: diacylglycerol *O*-acyltransferase; UCP3: uncoupling protein-3; GS: glycogen synthase; LCAD: long-chain acyl-CoA dehydrogenase; mtGPAT: mitochondrial glycerol-3-phosphate acyltransferase; ASO: antisense oligonucleotide. (Erion and Shulman, 2010).

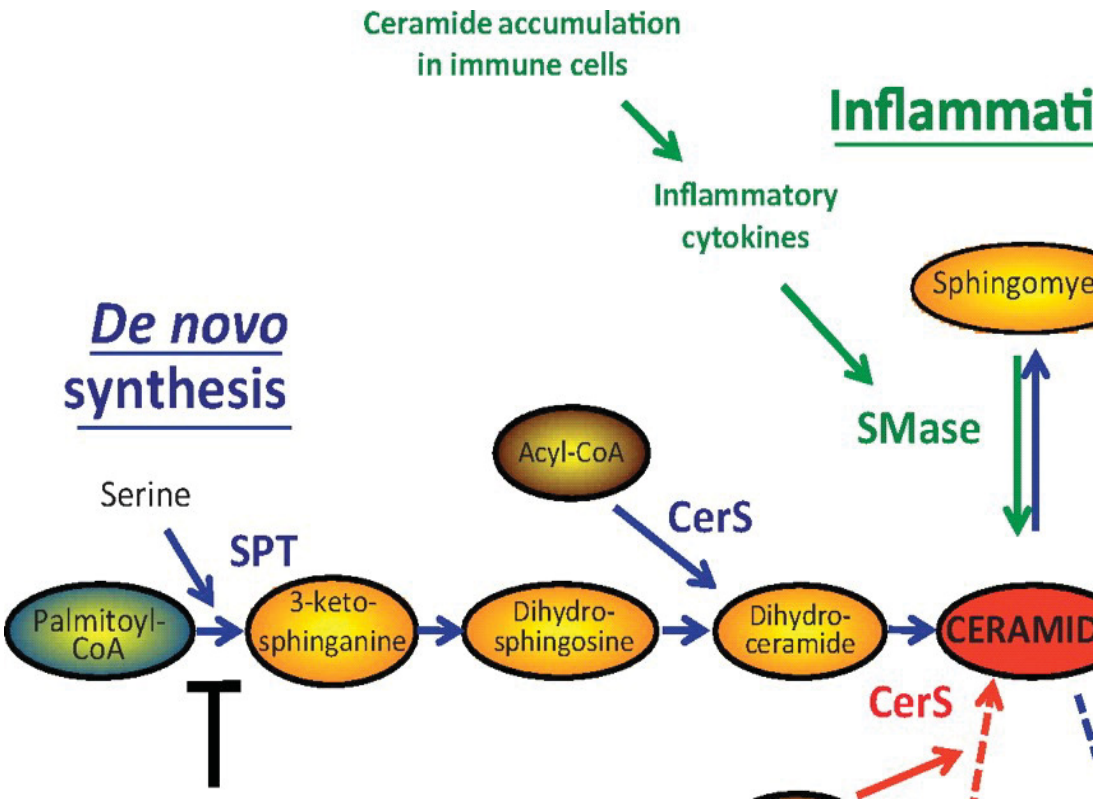

Fig. 3: Metabolism of ceramide and ceramide induced insulin resistance

Ceramide induced insulin resistance (Fig. 3: Metabolism of ceramide and ceramide induced insulin resistance):

Ceramides have a direct effect on insulin signaling pathway. Via activation of phosphatases or atypical PKCζ, Akt (or PKC beta) is inhibited. Thus, insulin resistance is induced (Hajduch et al., 2008), (Schmitz-Peiffer, 2010).

De novo synthesis of ceramide: serine and palmitoyl-CoA are connected by SPT (serine palmotyltransferase). Then saturated and unsaturated fatty acids (acyl-CoA) are included by CerS (LASS) isoforms.

The salvage pathway: By CerS activity ceramide accumulate via fatty acid oversupply. Sphingomyelin hydrolysis: ceramide dissolve from the cell membrane. Inflammation can induce ceramide release from sphingomyelin. Therefor through SMase is activated by cytokines.

Studies allude that ceramide (possibly also complex sphingolipids) impair insulin action by inducing further stress signaling and mitochondrial dysfunction or by direct interaction on insulin signal transduction (Schmitz-Peiffer, 2010).

1.3. Subcellular lipid metabolites

- ### Diacylglycerol

Diacylglycerol (1,2-Diacyl-sn-glycerin) is an intermediate product of the triacylglycerin synthesis. It includes two fatty acids bonded via ester compound to glycerine. It acts as second messenger with different functions. DAG are generated via hydrolysis of the phospholipid phosphatidyl-inositol-bisphosphate (PIP2) induced by Phospholipase C (Kitatani et al., 2008).

- ### Ceramides

The ceramide complex is composed of sphingosine and free fatty acid. It is a necessary part of the mammalian cell membrane. Further operations as a cellular signaling molecule are the regulation of the differentiation, apoptosis, and proliferation. It is assumed that ceramide takes part in the development of different diseases (cancer, inflammation, diabetes, obesity, neurodegeneration). Via Sphingomyelin hydrolysis (unhinge from the cell membrane), de novo generation (catalyzed by the enzyme serine palmitoyl transferase) and salvage pathway (unhinge from complex molecules) ceramide can be built (Kitatani et al., 2008).

Until now, there was no evidence in humans, which lipid metabolite, DAG or ceramides mediate lipid-induced insulin resistance. Furthermore, it was yet unclear which subcellular fraction of DAG (i.e. membrane vs. cytosol) play a role. In addition, the sequence of events leading to the intracellular inhibition of insulin signal transduction were not well investigated.

- ### PKC isoforms

PKC is a group of proteins with related structure and function. It is composed of a polypeptide with a regulatory N-terminal and a catalytic C-terminal. PKC are very important for the signal transduction in the cell (Uemura et al., 2005).

12 isozymes of PKC are described, which are divided in three categories: classical (cPKC α, βI, βII, γ), novel (nPKC δ, ε, θ, μ, η) (Newton 2001) and atypical isoforms (aPKC ζ, ι/λ) (Newton, 2003), (Timmers et al., 2008). The function of the PKS isoforms is the regulation of diverse signaling pathways. Therefore, their downstream kinases as well as the substrate proteins are phosphorylated. A translocation of the PKC can be made during the activation from the cytosol to the cell membrane. The activation of PKS can be

measured by analyzing the amount of the PKS isoform in the membrane (Timmers et al., 2008).

2. Hypotheses and aim of the study

The questions addressed and investigated here are:

1. Which subcellular lipid metabolites mediate short-term lipid-induced insulin resistance?
2. What is the sequence of cellular events during induction of short-term lipid-induced insulin resistance, regarding the concentrations of lipid metabolites and activation of specific PKC isoforms?

The experiments of this thesis aimed to test the following hypotheses:

- Increased availability of FFA induces activation of PKC θ and insulin resistance in skeletal muscle after 4 hours of lipid infusion.
- Induction of insulin resistance is related to increased membrane fractions of DAG but not related to ceramide contents.

3. Methods

3.1 Study design

This study was carried out at the research group of the department for Clinical Diabetology of the German Diabetes Centre (DDZ), Heinrich Heine University Düsseldorf.

All volunteers had to give a written consent. The experiment was performed in accordance with the principles of the Declaration of Helsinki, and was authorized and registered (ClinicalTrials.gov Identifier number: NCT01229059) by the local institutional ethics board Düsseldorf.

In order to perform the study, muscle biopsies have been sampled under standard conditions at the German Diabetes Centre in Düsseldorf. A complete description of study protocol can be found in paragraph 3.3. Afterwards the muscle biopsies were sent to the laboratory of Dr. G. I. Shulman, Yale School of Medicine, Yale University, New Haven, Connecticut, USA, for chemical analysis.

The following figure (figure 4) shows an overview of the study:

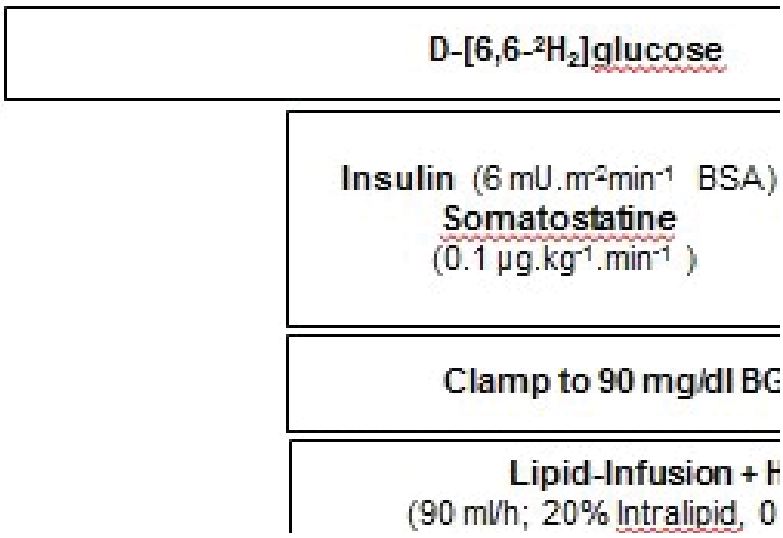

Fig. 4: Study design

There were two study designs with the only difference of an additional muscle biopsy after 2.5 h lipid infusion.

Study with three muscle biopsies: at baseline, after 2.5 hours and 4 hours lipid infusion (n=8)

Study with two muscle biopsies: at baseline and after 4 hours lipid infusion (n=8).

3.2 Volunteers

All subjects were recruited using local and online advertisement by the German Diabetes Center Düsseldorf.

The research volunteers who participated in these studies included healthy, non-diabetic, normal weight untrained individuals (2 women and 10 men) with an age from 20-55 years and a BMI within 20-25 kg/m². Four recruited male subjects, who already received 2 muscle biopsies, were additionally studied again with 3 muscle biopsies. Finally 16 experiments were done.

- Inclusion criteria

The subjects were prescreened to confirm that they were in excellent health, lean, nonsmoking, non-diabetic, without a family history of diabetes, any cardiovascular disease, arterial hypertension, thyroid disorder and taking no medications.

The subjects were instructed to eat on a regular basis a weight-maintenance diet containing at least 150 g of carbohydrate per day, thus, three days before admission. All subjects were instructed not to perform any exercise other than normal walking for the three days before the study. The female subjects were studied during the follicular phase (days 0 through 12) of the menstrual cycle in order to minimize changes in glucose metabolism resulting from ovarian hormonal effects.

Subjects were fasted overnight prior to the experiment. They continued to fast until the completion of the study.

- Exclusion criteria

The exclusion criteria are: acute illness within the last two weeks, autoimmune disease (leucocyte < 5000/ µl), renal insufficiency (creatinine > 1.5 mg/dl), cardiovascular diseases, anemia (Hb< 12 g/l), donation of blood within the last four weeks before the study, thyroid diseases, pregnancy, lactation period, smoking, night-shift working (disturbed rhythm), defective coagulation or wound healing, cancer, allergy against soya products, risk for or apparent of AIDS/HIV, under medication treatment, participation in another study within two month before the examination.

- Preliminary inquiry

The subjects underwent a standard three-hour oral glucose-tolerance test (with a 75g oral glucose load), a complete medical history check, an electrocardiogram and a physical examination combined with blood tests to verify that the following values were normal: blood and pallets counts, fasting plasma glucose, glycosylated hemoglobin A 1c, concentration of electrolytes, asparate aminotransferase, alanine aminotransferase, blood urea nitrogen, creatinine, cholesterol, and triglycerides, prothrombin time, and partial-thromboplastin time. The body height, weight, hip size and waist circumference were also measured.

Physical activity index was assessed with a self-administered questionnaire of habitual physical activity according to Baecke et al. Average physical activity was calculated 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) (Baecke et al., 1982).

Anthropometric and laboratory characteristics are given in table 1 (“Methods”).

- Preparation of volunteers

The subjects were elucidated and instructed again about the procedure. Following conditions have been checked: - nutrition- and liquid abstention more than 10 hours before the examination (exception: water and mineral water), no hard physical activity within more than 20 hours and no alcoholic addiction/drinking of alcohol the day before, no smoking more than 3 hours.

Before the calculation the parameters of the subject: name, first name, age, sex, height, weight, nutritional status was determined and recorded.

3.3 Experimental protocol

FFA-IR Study

This study takes place once the screening examination described in the "Preliminary inquiry" has been done and the subject chosen.

An all-day examination was performed with the subject, lying in a bed, started at 7:00 a.m. and ended at 3:30 p.m. with an observation time until 5:30 p.m.

Catheters (Vasifix; Braun, Melsungen, Germany) were inserted in antecubital veins of both arms. On one vein blood samplings were periodically taken to ascertain the laboratory values and the blood glucose. The blood glucose was measured twice every five minutes with a Beckmann glucose analyzer (Fullerton, CA). The other inflow was used to give infusions for the standardization of the experiment conditions and for the determination of the whole body and liver insulin sensitivity.

A primed-continuous infusion of a glucose isotope solution (start at 7:10 a.m.:5 min: 3.6 mg.kg body weight-1.fasting glucose [mg/dl]/90 [mg/dl]; 465 min: 0.036 mg.min-1.kg body weight-1) of D-[6.6-2H2]glucose (98% enriched; Cambridge Isotope Laboratories, Andover, MA) was performed in order to calculate the endogenous glucose production (EGP).

Parallel to it, Somatostatine (120.-510.min: 0.1 µg/kg.min, UCB Pharma) was infused to suppress endogenous insulin production, and glucagon (Strowski et al., 2000).

Insulin (Actrapid, Novo Nordisc, Bagsvaerd, Denmark) was administered as a primed-continuous infusion with low dose Insulin (120.-360.min: 0.1 mU kg^{-1}.KG.min^{-1}, ratio 10 min: 40 ml/h then 15 ml/h) and subsequently the clamp test was started with high dose

Insulin (360.min -510.min: 40 mU.m^{-2}.min^{-1}, ratio: 10 min: 40 ml/h then 20 ml/h) to create condition of hyperinsulemia. At the same time to insulin infusions a 20% dextrose infusion (solution 20 %) labeled with D-[6.6-$^{2}H_2$]-glucose (2% enriched) according to the hot glucose infusion (GINF) protocol was adjusted at 5 minutes intervals based on the actual glucose concentration to maintain euglycemia (120.-510.min).

Lipid induced insulin resistance is effected by lipid infusions, (Lipovenös 20%, Fresenius® Kabi GmbH Bad Homburg, Germany); 120.-510.min: 0.2mU.kg^{-1}.min^{-1}, ratio: 10 min: 30ml/min then 90 ml/min) with combined heparin (Heparin-Natrium, ratiopharm®, Ulm; Germany; 0.2 mU.kg^{-1}.min^{-1}) infusion. 1000 ml of lipid solution contains: soya bean oil: 200 g, linoleic acid: 87.5-117.2 g, z-linolenic acid: 9.06-22 g; glycerol: 25 g; phospholipid: 12 g, and a total energy of 8400 kJ/ 2000 kcal/l. Heparin induce to a disentanglement of lipoprotein lipase from its proteoglycan bonding and so to its higher activity. It splits in blood soluted triglyceride in fatty acids and glycerin (Chevreuil et al., 1993).

Whole body insulin sensitivity was assessed from steady-state glucose infusion rates (M-values) during the last 20 min of the clamp test.

Muscle biopsies of the vastus lateral muscle were obtained at its baseline and after 4 hours lipid infusion. From the ninth experiments to the last one, a supplementary muscle biopsy was taken after 2,5 lipid infusion. The increase of subcellular lipid metabolites (diacylglycerols, ceramides) as well as the activation of mediators (PKC) that impart the inhibition of Insulin signaling were analyzed in muscle biopsies during the time prior and during lipid donation.

Furthermore, indirect calorimetry examination was performed before and at the end of the experiment to evaluate the metabolized substrate.

3.4 Parameters and analytical procedures

3.4.1 Plasma hormones and metabolites

Plasma glucose concentrations were assayed with the glucose oxidase method (Glucose analyzer II, Beckman Coulter,Fullertan, CA, USA).

Venous plasma samples were placed on ice, centrifuged at 4°C, and separated and stored at -20°C until assay.

Plasma concentrations of FFA were measured by microfluorimetric method (WAKO, Neuss, Germany). None esterified FFA were turned enzymatic by Acyl-Synthase to Acyl-CoA that was quantified with Acyl-CoA oxidase and 3-Methyl-N-Ethyl-N-Hydroxyethyl-Anilin as violet coloured complex. In vitro lipolysis was prevented by collecting blood into vials containing orlistat and rapid centrifugation of the samples.

Plasma insulin and C-peptide were determined using double antibody radio-immunoassay kits (RIA), (Millipore, St. Charles, Miss, USA). The disjunction between the antigen-antibodycomplex and the free antigen is effected per second antibody in Polyethylenglycol. The supernatants decant complex is used for the analysis. EDTA ampules were utilized for blood collection.

Concentrations of triglycerides were measured by a peroxidase-coupled colorimetric assay (Roche/Hitachi, Roche Diagn Mannheim, Germany). Triglycerides were hydrolysed by lipoprotein lipase to glycerol and free fatty acids. Glycerol oxidizes to Dihydroacetonphosphat and hydrogen peroxide. Hydrogen peroxide creates with 4-aminophenzon and 4-chlorphenol a red coloring. The intensity of the colour is directly proportional to the triglyceride amount.

3.4.2 Plasma glucose isotopic enrichments

Marked Glucose with stable, not radioactive isotope deuterium D-[6,6-$^{2}H_2$] glucose was used. Deuterium is a hydrogen isotope that behaves quite similarly to ordinary hydrogen in chemical reactions, metabolic pathways and can be detected.

Plasma enrichments of D-[6.6-$^{2}H_2$] glucose was measured by gas chromatography-mass spectrometry (GC-MS; Hwlett Packard GC 5890, MS 5970®, Hewlett Packard, Palo Alto/USA). The plasma was deproteinized. Then, endogenous glucose and exogenous infused D2-Glucose was ivatized as the pentaacetat, following Ba(OH)2/ZnSO4 deproteinization and semi-purification by anion/cation exchange chromography.

Both masses were quantified by gas chromatography-mass spectrometry and as atom percent enrichment (APE) placed in rate. Subsequently, the endogenous glucose production could be calculated by inclusion of the infused D2-glucose.

3.5 Hyperinsulinemic-euglycemic clamp

The tissue sensitivity to insulin was performed by euglycemic-hyperinsulinemic clamp technic (related approximately to fasting plasma glucose and postprandial peripheral insulin concentrations).

The glucose infusion rate that is dispensed to maintain constant glucose levels of euglycemia (steady-state conditions) corresponds to glucose uptake of all the tissues in the body. Thus, that is a method to determinate the tissue sensitivity to exogenous insulin (DeFronzo et al., 1979).

The pancreatic clamp test was performed as following:

A 4 h period (120.-360.min) of insulin (Insuman ®Rapid, sanofi aventis, Frankfurt am Main, Germany) infusion with fasting insulin levels (0.1.mU.kg^{-1}·min^{-1}) (infusion rate: after a 10 min bolus of 40 ml/h, 15 ml/h) was dispensed at first. Subsequently at 1 p.m. the hyperinsulinemic clamp with a 2.5 h period (360.-510.min) of high dose insulin (40 mU.m^{-2}.BSA·min^{-1}) (infusion rate: after a 10 min bolus of 40 ml/h, 20 ml/h) was performed.

Simultaneous to insulin a variable rate of 20% glucose infusion labelled with D-[6.6-2H2] glucose (2% enriched) was dispensed in pursuance of the hot glucose infusion (GINF) protocol to create a standardized condition of normoglycemia with clamping the plasma glucose at 90 mg/dl. Thus, the glucose infusion was adjusted at 5-minute intervals according the actual plasma glucose concentration that was measured at the same time twice from the antecubital vein. Conditions of euglycemic-hyperinsulinemia were so induced by infusions of regular human insulin and a variable 20 % glucose infusion.

A Somatostatine (Somatostatin HEXAL®, Holzkirchen, Germany) infusion was given (0.1 µg/kg.min) in combination to insulin to inhibit secretion of hormones with effect on glucose metabolism (DeFronzo et al., 1979), (Ferrannini and Mari, 1998).

Own blood (2ml blood to 48ml solution) was added to insulin infusion to have a better insulin bond in the solution. During the test, blood was periodically collected for determination of plasma glucose, insulin, c-peptide, FFA and triglycerides.

3.6 Indirect calorimetry

It is a non-invasive method to measure the respiratory quotient as well as the resting energy expenditure (REE). The rates were calculated during the last 20 minutes of the baseline period (no infusions were given) and again during the last 20 minutes of the clamp test with continuous indirect calorymetry. The calorimeter (System Vmax Encore 29n Viasys, type MBM-100-33-00, Ser-Nr. 65178) gives information about manner and extends of metabolized substrates in vivo. It yields the rates of substrate utilization, and energy metabolism in vivo by gas exchange measurements. Thereby oxygen (02) consumption and carbon dioxide (CO2) production are measured. The respiratory quotient was determined using the ratio of oxygen consumption (VO_2) and carbon dioxide production (V CO_2) respectively to know which substrate was metabolized in vivo; protein has an RQ= 0,81, fat: 0,7, and carbohydrate: 1,0; and the whole body energy expenditure in rest, the REE was assessed automatically with the Weir equation ($3{,}941*VO_2+1{,}11*VCO_2$). The urea nitrogen in the 24 h urine (g/h) was additionally considered for the determination of protein oxidation. Resting energy expenditure represents the amount of calories required for a 24-hour period by the body during a non-active period (Ferrannini, 1988), (Simonson and DeFronzo, 1990), (Westerterp, 2017).

In the experiment, indirect calorimetry was performed during euglycemic-hyperinsulinemic clamp technique in comparison to baseline state.

Insulin excite carbohydrate metabolism to accumulate glucose. But elevated plasma free fatty acid levels via lipid infusion affect in lean subjects glucose oxidation and glucose storage during euglycaemic insulin clamps (Jequier and Felber, 1987).

- RQ-value

The respiratory coefficients for organisms range generally from 1.0 to 0.7. RQ can be more than 1.0. This indicates for an anaerobic respiration like physical stress, also during growth phase or feeding huge carbohydrate amount a higher RQ is found. The RQ depends on which substrate is metabolized as an energy source during cell respiration.

RQ is at 1.0, when carbohydrates are the only substrate being oxidized, at 0.7 when only pure fatty acids are oxidized (RQs for oxidation of palmitate: 0.696, stearate: 0.692, or oleate: 0.706), and around 0.8, when only proteins are oxidized. Mixed meal provides a RQ at 0.85 (Simonson and DeFronzo, 1990).

- REE

Resting energy expenditure is the energy requirement for the organism to sustain normal body fuel economy during a 24 h non-active-period. It is around 50-70 % of the whole energy requirement (Simonson and DeFronzo, 1990), (Zurlo et al., 1990).

- Examination/calculation

The subject was supine in a quiet atmosphere with no acousto-optical deflexion. After a 10-minute rest period the respiration cover with inspiration and expiration adapter, adherent cling wrap and respiration gas hose was donned. The adherent cling wrap was placed into the correct position, so suction of the ambient air could be prohibited. Then a 10 minutes equilibration phase was done under standard- analysis conditions but the respiration gas exchange was unregistered.

Volume- (with air pressure, temperature, air humidity) and then gas (O_2, CO_2) calibration were executed. Afterwards the measurement started. The concentration of O_2 and CO_2 were performed continuous in inspiration and expiration air (breathy by breath) using a tight lapped mask for nose and mouth. The concentration differences of respiration and known stable ambient air were detected. The rates (RQ, REE) were calculated during the last 20 minutes of the measurement. Noticeable problems (activity, cough) were noted.

At the end of the measurements an individual recalibration was made using pure gases.

3.7 Measurement of endogenous glucose production

Endogenous glucose production is at average ~1.8 $mg \cdot kg^{-1} \cdot min^{-1}$ in an overnight fasted healthy adult. The amount is provided from liver ~95% and kidneys ~5%. (Cobelli et al., 1987), (Gastaldelli et al., 1999)

Therefore, subsequent procedure was performed:

A primed-continuous infusion (start at 7:00 a.m.: 5 min: 3.6 mg.kg.bodyweight$^{-1} \cdot$fasting glucose [mg/dl]/90 [mg/dl]; 380 min: 0.036 mg.min^{-1}.kg body weight^{-1}) of D-[6.6-2H2]glucose (98% enriched; Cambridge Isotope Laboratories, Andover, MA) was administered from the beginning of the experiment until the end of the clamp test to define the endogenous glucose production (EGP). The total glucose production rate was determined from the isotope enrichment in different venous collected blood samples. The difference of total glucose production and infusion rate of not marked glucose show the endogenous glucose rate.

3.8 Skeletal muscle biopsy

Following approach was performed to attain a muscle biopsy:

After re-elucidation about the accomplishment and checking the exclusion criterias (intolerance to local anesthetics, anticoagulant treatment, well-known coagulation disorder) only baseline sample prior lipid infusion (90. min) and after 4 hours lipid infusion (360.min) were taken in the first eight studies. In the following eight experiments, a third muscle biopsy was obtained additionally after 2.5 hours lipid infusion (270. min).

The biopsy was extracted from the external vastus lateralis muscle with changing every biopsy to the contralateral thigh side. A following incision at the same side was made proximately 4 cm away from the former. In case there were not enough tissue the operation has been repeated with the accordance of the subject at through the same incision as before.

For this purpose, the subject was supine and resting with a pad under the knee. The location of the puncture was defined on contracted vastus lateralis muscle 15 cm above the patella. If necessary, the area was shaved with a disposable shaver.

The muscle was prepared sterilely and under fan-shaped subcutaneously injection of local anesthesia (Xylocain®, 2%), with a percutaneous needle. An incision into the anaesthetized muscle was done by scalpel. Then muscle samples (250 mg minimum each) were taken using a modified Bergström biopsy needle connected over a line and a threeway cock to a syringe. An assistant has performed suction via the syringe (pull of the plunger). Ideally, muscle tissue appeared in the tubing system, stained free of blood, fat and connective tissue. The vacuum was finished by opening the threeway cock at the line. Then the biopsy needle could be removed.

The location of the puncture was immediately compressed with sterile swabs during at least 10 minute. Afterwards the cut was bandaged with patch strip, sterile compress, and a compression bandage. The patient had to check the wound for about two weeks.

All samples were snapping frozen within 30 s in liquid nitrogen and kept at -80 °C until analysis. biopsies were homogenized in six volumes of lysis buffer containing 50 mmol/l HEPES, pH 7.5, 137 mmol/l NaCl, 1 mmol/l CaCl2, 1 mmol/l MgCl2, 10% glycerol, 2 mmol/l EDTA, 10 mmol/l NaF, 2 mmol/l Na3VO4, and protease inhibitor cocktail.

Muscle homogenates were solubilized in 1% NP-40 for 1 h at 4°C and centrifuged at 14,000g for 10 min. The supernatant was used for insulin signaling studies.

- Isolation and preparation of muscle fibers

Muscle biopsy samples were directly dissected free of fat and connective tissue. Then a small sample of ~30 mg was mounted in ice-cold biopsy containing preservation medium (biops; oroboros Instruments, Innsbruck, Austria). Muscle fibers were made permeable with saponin. So diffusion limited was excluded, substrate dependent respiration. Afterwards muscle fibers were transferred into ice-cold mitochondrial respiration buffer (MiRO5; OROBOROS Instruments). Subsequently, the muscle fibers were placed to the oxygraph to detect high-resolution respirometry corrected for wet weight.

3.8.1 Myocellular lipid metabolites

The muscle biopsies were sent to the laboratory of Dr. G.I.Shulman, Yale University in New Haven, Connecticut, USA. The lipid metabolites DAG, Ceramides and PKC activation were analyzed there.

- Long chain fatty acyl-coenzyme a (lcfa- coa)

Long chain fatty acyl-coenzyme A was disconnected from biopsies sample by solid-phase extraction, and C17 CoA was put as internal standard. A tandem mass spectrometer (API3000; PerkinElmer Sciex) linked with a turboIonspray ionization source was utilized for mass spect analysis. LCFA-CoA was calculated as sum of LCFA-CoA species measured.

- DAG and ceramide

At the beginning the amount of total membrane and cytosol DAG were determined. Thereafter the DAG fragmented membrane and cytosol species were analyzed including PO, AE, PA, AS, SS, LS, OS, PP, SP, OO, OL, LL and PL.

DAG is built of glyceride and two fatty acid chains. Each fatty acid chain is covalently bonded to a glycerol molecule. This is effected via ester compounds. The different fatty acids are caproid acid, oleic acid, palmitic acid, arachidonic acid, and stearic acid. DAG is an intermediate of the TAG synthesis and a product of scission of PIP2.

DAG acts as second messengers, among other things as activator of protein kinase C.

Just as before after calculation of the amount of total membrane and cytosol ceramides the ceramides species were identified including C16, C18, C20, C22, C24:1, C24 and C17.

Ceramide are sphingosine lipid. That means a sphingosine molecule, a C18-Aminoalkohol that is bonded to a fatty acid through an amide compound. The free fatty acid part has between 16-24 hydrocarbons atoms.

Free ceramides are found in high concentrations in the skin stratum corneum and in smaller proportions in many cell membranes (Kolesnick et al., 2000).

For the analysis, subsequent methods were used:

Diacyl glycerols and ceramide content were determined after chloroform: methanol:PBS + 0.2% SDS (1:2:0.8) extraction of biopsies. Then diacyl glycerol kinase and [γ-32P] ATP (15 μCi/μmol cold ATP) was given to extracts. The reaction was stopped by adding chloroform:methanol (2:1). Samples were run on thin-layer chromatography plates in chloroform:acetone:methanol:acetic acid:water (100:40:20:20:10). Thereupon DAG and ceramide bands were automatically counted.

3.8.2 Muscle protein kinase C activation

In this experiment, we analyzed the DAG-sensible protein kinase C isoforms (PKCθ, δ, β, ε). Activation of PKC was represented as translocation of PKC from cytosol to plasma membrane. PKC translocation was shown as the ratio of arbitrary units of membrane bands over cytosol bands.

Total cellular PKC was determined from prepared lysates in a solubilization buffer. Proteins were electro blotted onto polyvinylidine difluoride membranes (Amersham Life Sciences), which were then be explored with rabbit anti-peptide antibodies specific for PKC isozymes, followed by horseradish peroxidase–linked donkey anti-rabbit antibody.

Membrane band density was corrected by Na+/K+-ATPase band density and cytosolic band density was corrected by glyceraldehyde 3-phosphate dehydrogenase band density.

3.9 Calculation

3.9.1 Rates of endogenous glucose production

EGP is measured by the aid of labeled glucose that is given as a primed-continuous infusion initiated at ~120 min before and during the clamp. The variable glucose is also labeled to avoid a mixture of the tracer (HOT-GINF protocol).

For the calculation following values were used:

Tracer-to-tracee-ratio (TTR): basal (100.-120. min) at the end of the clamp test after 6.5 h(490-510 min=steady state)

Tracer-to-tracee-ratio (TTR): d2-in infusion and hotginf

d2-Infusionrate (converted in mg/kg.min), subsequently needed: d2-conzentration in Infusion solution(mg/ml), d2-infusion rate (ml/h), body weight (kg):

M-Value (mg/kg.min) under steady state condition (Calculation s. next site)

Tracers to trace ratios were measured at each sampling time.

$$d2-Infusion[mg/kg\times\min]=\frac{d2-Infusionrate[ml/h]\times d2-concentration-Infusion[mg/ml]}{60\min\times bodyweight[kg]}$$

Formula 1 D2 infusion rate

$$APE=\frac{TTR\cdot 100}{(TTR+100)}$$

Formula 2 APE (atom excess percent)

$$R_a=\frac{M_{body\ weight}\cdot APE_{hotg\,\inf}+d2-Infusion[mg/kg\cdot\min]\times 98}{ttr_{Steady\ state}}$$

Formula 3 Rate-of-appearance (Ra)

EGP was calculated in assumption of steady state conditions.

Basal endogenous glucose produktion (EGPb):

Before the clamp, baseline EGP ($mg\times kg^{-1}\times min^{-1}$) rates were calculated. Therefore, D-[6.6-2H2]glucose infusion rate($mg.kg^{-1}.min^{-1}$)corrected for tracer purity (98%) was

divided by the average tracer to trace ratio above natural of D-[6.6-2H2]glucose (TTR DD) measured in blood samples before the start of the clamp.

$$EGP_b = \frac{d2 - Infusion[mg/kg \cdot \min] \times 98}{meanvalue(ttr_{basal})}$$

Clamp test endogenous glucose production (EGPi):

The EGP in an insulin suppressed condition. The M-Value of the body weight was subtracted from the rate-of-appearance (Ra):

$$EGP_i = R_a - M_{body\ weight}$$

(Cobelli et al., 1987), (Gastaldelli, Coggan et al., 1999)

3.9.2 M-value

M-Values (mg glucose.kg^{-1}.min^{-1}) was assessed from steady-state glucose infusion rates (M-values) during the last 20 min of the clamp test, corrected for changes of glucose mass glucose space (space correction) and urinary glucose loss. The urea glucose was detected in the collecting urine. Under steady-state conditions, the total infused glucose amount (exogenous glucose rate) corresponds to the glucose amount that is metabolized in the whole body tissue. So whole body insulin sensitivity was quantified. The more glucose was given the more sensitive the subject responds to insulin.

Space correction (SC 20min) for 20-minute interval was determined as follows:

SC 20min (mg.kg^{-1}.min^{-1}) = (G2-G1)×10×(0,19× bodyweight) / 20× bodyweight

G1 and G2: plasma glucose concentration (mg/dl) at the beginning and at the end of the 20-minute interval,

Factor 10: converts the unit mg/dl of (G2-G1) to mg/l

(0,19× bodyweight) gives the accessible whole body glucose space [0,19 liter/kg bodyweight), divided by 20 (time interval between G2 and G1 in minutes) and bodyweight (kg).

M-Value was additionally detected as:

M (mg.kg^{-1}.min^{-1})= GIR- SC20min+EGP –UGL

The EGP is added to mean glucose infusion rate corrected with the space correction and urinary glucose loss.

GIR ($mg.kg^{-1}.min^{-1}$) : mean glucose infusion rate during this 20 min interval

SC 20 min ($mg.kg^{-1}.min^{-1}$): space correction

EGP ($mg.kg^{-1}.min^{-1}$): endogenous glucose production during the specific time interval

UGL ($mg.kg^{-1}.min^{-1}$): urinary glucose loss detected as glucose concentration

M/I-ratios (M/I) as an index for insulin sensitivity was calculated as follows:

$$M/I = 100 \times \frac{M(mg \times kg^{-1} \times \min^{-1})}{Ins\left(\mu U \times ml^{-1}\right)}$$

M: steady state M value (mg glucose.min^{-1}.kg^{-1})

Ins: plasma insulin concentration

(Cobelli et al., 1987), (Gastaldelli et al., 1999)

3.10 Statistical analysis

Data are reported as means ± SD (standard deviation) or ± SEM (standard error of the mean). Significance of differences between two groups were assessed by the paired two-tailed Student`s test. Comparisons were considered statistically significant at p-values < 0.05. Statistical analyses were performed using Microsoft®Excel 2010 and SPSS 6.0software (SPSS Inc., Chicago, IL, USA).

4. Results

4.1 Anthropometric and laboratory characteristics

The following table 1 provides information about the average type of subjects that have been studied.

male/female	10/2
Age (years)	30 ± 5
BMI (kg/m²)	24 ±2
Fasting plasma glucose (mg.dl^{-1})	81 ± 9
HbA1c (%)	5.3 ± 0.3
Triglycerides (mg/dl)	107 ± 26
Total cholesterol (mg/dl)	189 ± 28
HDL cholesterol (mg/dl)	53 ± 16
LDL cholesterol (mg/dl)	120 ± 31
AST (U·L^{-1})	27 ± 5
ALT (U·L^{-1})	26 ± 12
Creatinine (mg/dl)	0.9 ± 0.2
Hb (g/dl)	15 ± 1.2
TSH (µlU/ml)	2.4 ± 1.1
Quick (%)	100 ± 9
(PW	2.3 ± 0.5
PS	2.9 ± 0.8
PL	2.7 ± 0.3
Physical activity index , mean value: AAI+SAI+FAI	2.6 ± 0.4
(summation: AAI+SAI+FAI)	7.9 ± 1.3
Abbreviations: BMI, body mass index; HbA1c, glycosylated hemoglobin A 1c (normal range, 4.8-5.9 %);	
AST, aspartate transaminase (normal range, < 31 U·L^{-1});ALT, alanine transaminase (normal range, < 38 U·L^{-1});	
Hb, hemoglobin (normal range, 12-16 g/dl); TSH, thyroid-stimulating hormone (normal range, 0.27-4.20 µlU/ml),	

Physical activity index: mean of the indices PW, physical activity at work; PS, sport during leisure time; PL, physical activity during leisure time excluding sport. Values range from 1.7 to 3.3 with higher values indicating higher activity.

Table 1. Anthropometric and laboratory characteristics of volunteers

Mean values ± SD. Clinical characteristics as well as fasting plasma/ serum laboratory parameters of healthy study participants with 2 and 3 muscle biopsies.

4.2 Plasma metabolites and hormones time response

Aim of this part is to validate the experimental condition described above: constant glucose concentration, Plasma insulin increase under clamp situation, constant increase of FFA until 2.4 mmol/l (mv : 1.68 ± 0.75mmol/l) as well as for the triglycerides (190 ± 60 mg/dl).

The background shades of the graphics (figure 5-8) indicate the several investigation parts of the experiment. At first (dark background, 0.-125.min.) no infusion were dispensed. Afterwards (white background, 125.-360.min) fasting insulin levels, glucose, high dose lipid, somatostatin and heparin infusions were administered. During the last part (grey background, 360.-510.min) high dose of insulin and with it depended glucose were given in comparison to before.

Mean values ± SEM (standard error of the mean) were used for all figures.

The following figure (figure 5) shows the evolution of the plasma glucose concentration in the blood during the time of the experiment as described before:

- Plasma glucose

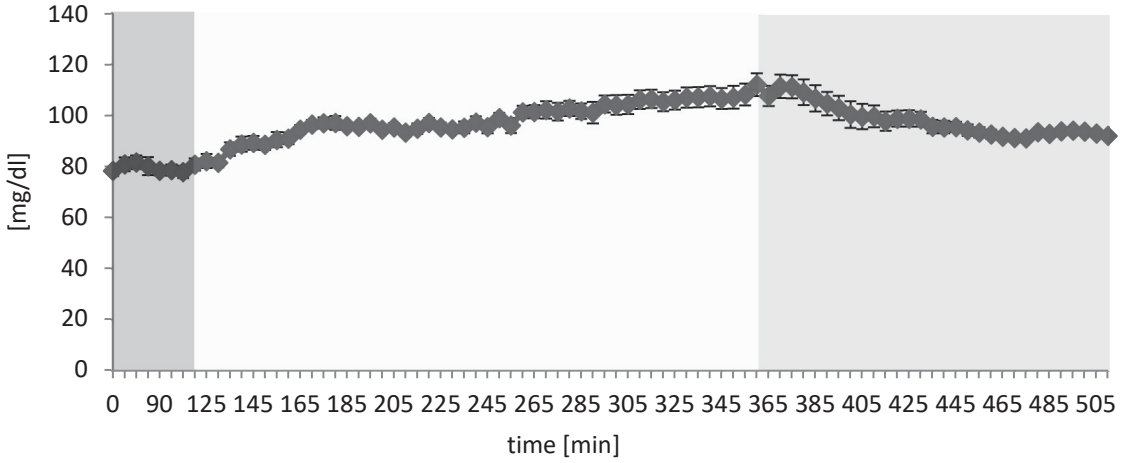

Fig. 5: Plasma glucose profile 0.-510 min

Blood glucose concentration is steady around 90 mg/dl (95 ± 4mg/dl) during the experiment (infusion with insulin, glucose, somatostatin, heparin and lipid infusion). In the first part of the experiment, the mean value of blood glucose is in the fasting glucose array (80 ± 1.4 mg/dl). Then, during the second part, a low increase of the blood is observed because of gluconeogenesis (98.4 ±7 mg/dl). With the start of the clamp test, the blood glucose settle down to values around 90 mg/dl (98 ± 6 mg/dl). Thus, the aim to create a condition of normoglycemia was accomplished.

The following figures (figure 6-8) show the time course of plasma concentration of plasma insulin, FFA and TG as described before. Different infusions (insulin glucose, lipid, somatostatine and heparin) were given at certain times (see experimental protocol).

- Plasma insulin

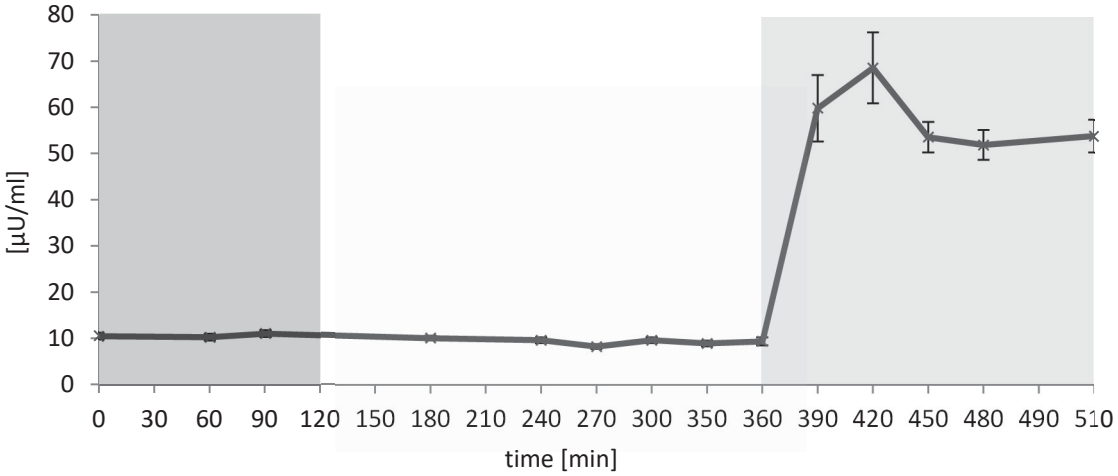

Fig. 6: Plasma insulin concentration 0.-510 min

Plasma insulin concentration was in the first part of the experiment (0.-360.min) around 9.8 ±0.87 µU/ml. During this time only fasting levels of Insulin (0.1 $mU.kg^{-1}\cdot min^{-1}$) were administered. After high dose insulin (40 $mU.m^{-2}$ $BSA\cdot min^{-1}$) infusion application (360.-510.min) the insulin level increased 484% to values of 57.2 ±µU/m. Condition of hyperglycemia was so induced.

- Plasma free fatty acids

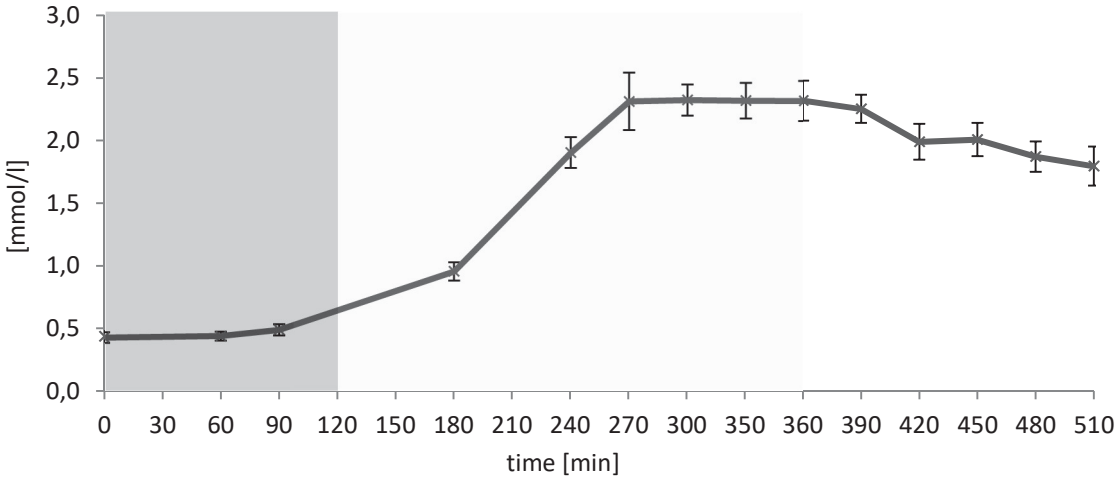

Fig. 7: Plasma free fatty acids concentration 0.-510 min

- Plasma triglycerides

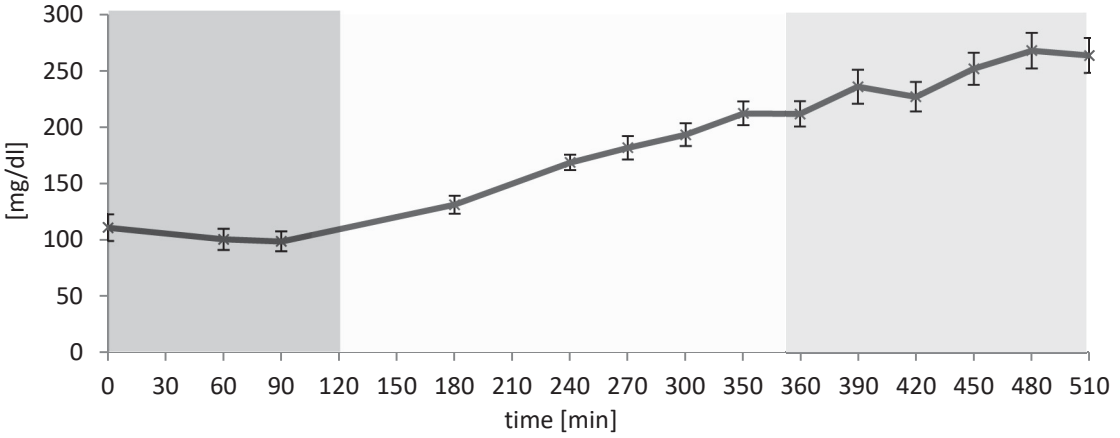

Fig. 8: Plasma triglycerides concentration 0.-510 min

Plasma lipid concentration of free fatty acids and triglycerides (figure 7-8) raised remarkable continuously corresponding to the time of lipid infusion application with Lipovenös 20%.

4.3 Plasma metabolites and hormones during the muscle biopsy

The following graphics (figure 9-11) show any influence on the blood values of insulin, free fatty acids and triglycerides during the muscle biopsies. The correlation between values in blood and intracellular in muscle biopsies can be demonstrated.

At first two muscle biopsies have been performed at baseline and after 4 hours lipid infusion (n=8). From the ninth experiments to the last one, a supplementary muscle biopsy was taken after 2.5 lipid infusion (n=8).

The laboratory mean values are taken from the time period around the muscle biopsies. That means, mean value of 60. and 90 minute, 270. and 300.min and 350. and 360. min values are used.

All figures contain mean value ±SEM.

The following figures show Plasma insulin, FFA and TG during the muscle biopsy at baseline (black columns), after 2.5 h lipid infusion (dark grey columns) and after 4 h lipid infusion (white columns):

- Plasma insulin

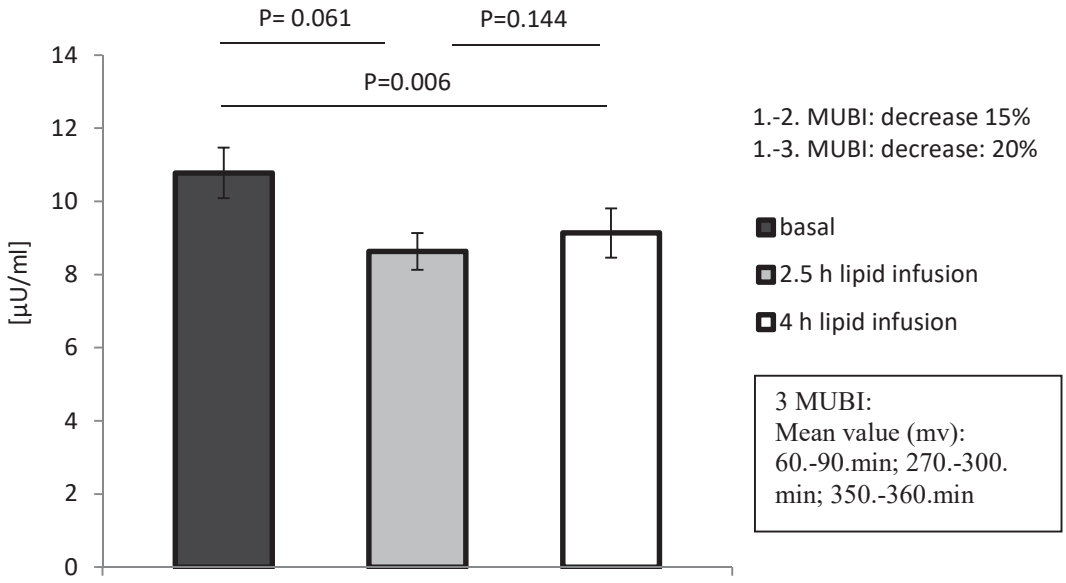

Fig. 9: Plasma insulin concentration during muscle biopsies

Plasma insulin concentration in young lean healthy participants at baseline (black column), after 2.5 h (grey column) and 4 h (white column) of lipid infusion.

Insulin level decrease after the first muscle biopsy. There is a significant difference between first and third muscle biopsy.

Data are given as means ±SEM. Significance of difference between two groups were determined using paired two-sample t-test. Differences were considered significant at the 5% level.

- Plasma free fatty acids

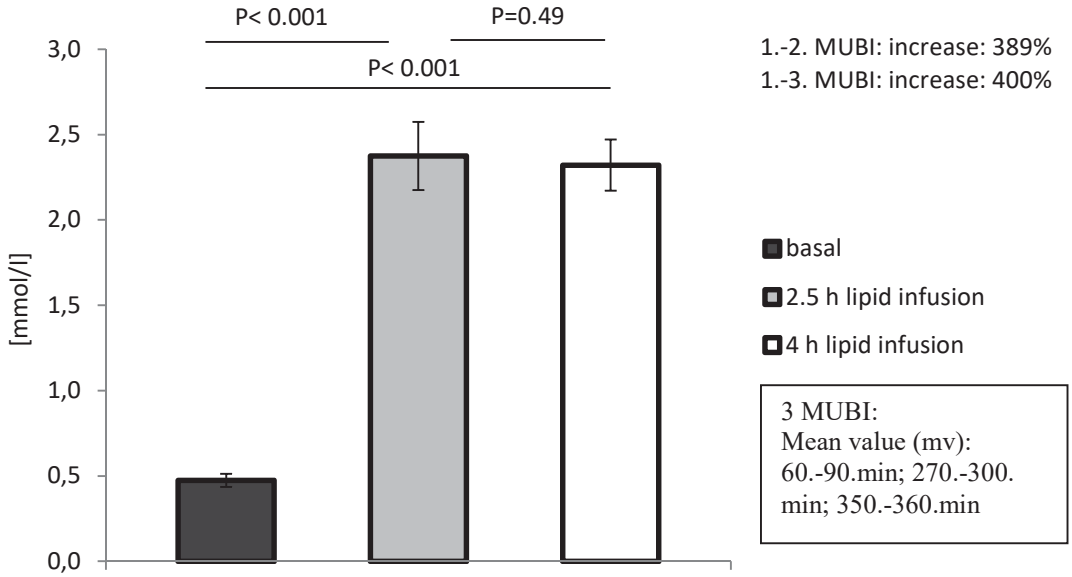

Fig. 10: Plasma free fatty acid concentration during muscle biopsies

Plasma free fatty acid concentration in young lean healthy participants at baseline (black column), after 2.5 h (grey column) and 4 h (white column) of lipid infusion.

FFA increase enormously after the first to the second muscle biopsy and stay above. A grand significance is remarkable between first and third FFA mean value and first and second one.

Data are given as means ±SEM. Significance of difference between two groups were determined using paired two-sample t-test. Differences were considered significant at the 5% level.

- Plasma triglycerides

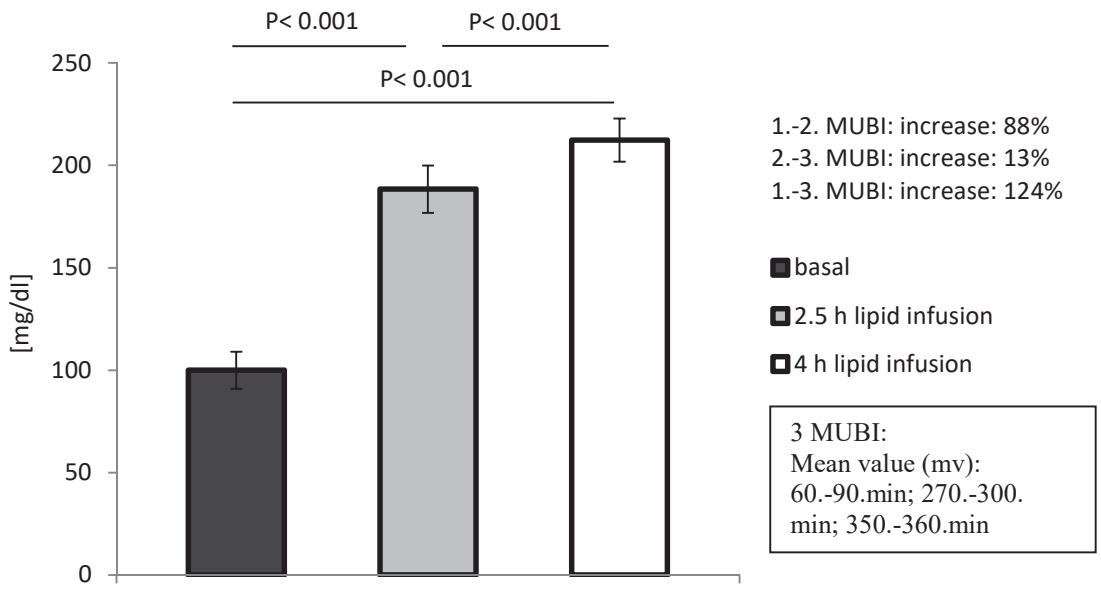

Fig. 11: Plasma triglyceride concentration during muscle biopsies

Plasma triglyceride concentration in young lean healthy volunteers at baseline (black column), after 2.5 h (grey column) and 4 h (white column) of lipid infusion.

Triglycerides ascend continuously. Very high significance of Triglycerides values is detectable.

Data are given as means ±SEM. Significance of difference between two groups were determined using paired two-sample t-test. Differences were considered significant at the 5% level.

4.4 Indirect calorimetry

The RQ-and REE values were measured by indirect calorimetry at baseline and during the clamp test.

Enclosed graphics of RQ values and REE at baseline (dark columns) and during the clamp test (white columns).

- Respiratory quotient (RQ)

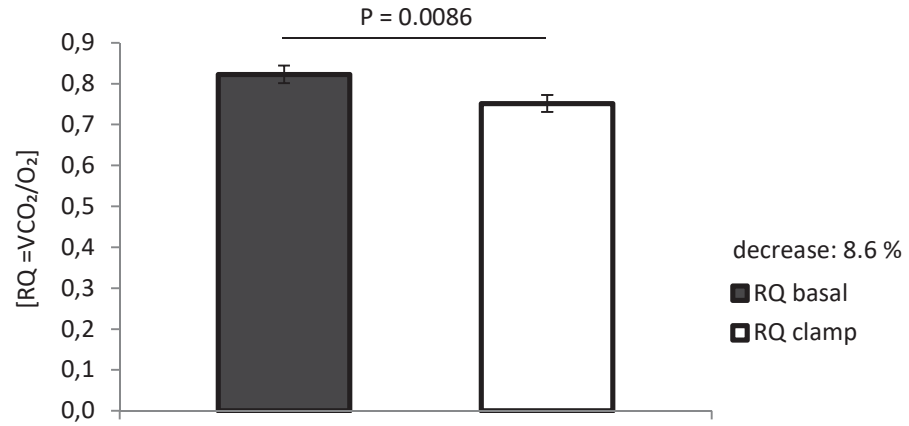

Fig. 12: Respiratory quotient at baseline and during clamp test

At baseline RQ mean value (figure 12) is equals to 0.82 that describes an overnight fasting situation with a metabolism of mixture substrates. The difference of RQ under clamp condition after high lipid infusion donation is significant (p = 0.0086, decrease: 8.6%). The second RQ mean value is at 0.75 that correspond approximately with lipid metabolism and show so a switch to fat consumption.

Data are given as means ±SEM. Significance of difference between two groups were determined using paired two-sample t-test.

- Resting energy expenditure (REE)

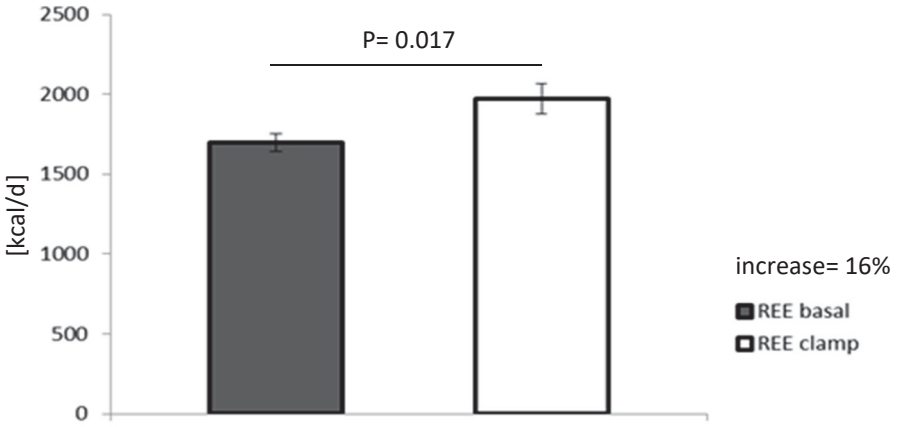

Fig. 13: REE at baseline and during clamp test

The REE increased (+16%) significantly during the clamp test that argue for a higher caloric intake (figure 13).

Data are given as means ±SEM. Significance of difference between two groups were determined using paired two-sample t-test.

4.5 Rates of endogenous glucose production and M-value during the clamp test

The following figure (figure 14) shows the measured EGP at baseline (dark columns) and during clamp test (white columns):

- Endogenous glucose production (EGP)

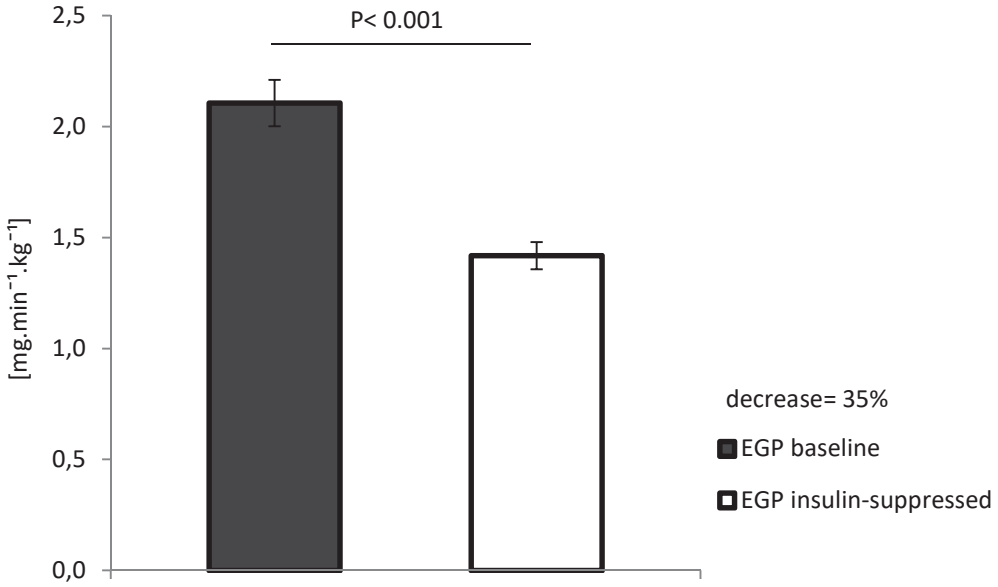

Fig. 14: EGP at baseline and during clamp test

The EGP abated 35% under clamp condition. An impaired suppression of EGP is remarkable with a grand significant difference (figure 14).

Data are given as means ±SEM. Significance of difference between two groups were determined using paired two-sample t-test. Differences were considered significant at the 5% level.

The M-value during clamp condition is at 3.25 ±0.8 mg.kg^{-1}.min^{-1}.

4.6 Lipid metabolites in human skeletal muscle biopsies during lipid infusion

Below are figures (figure 15-16) of diacylglycerol membrane and cytosolic fractions as well as the DAG species at baseline (dark columns), after 2.5 h lipid infusions (dark grey columns) and after 4 h lipid infusions (white columns)

4.6.1 Diacylglycerols (DAG) and species

- DAG membrane fraction and cytosolic fraction

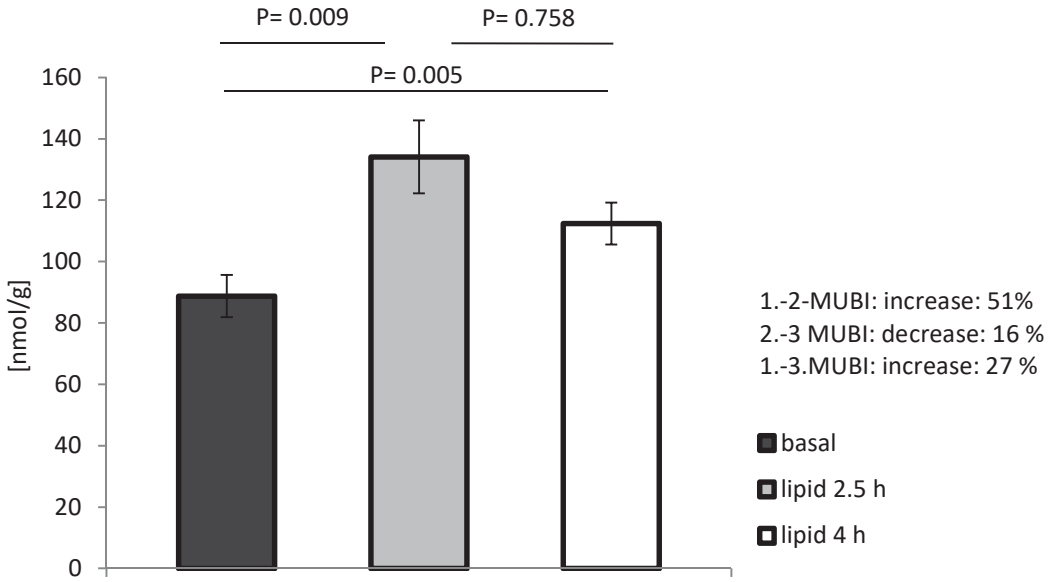

Fig. 15: DAG membrane fraction in muscle biopsies

Myocellular concentration of DAG membrane fraction in young lean healthy participants at baseline (black column), after 2.5 h (grey column) and 4 h (white column) of lipid infusion.

DAG membrane fraction first increase very significantly (p = 0.009, increase: 51%) after 2.5 hours lipid infusion. But subsequently after 4 hours lipid infusion DAG membrane decline (p=0.758, decrease: 16%). Grand Significant change is found from 1. to 2. muscle biopsy and 1. to 3. mubi (figure 15).

Data are given as means ±SEM. Significance of difference between two groups were determined using paired two-sample t-test. Differences were considered significant at the 5% level.

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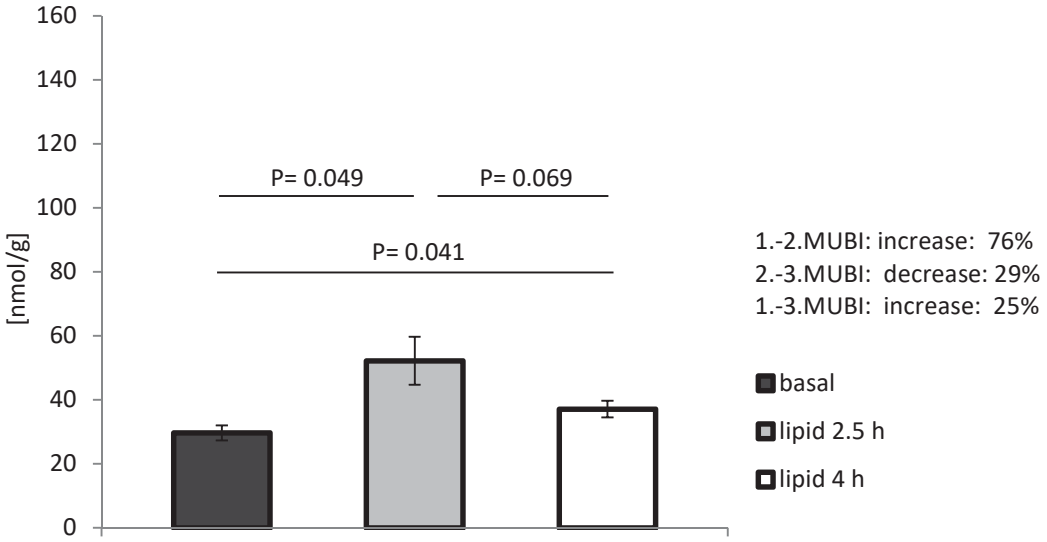

Fig. 16: DAG cytosolic fraction in muscle biopsies

Myocellular concentration of DAG cytosolic fraction in young lean healthy participants at baseline (black column), after 2.5 h (grey column) and 4 h (white column) of lipid infusion.

DAG cytosol fraction followed a similar pattern. After 2.5 hours DAG cytosol fraction increase (P = 0.049, increase: 76%) and after 4 hours decrease (p= 0.069, decrease: 29%) (figure 16).

Data are given as means ±SEM. Significance of difference between two groups were determined using paired two-sample t-test. Differences were considered significant at the 5% level.

- Cytosolic and membrane DAG species

The DAG species palmitate (P) [C16H32O2], stearate (S) [C18H36O2], linoleate (L) [C18H32O2], arachidonate (A) [C20H32O2] and oleate (O) [C18H34O2] were analyzed in the membrane and cytosol fraction.

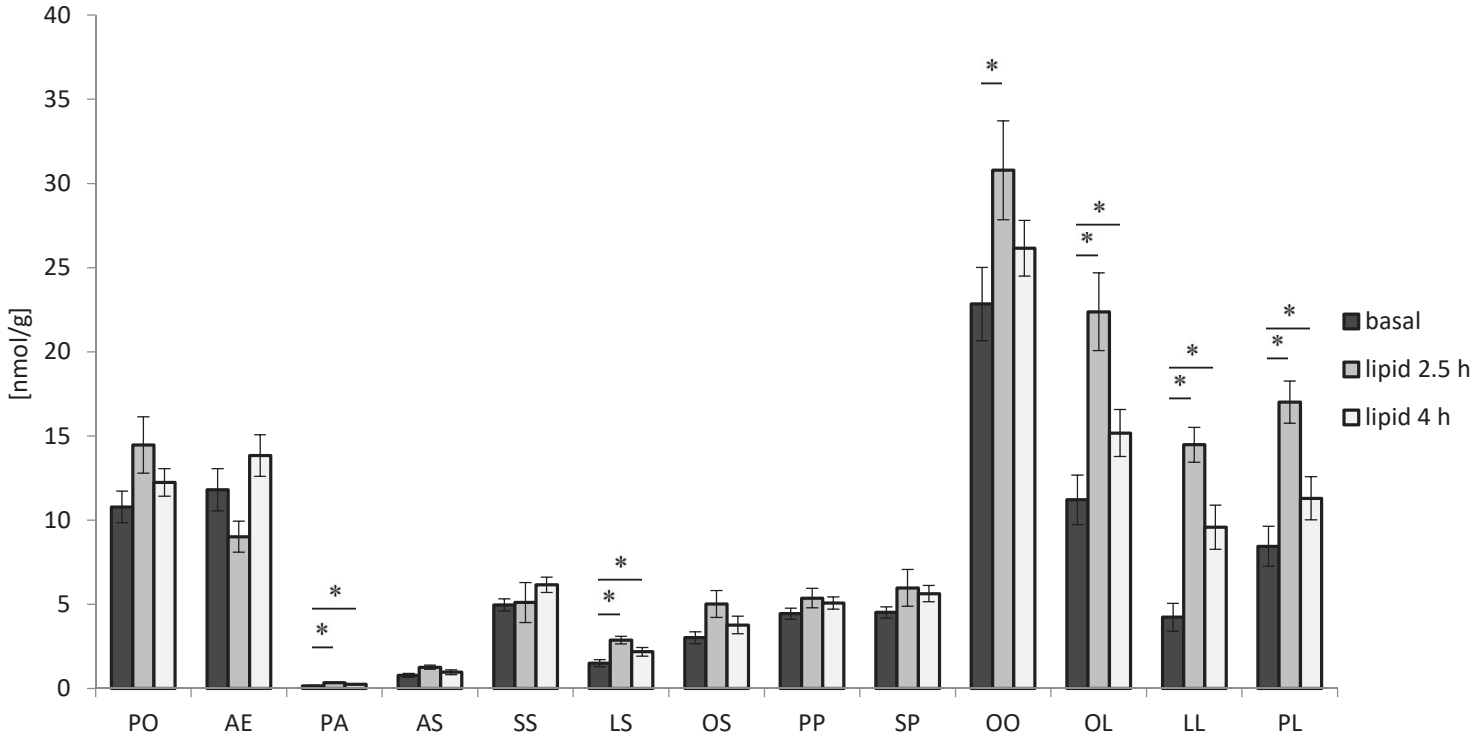

Fig. 17: DAG membrane species in muscle biopsies

Concentration of membrane DAG species in young lean healthy participants at baseline (black columns), after 2.5 h (grey columns) and 4 h (white columns) of lipid infusion. Stars indicate significant differences of DAG membrane species during lipid infusion

After 2.5 h lipid infusion, membrane fractions of DAG species increased and have a significant change (figure 17).

Data are given as means ±SEM. Significance of difference between two groups were determined using paired two-sample t-test. Differences were considered significant at the 5% level. *$p<0.05$.

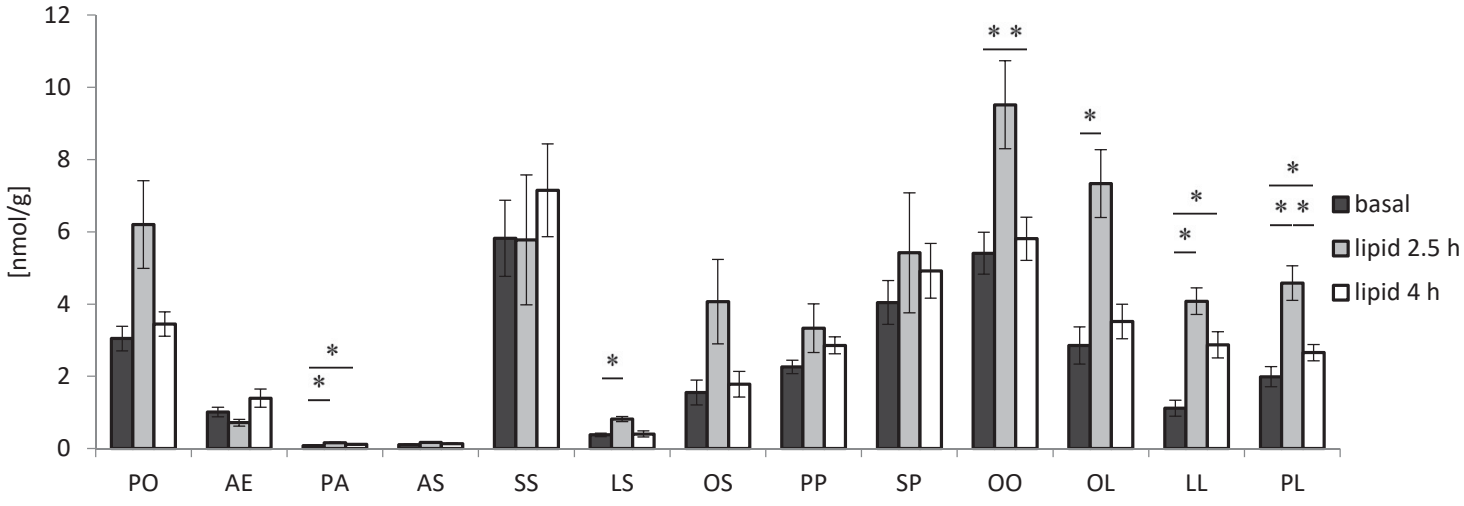

Fig. 18: DAG cytosolic species in muscle biopsies

Concentration of cytosolic DAG species in young lean healthy participants at baseline (black columns), after 2.5 h (grey columns) and 4 h (white columns) of lipid infusion.
Stars indicate significant differences of DAG cytosolic species during lipid infusion. Some DAG cytosolic species present as well a significant difference.
Data are given as means ±SEM. Significance of difference between two groups were determined using paired two-sample t-test. Differences were considered significant at the 5% level. *$p<0.05$.

DAG membrane and cytosol species behave similar with a significantly change of palmitate/arachnidonate (PA), linoleate/stearate (LS), oleate (OO), oleate/linoleate (OL), linoleate (LL) and palmitate/linoleate (PL).

These species increase after 2.5 lipid infusion and decrease after 4 hours lipid infusion.

Membrane species: PA 1.-2 .mubi: p=0.0006; 1.-3. mubi: p=0.000043; LS: 1.-2. mubi: p=0.003; 1.-3. mubi: 0.0009; OO: 1.-2.mubi: p=0.013; OL: 1.-2. mubi: p=0.004; 1.-3. mubi: p= 0.003; LL: 1.-2. mubi: p=0.000006; 1.-3. mubi: p=0.000001; PL: 1.-2. mubi: p=0.002; 1.-3.mubi: p=0.0009

Cytosol species: PA: 1.-2. mubi: p=0.049; 1.-3. mubi: 0.0046; LS: 1.-2. mubi: p=0.026; OO: 1.-2. mubi: p=0.006; 2.-3. mubi: p=0.028; OL: 1.-2. mubi: 0.0024; LL: 1.-2. mubi: p=0.00026; 1.-3. mubi: p=0.000048; PL: 1.-2. mubi: p= 0.0082; 2.-3. mubi: p=0.021; 1.-3.mubi: p= 0.012.

4.6.2 Ceramides and ceramides species

- Ceramides

Next figures (figure 19-20) demonstrate ceramides and the specie in muscle biopsies during lipid infusion:

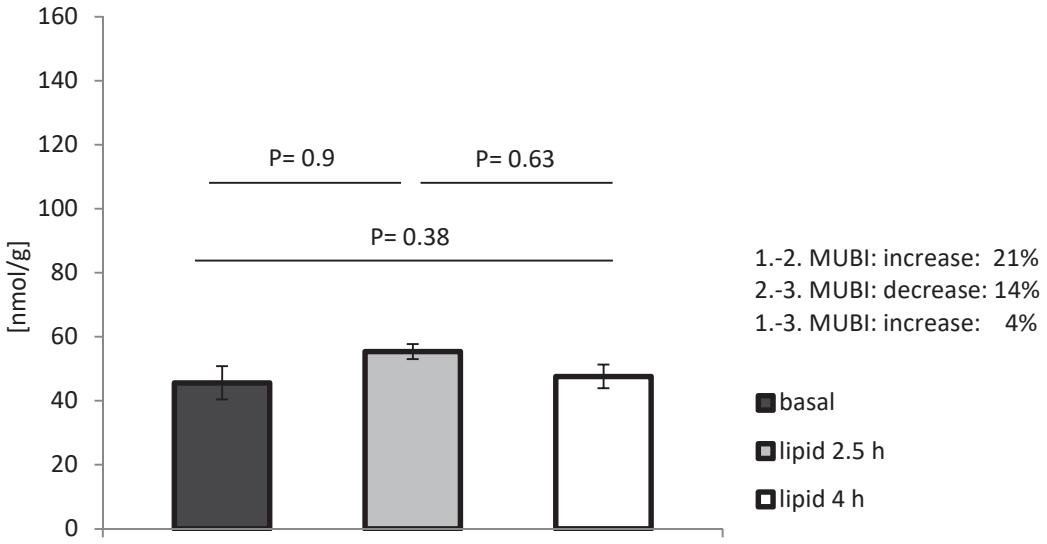

Fig. 19: Ceramides concentration in muscle biopsies

Myocellular concentration of ceramides in young lean healthy participants at baseline (black column), after 2.5 h (grey column) and 4 h (white column) of lipid infusion.
Ceramid levels remain unchanged after 2.5 h und 4 h lipid infusion (figure 19).
Data are given as means ±SEM. Significance of difference between two groups were determined using paired two-sample t-test. Differences were considered significant at the 5% level.

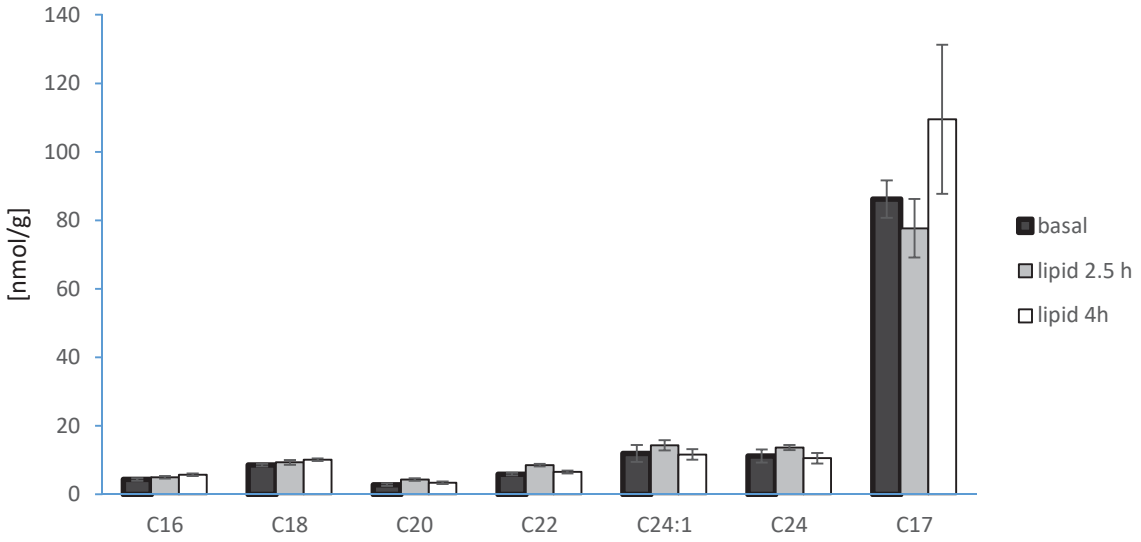

Fig. 20: Ceramide species in muscle biopsies

Concentration of ceramide species in young lean healthy participants at baseline (black column), after 2.5 h (grey column) and 4 h (white column) of lipid infusion.

The species of ceramides show also no difference.

C16: N-palmitoyl-D-erythro-sphingosine,C17: N-heptadecanoyl-D-erythro-sphingosine, C18: N-stearoyl-D-erythro-sphingosine, C20: N-arachidoyl-D-erythro-sphingosine, C22: N-behenoyl-D-erythro-sphingosine, C24:1:N-nervonoyl-D-erythro-sphingosine, C24: N-lignoceroyl-D-erythro-sphingosine (figure 20).

Data are given as means ±SEM. Significance of difference between two groups were determined using paired two-sample t-test. Differences were considered significant at the 5% level.

4.6.3 Protein kinase C activation

- **PKC theta in human muscle**

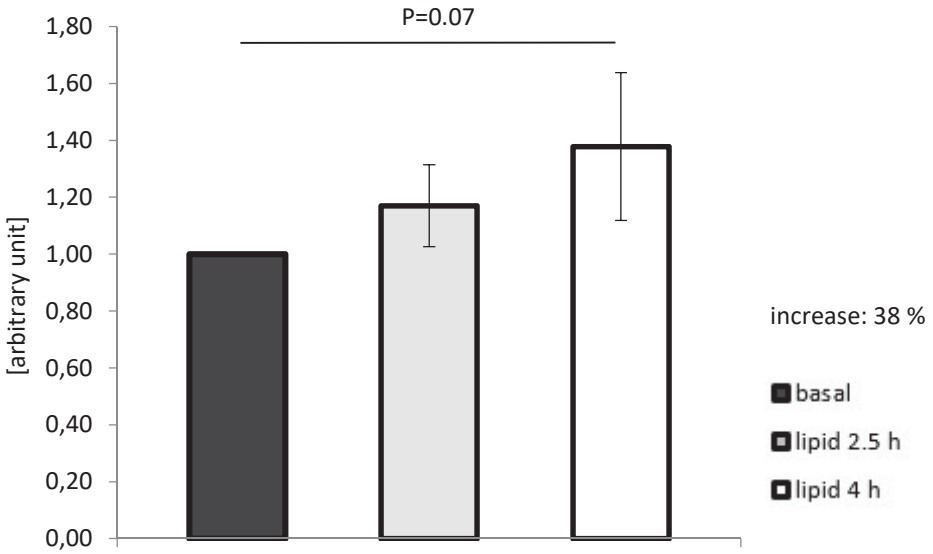

Fig. 21: Activation of PKC θ

Activation of myocellular PKC θ in young lean healthy participants at baseline (black column), after 2.5 h (grey column) and 4 h (white column) of lipid infusion.

PKC theta rise after 4 h lipid infusions and tended to be significant (p=0.07 versus basal). Data are given as means ±SEM. Significance of difference between two groups were determined using paired two-sample t-test. Differences were considered significant at the 5% level.

- **PKC beta in human muscle**

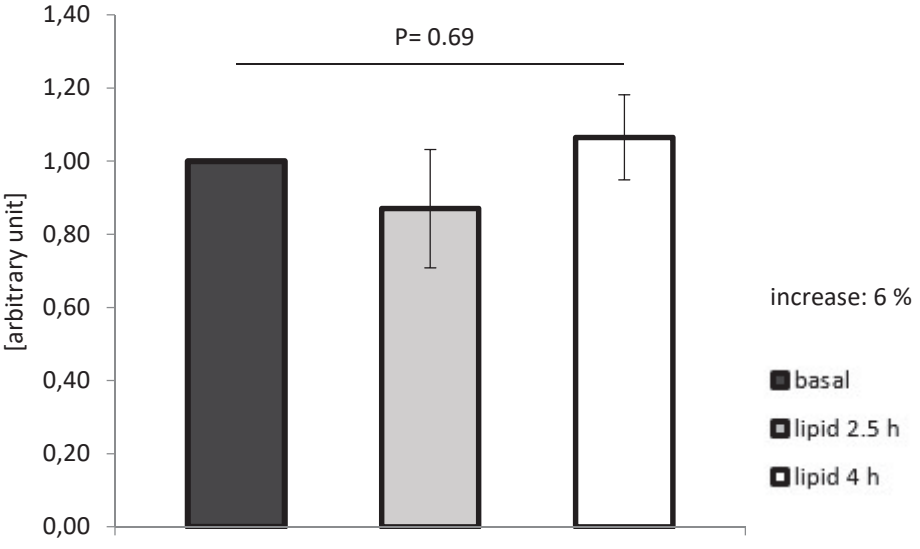

Fig. 22: Activation of PKC β

Activation of myocellular PKC β in young lean healthy participants at baseline (black column), after 2.5 h (grey column) and 4 h (white column) of lipid infusion.
Lipid infusions have no influence on PKC beta.
Data are given as means ±SEM. Significance of difference between two groups were determined using paired two-sample t-test. Differences were considered significant at the 5% level.

- **PKC delta in human muscle**

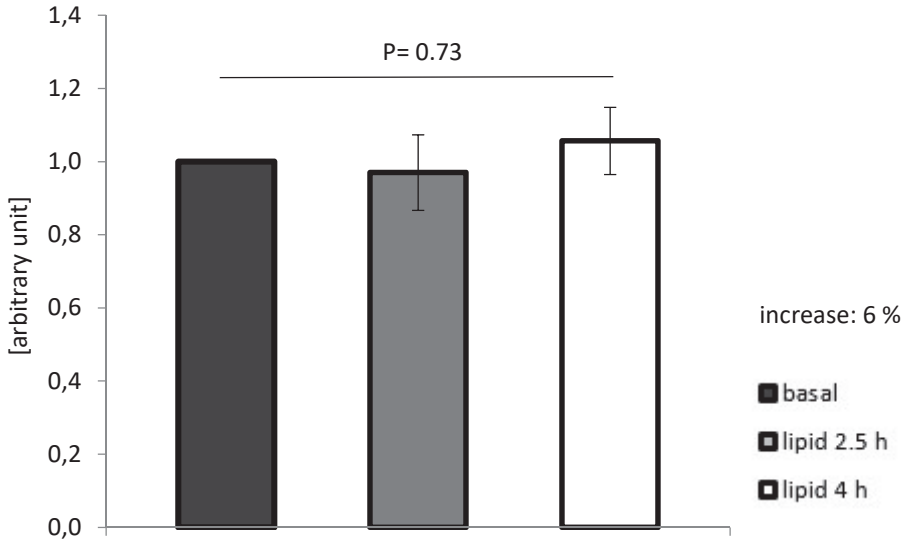

Fig. 23: Activation of PKC δ

Activation of myocellular PKC δ in young lean healthy participants at baseline (black column), after 2.5 h (grey column) and 4 h (white column) of lipid infusion.
PKC delta shows no change after lipid infusions.
Data are given as means ±SEM. Significance of difference between two groups were determined using paired two-sample t-test. Differences were considered significant at the 5% level.

5. Discussion

The experiments of this thesis corroborated results of previous studies showing that infusion of intralipid elevate plasma FFA concentrations to ~2.4 mM in young lean healthy subjects and induce insulin resistance.

This was reflected by low glucose disposal and impaired suppression of EGP during hyperinsulinemia.

The molecular sequence in the skeletal muscle cell that induces insulin resistance via inhibition of insulin signal transduction was analyzed. Therefore, the distribution and amount of the lipid metabolites DAG and ceramide and the activation of PKC isoforms were determined.

Activation of PKC θ remained unchanged after 2.5 hours, but increased by 38 % after 4 hours lipid infusion and tended to be significant (p=0.07). Membrane and cytosolic fractions of total DAG concentrations increased within 2.5 h lipid infusion (DAG: membrane: p=0,009; cytosol: p= 0,049).

Thus, this allows concluding that PKC θ activation at 4 h was followed after myocellular increase of DAG content induced by lipid infusion with high levels of plasma FFA.

Some but not all specific DAG species increased 2-3 folds at 2.5 hours of lipid infusion and decreased upon prolonged lipid infusion for 4 hours. Apparently, the DAG isoforms such as linolate, palmitate and oleate are here relevant for the mechanism of lipid induced insulin resistance.

Total Ceramide levels remained unchanged and thus do not seem to play a role for the induction of short-term lipid-induced insulin resistance in skeletal muscle.

The article published by the German Diabetes Center Düsseldorf 2014 with lean healthy young humans confirm the results, that elevation of DAG membrane and cytosol fraction and therewith associated activation of PKC θ play an important role in the signaling pathway of lipid induced insulin resistance in skeletal muscle. Furthermore, the experiment was followed up with healthy insulin-resistant obese subjects and obese type 2 diabetic subjects under same conditions. The findings were similar and it was also shown an association of DAG activation of PKCθ in the mechanism of lipid induced muscle insulin resistance in obese and type 2 diabetic human volunteers (Szendroedi et al., 2014).

5.1 Plasma metabolites and hormones

By the questionnaire of Baecke et al., the habitual physical activity was determined showing that the subjects were not extremely trained (Baecke et al., 1982).

- Plasma glucose and insulin:

The conditions of hyperinsulinemic-euglycemic clamp are given (DeFronzo et al. 1979).

The blood glucose concentrations are steadily normoglycemic with values around 95 mg/dl (95 ± 4mg/dl). The plasma insulin levels were around 9.8 ± 0.87 µU/ml in the first part of the experiment (0.-360.min). During this time fasting levels of insulin (0.1 $mU.kg^{-1} \cdot min^{-1}$) were applied. Subsequent higher dose insulin (40 $mU.m^{-2}$ BSA$\cdot min^{-1}$) was administered (360.-510.min) and insulin levels increased to values of 57.2 ±µU/ml.

- Plasma free fatty acids and triglycerides:

During the application of Lipovenös 20% and in parallel with heparin a constant increase of plasma FFA up to 2.4 mmol/l (mv : 1.68 ± 0.75mmol/l) as well as of plasma triglycerides (190 ± 60 mg/dl) was detectable.

The aim to raise plasma lipid or rather the FFA level was gained.

5.2 Plasma metabolites and hormones during the muscle biopsies

Laboratory mean values of free fatty acids (FFA), triglycerides (TG) and insulin are determined from the time period around the muscle biopsies to correlate between values in blood and intracellular in muscle biopsies.

Insulin decreased marginally but significantly between the first and third muscle biopsy. ($p= 0.006$, 15% decrease). In this period, we aimed to inhibit lipid-induced insulin secretion by administration of somatostatin and low dose insulin to maintain fasting insulin levels because we intended to study the pure lipid-induced effects on muscle metabolism.

FFA increased strongly and highly significant from the first to the second muscle biopsy. Then the level remained elevated ($p < 0.001$, increase: 389%).

TG elevate in the course continuous from the first to the third muscle biopsy 124% ($p < 0.001$, increase: 124%).

5.2 Indirect calorimetry

- REE

The REE increase significantly ($p = 0.017$) by 16% from the baseline value 1696.87 ± 57 to the clamp value 1972.2 ± 96 kcal/d.

This increase reflects higher energy input applied via lipid infusion.

5.3 Rates of endogenous glucose production and M-value during the clamp test

It is known that insulin suppresses endogenous glucose production so that under euglycemic-hyperinsulinemic clamp conditions EGP is diminished reflecting insulin sensitivity of the liver. It has been shown that high FFA levels induce hepatic gluconeogenesis and induce insulin resistance in the liver (Ferrannini et al. 1983), (Roden al. 1996).

As aforementioned Somatostatin was given to inhibit endogenous insulin secretion.

EGP decline very significantly ($p < 0.001$) but decrease only 35%. The value at baseline is 2.2 ± 0.3 and during clamp condition at 1.4 ± 0.3 $mg.kg^{-1}.min^{-1}$.

The M-value is under hyperinsulinemic euglycemic clamp test in healthy lean subjects around 7.1±2.1 $mg.kg^{-1}.min^{-1}$ (Ferrannini et al., 1997) and type 2 diabetic patient with poor glycemic control at 2.9±0.2 $mg.kg^{-1}.min^{-1}$ (Anderwald et al., 2002).

In the study of this thesis, the M-value is determined at 3.25 ±0.8 $mg.kg^{-1}.min^{-1}$. It is detected under steady state during clamp condition plus lipid/heparin infusion (Krssak et al., 1999). In comparison with healthy subjects without lipid infusion, the value is lower and indicates abated insulin sensitivity.

This result is in good accordance to previous studies (Krebs and Roden, 2005).

5.4 Lipid metabolites in human skeletal muscle biopsies during lipid infusion

- DAG content, species and protein kinase C

It is suggested that DAG, an intermediate in the synthesis of Triglyceride is associated with the degree of insulin resistance. Increased DAG levels might induce protein kinase C activation and a reduction in both insulin-stimulated IRS-1 tyrosine phosphorylation and PI3 kinase activity (Timmers et al., 2008), (Erion and Shulman, 2010).

An increased activation of PKC theta was referred in several animal and human studies (Coffer et al., 1998), (Qu et al., 1999), (Griffin et al., 1999), (Schmitz-Peiffer, 2000), (Chalfant et al., 2000), (Itani et al., 2000), (Parekh et al., 2000), (Kim et al., 2003), (Gray et al., 2003), (Li et al., 2004), (Krebs and Roden, 2005), (Roden, 2005), (Szendroedi et al. 2011).

The following description will highlight the different studies about the topic and show how different are their conclusions.

Firstly, Itani reported in a study of 2000 that in skeletal muscle from obese insulin-resistant subjects membrane-associated PKC-beta protein was raised under basal conditions and membrane-associated total PKC activity was increased under insulin-stimulated conditions (Itani et al., 2000).

Later on Itani et al. (2001) referred about PKC isoforms (beta, theta, epsilon, delta, mu, and zeta) that were measured in human rectus abdominus skeletal muscle from obese (hyperinsulinemic, normoglycemic) and obese diabetic (hyperinsulinemic, hyperglycemic) volunteers.

PKC theta protein content and activity were significantly increased in the muscle from diabetic patients compared with the nondiabetic control subjects.

Most of the PKC theta protein were located in the cytosol. But no change was detected in cytosolic PKC theta protein content in muscle from diabetic patients compared with muscle from nondiabetic control subjects. These results underline the importance of the translocation and therewith activation of PKC theta rather than an increase in protein mass (Itani et al., 2001).

In a further experiment, Itani et al. (2002) detected changes in DAG content and PKC activity after 6 hyperinsulinemic-clamp condition and simultaneous elevated FFA level.

Vastus lateralis muscle biopsies were also obtained before, but at different times: after 2 hours and 6 hours hyperinsulinemic-euglycemic clamp test from lean healthy subjects either with low-plasma FFA levels or increased plasma FFA levels induced by lipid infusion simultaneous to the clamp test.

In contrast with this thesis, DAG content or PKC activity did not change after 2 hours lipid infusion (in our thesis 2.5 h). But after 6 h (our thesis: 4 h) lipid infusion DAG increased fourfold as were the amount of membrane-associated PKC-βII and -δ without change in ceramide content. Membrane-associated PKC-βII increased already after 2 hours lipid infusion threefold without significance. No significant alterations in PKC-ε or θ was detected (Itani et al., 2002).

According to our results diacylglcerol increased, but PKC theta activation did no show a difference.

Yu et al. presented 2002 a study with wistar rats. Either lipid, triglyceride or saline infusion was given during different times. Soleus muscle biopsies were obtained. Intracellular C18:2 (linoleate) CoA increased 6 fold by 5h lipid infusion, but any other acyl-CoA remained unchanged. Interestingly DAG increased after 3-4h lipid infusion and then decreased to basal concentrations. There was no difference in intracellular ceramide or triglycerides concentration during lipid infusion detectable. PKC theta: (membrane associated/cytosol) fraction elevated significantly after 5 h lipid infusion.

The results approve the supposition that increased plasma FFA leads to an increase in intracellular fatty acyl-oA and DAG concentration. This in turn activates PCK theta (Yu et al., 2002).

In a study, it was shown that in vitro activation of different PKC isoenzymes vary in response to different DAG species at different times. That could explain the different in vivo effects (Madani et al., 2001).

Another study of 2009 also indicates that intramyocellular DAG might have the main effect on insulin resistance. Thereby DAG is independent from markers of adiposity, but relate to lipolytic enzymes activity in skeletal muscle. Sedentary healthy and diabetic subjects participated in this study. By NMR spectroscopy of soleus and tibialis anterior

muscles and by muscle biopsy before start of the clamp vastus lateralis muscle were analyzed. A hyperinsulinemic-euglycemic clamp was performed during 2 hours (Moro et al., 2009).

Liu et al. showed in a study that increased activation of myocellular diacylglycerol o-acyltransferase 1 (DGAT1) and thus increased triglyceride (TG) synthesis in skeletal muscle also reduce insulin resistance. Wild type C57BL/6J mice and DGAT1-knockout C57BL/6J mice were fed with high fat diet or normal caloric diet and an exercise training during one week was performed. Exercise induced in soleus muscle biopsies an elevated diacylglycerol (DAG) acyltransferase (DGAT) activity. Transgenic overexpression of DGAT1 in mouse skeletal muscle induced an augmented build of fatty acid substrates into TG and that resulted in decreased DAG and ceramide levels and improved insulin sensitivity. DGAT1 deficiency in turn worsened insulin resistance (Liu et al., 2007).

Zhang et al propose a later experiment 2010 with mice that were fed either a low-fat diet or a high-fat diet for 3 or 10 weeks. It is suggested that cardiac diacylglycerol accumulation in high fat-fed mice is correlated with insulin resistance. But long-chain acyl CoA, ceramide, or triacylglycerol do not show referring to this an influence (Zhang et al., 2010).

In another experiment liver, epididymal fat and soleus muscle of lean and obese Zucker rats in an overnight fasted situation were analyzed. Hepatic PKC show a higher activity in obese rats under basal fasting conditions without any differences in muscle and fat. The fed state was linked up with elevated DAG levels and in turn higher PKC activity in muscle tissue of obese rats, and increased expression of the major muscle isoforms, PKC-theta and PKC-epsilon (Qu et al., 1999).

In a further report, the effects of saturated fatty acids on insulin signaling and ceramide and diacylglycerol accumulation in 3T3-L1 adipocytes and C2C12 myotubes were examined in a study. Therefore, 3T3-L1 adipocytes or C2C12 myotubes were treated with either the saturated FFA palmitate (C16:0) or the monounsaturated FFA oleate (C18:1). In C2C12 myotubes, palmitate reduced insulin-stimulation of glycogen synthesis and its activation of Akt/Protein Kinase B (PKB). Oleate did not show this effect. Palmitate also increased ceramide and diacylglycerol contents. Whereas in 3T3-L1 adipocytes palmitate or oleate did not inhibit glycogen synthesis or Akt/PKB activation and they did not lead to ceramide or DAG synthesis.

Furthermore, the myotubes were tested with other saturated fatty acids. The long-chain fatty acids stearate (18:0), arachidate (20:0), and lignocerate (24:0) had the same effect as palmitate. But saturated fatty acids with shorter hydrocarbon chains [i.e., laurate (12:0) and myristate (14:0)] do not affect ceramide and diacylglycerol contents. The findings show the particular importance of excess accumulation of long-chain fatty acids in the progress of insulin resistance resulting from lipid oversupply to skeletal muscle (Chavez and Summers, 2003).

Bergman et al published 2012 a study with healthy sedentary obese controls, individuals with type 2 diabetes and lean endurance-trained athletes. Insulin sensitivity was exanimated via an Intravenous Glucose Tolerance Test. Muscle biopsy samples were obtained after an overnight fast.

The results of this experiment suggest that only saturated DAG in skeletal muscle membranes are related to insulin resistance in humans. Of the sixteen quantified membrane species Di-C18:0 (Stearate) was significantly associated to insulin sensitivity (Bergman et al., 2012).

In a recent study (Perreault et al. 2018) the subcellular localization of skeletal muscle DAGs and sphingolipids in relation to insulin sensitivity in human skeletal muscle were detected. Lean individuals, endurance-trained athletes, and obese subjects with and without type 2 diabetes participated. The muscle biopsies were fractionated into sarcolemmal, cytosolic, mitochondrial/ER, and nuclear compartments.

In this experiment greater PKCε membrane/cytosol ratios were found in obese and T2D compared with lean and athletes ($P = 0.03$). PKCθ, PKCδ, or PKCβII did not show significant differences. Furthermore, a significant positive association between PKCε and sarcolemmal 1,2 DAG C16:0/C18:2 ($P = 0.03$) was indicated. There were no significant relationships between 1,2-DAGs in any other compartment and PKCε. No significant relationships were found between sarcolemmal 1,2-DAGs and PKCθ, PKCδ, or PKCβII.

In another experiment the relation of protein kinase Cε translocation and increased C18:1-diacylglycerol content in hepatic insulin resistance was observed.

They examined the mechanism of hepatic insulin in mice with NAFL. Ceramides were not associated with hepatic insulin resistance (Jelenik et al., 2017).

The strongly association of hepatic lipid accumulation with hepatic insulin resistance is known, but the mechanism is still incompletely understood and controversial. Ceramides and diacylglycerols (DAGs) play a potential mechanistic role to mediate lipid-induced hepatic insulin resistance (Petersen and Shulman, 2017).

- Ceramides and ceramides species

In the experiment of this study Ceramide and the ceramide indicate no significant difference after lipid infusion. This leads to the suggestion that ceramide do not play a role for the inducing of insulin resistance after 4 h lipid infusion.

However, contradictory results to ceramide and their role for insulin resistance have been reported in the literature.

In an animal mice experiment of Ussher et al. 2010 the role of de novo ceramide synthesis in mediating muscle insulin resistance was examined. In this experiment diet-induced obesity (DIO) mice with high fat therapy for 12 weeks following treated with an inhibitor of serine palmitoyl transferase-1 (SPT1) (myriocin, 4 weeks) showed reversal of glucose intolerance and insulin resistance with enhanced insulin-stimulated Akt and glycogen synthase kinase 3β phosphorylation in gastrocnemius muscle. This indicates a strongly correlation of intramyocellular accumulation of ceramide with the development of insulin resistance. DAG content remained unchanged.

Accordingly, Jocken et al. (2010) suggested via human experiments that total muscle DAG contents do not play a role in the development of insulin resistance in obese men. That was detected in vastus lateralis muscle biopsies of lean, obese, nonobese type 2 diabetic (T2D), obese T2D, and healthy normoglycemic men.

Contrarily, it is supposed that myriocin might have an indirect influence on muscle insulin action. Because chronic inflammation plays a role for insulin resistance and an effect of myrocin is reducing the activation of macrophages (Schmitz-Peiffer, 2010). The sphingolipid ceramide might be a supposable link of excess ingestion of FFA and inflammatory cytokines to cause insulin resistance (Summers, 2006). Ceramide also seemed to be involved in the regulation of myogenic differentiation. (Bruni and Donati, 2008).

Coen et al. (2010) present a study that was performed with obese insulin sensitive and insulin resistant subjects. In a vastus lateralis muscle biopsy insulin resistance is

associated to greater IMTG content in type I but not type II myocytes and show a higher ceramide content.

Adams et al. (2004) performed an experiment with lean and obese subjects under euglycemic-hyperinsulinemic clamp conditions. Vastus lateralis muscle biopsies obtained at baseline, after 30 min, 40min and 60 min showed a higher ceramide content in obese subjects. Also Ceramide species were similarly increased. There was a significant correlation of muscle ceramide content with the plasma free fatty acid concentration detectable. This data suggest that ceramide may be involved in the development of insulin resistance (Adams et al., 2004).

Lean healthy subjects were examined in an experiment 4 h under hyperinsulinemic-euglycemic clamp condition with a simultaneous administration of a lipid/heparin infusion. The lipid infusion started one hour before clamp and was given throughout the experiment. Content and composition of fatty acids in ceramide and sphingomyelin were analyzed in vastus lateralis muscle biopsies before the clamp, after 4-h clamp and after clamp. The results show a relation between total ceramide content as well as the ceramide consisting of palmitic, palmitoleic, mirystic, and nervonic acid to insulin sensitivity (Straczkowski et al., 2007).

Holland et al. (2007) demonstrate in a mice experiment that the inhibition of ceramide synthesis improved the insulin sensitivity. In this experiment, the mice were treated with control injections (normal saline) or myriocin. Then they were distributed in 6 groups and got either glycerol or 20% lard-oil or 20% soy-oil emulsions. After 4.5 hours of lipid administration a hyperinsulinemic-euglycemic clamp was performed.

Skovbro et al. 2008 present human experiment with four groups: type 2 diabetes, overweight with impaired glucose tolerance, healthy sedentary and endurance-trained participants. Under hyperinsulinemic-clamp conditions, 2 vastus lateralis muscle biopsies after 2 h and 3.5 h clamp.
However, the results here indicate that total muscle ceramide content was similar between individuals showing marked differences in insulin sensitivity. This concludes that ceramide are not important for muscle insulin resistance. However, a positive correlation was presented between muscle ceramide content at baseline and insulin sensitivity.

Bruce et al. (2006) propose that the reduction in muscle ceramide and DAG content play a role in muscle glucose sensitivity. This result was independent of any change in muscle

TAG content. In this experiment, obese subjects underwent an 8 week of ergometer cycling training program. Three vastus lateralis muscle biopsies were performed one week prior to training, and 36–48 h after completion of the training program.

In a further study muscles from three rat groups were performed. The groups were divided in: 1. Control, fed standard diet, 2 HFD, fed high fat diet, 3. HFD/Myr, fed HFD and treated with myriocin (Myr) (an inhibitor of ceramide de novo synthesis). A palmitate isotope tracer infusion was used and mass spectrometry to measure content and synthesis rate of muscle long-chain acyl-CoA (LCACoA), diacylglycerols (DAG) and ceramide (Cer). It was shown that decreased muscle stearoyl- and oleoyl-ceramide content was related with increased Insulin sensitivity (Blachnio-Zabielska et al., 2016).

Published articles by the German Diabetes Center Düsseldorf about some results of this thesis with lean healthy young humans did no show a difference in ceramide content during short-term lipid-induced insulin resistance (Szendroedi et al., 2011), (Szendroedi et al., 2014).

The results of the data records relating to the mechanism of lipid mediated insulin resistance are different probably due to differences in animal models, type, dosage, and duration of interventions. However, lipid metabolites play a crucial role. In human muscle saturated and unsaturated fatty acids supported to stimulate the DAG-PKC signaling pathway. Especially the unsaturated fatty acids enriched DAG such as C18:1 and C18:2 DAGs are of importance. Still more research are needed (Ritter et al., 2015).

5.5 Conclusion

The observations, reported here, lead to following two conclusions:

I. 4 hours high FFA plasma levels induced by lipid infusion with a conditional state of insulin resistance are related with an activation of PKC θ. Moreover, this is observed with a preceded increase of DAG membrane and cytosol fractions after 2.5 hours lipid infusions.

II. Ceramide levels do not show a change during short-term lipid-induced insulin resistance.

The results support the assumption that lipid induced insulin resistance in skeletal muscle is associated with an elevation of DAG membrane and cytosol fraction and therewith related activation of PKC θ. Ceramide levels remain unchanged. Furthermore, the DAG isoforms e.g. linoleate and oleate seemed to be important in the mechanism of lipid-induced insulin resistance.

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