Aus dem Deutschen Diabetes-Zentrum

Leibnitz-Zentrum für Diabetes-Forschung

an der Heinrich-Heine-Universität Düsseldorf

Institut für klinische Diabetologie

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Central insulin regulation of hepatic glucose and energy metabolism in humans

Dissertation

zur Erlangung des Grades eines Doktors der Medizin

der Medizinischen Fakultät der Heinrich-Heine-Universität

Düsseldorf

vorgelegt von

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2019

Als Inauguraldissertation gedruckt mit Genehmigung der Medizinischen

Fakultät der Heinrich-Heine-Universität

gez.

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Zusammenfassung

Das Ungleichgewicht zwischen Energieaufnahme und Energieverbrauch führt bei Adipositas, Insulinresistenz und Typ-2-Diabetes (T2D) zu ektoper Lipidspeicherung in der Leber und so zur nichtalkoholischen Fettlebererkrankung (NAFLD), die häufigste Ursache einer chronischen Lebererkrankung in Europa und in den USA. Der hepatische Energiestoffwechsel, im Besonderen die mitochondriale Funktion, steht im engen Zusammenhang mit der Lipidspeicherung und Insulinsensitivität der Leber. Die Regulation des hepatischen Energiestoffwechsels bei gesunden Menschen und bei Patienten mit T2D war bisher aber nur wenig untersucht. In Mausmodellen reguliert Insulin im Hypothalamus nicht nur die Nahrungsaufnahme, sondern bewirkt auch Abnahme der endogenen Glukoseproduktion (EGP) und Unterdrückung der Lipolyse des Fettgewebes. Die Bedeutung der zerebralen Insulinwirkung am Menschen war bisher ebenso unklar. Diese Arbeit untersuchte folgende Hypothese: zerebrales Insulin verbessert den hepatischen Energiestoffwechsel und senkt die EGP bei Gesunden, nicht aber bei T2D und Adipositas.

Die hepatischen Konzentrationen von Triglyzeriden und Adenosintriphosphat (ATP) wurden mittels nicht-invasiver *in vivo* $^{1}H/^{31}P$ Magnetresonanzspektroskopie bestimmt. Die EGP wurde mittels stabiler Isotopenverdünnung vor und während eines hyperinsulinämisch-euglykämischen Clamptests gemessen. Die Untersuchung der Wirkung von zerebralem Insulin erfolgte mittels intranasaler Verabreichung von Insulin in randomisierten, Plazebo-kontrollierten Interventionsstudien an Menschen mit und ohne T2D und Adipositas.

Intranasale Insulingabe bewirkt bei gesunden Personen keine Veränderung der EGP unter Nüchtern-Bedingungen, steigert aber die hepatische Insulinsensitivität. Die hepatische Triglyzeridkonzentration sinkt um 35% und die ATP-Konzentration steigt um 18% an. Intranasales Insulin erhöht die Insulin-stimulierte Glukoseaufnahme im Skelettmuskel und verbessert dadurch die Glukosehomöostase. Bei Patienten mit T2D und bei adipösen Menschen hat intranasales Insulin aber keine Effekte auf den hepatischen Energiestoffwechsel und die Glukosehomöostase.

Diese Ergebnisse weisen darauf hin, dass der hepatische Energiestoffwechsel sowie die periphere (muskuläre) Glukosehomöostase auch beim Menschen zumindest zum Teil der Steuerung durch zentralnervöse Effekte von Insulin unterliegen. Die Störung dieser Steuerung bei T2D und Adipositas könnte durch eine Insulinresistenz bestimmter Hirnareale bedingt sein und zur Beeinträchtigung der Glukosehomöostase bei diesen Patienten beitragen. Diese Ergebnisse weisen den Weg zu neuen Strategien zur Therapie des Diabetes mellitus.

Summary

The imbalance between energy intake and energy expenditure leads in obesity, insulin resistance and type 2 diabetes (T2D) to ectopic lipid storage in the liver, termed non-alcoholic fatty liver disease (NAFLD), which is the most common liver disorder in Europe and the USA. Hepatic energy metabolism and mitochondrial function tightly relate to hepatic lipid accumulation and insulin sensitivity. However, the regulation of hepatic energy metabolism in healthy humans and patients with T2D remains not completely understood. Mouse model studies demonstrated that hypothalamic insulin action not only reduces food intake but also suppresses endogenous glucose production (EGP) and decreases lipolysis in adipose tissue. The role of brain insulin action in humans with and without T2D and obesity, however, is not completely understood. This work tested the hypothesis that central insulin action improves hepatic energy metabolism and reduces EGP in healthy lean humans, but not in T2D and obese individuals.

Hepatic triglyceride and adenosine triphosphate (ATP) concentrations were assessed using non-invasive *in vivo* $^{1}H/^{31}P$ magnetic resonance spectroscopy. Rates of EGP were measured using stable isotope dilution technique in fasting conditions as well as before and during hyperinsulinemic-euglycemic clamps. Investigation of central insulin action in humans was performed using intranasal insulin application in randomized, placebo-controlled, interventional studies in humans with and without T2D and obesity.

Intranasal insulin does not alter hepatic EGP in healthy humans in fasting conditions, but increases hepatic insulin sensitivity. Liver fat content is reduced by 35%, while hepatic ATP concentration increases by 18%. Insulin-stimulated skeletal muscle glucose uptake is elevated after intranasal insulin application thereby improving glucose homeostasis. However, the effects of intranasal insulin on hepatic energy and glucose homeostasis are absent in T2D and obese individuals.

These data suggest that hepatic energy metabolism and peripheral glucose homeostasis can be regulated at least in part by central insulin action in humans. Blunted central insulin effects in T2D and obese participants could be due to brain insulin resistance and likely contribute to glucometabolic perturbations in these patients. These results show the way to new therapeutic strategies for diabetes mellitus.

List of abbreviations

ATP - adenosine triphosphate

BMI – body mass index

CNS – central nervous system

EGP – endogenous glucose production

ETC – electron transport chain complex

fATP – flux through ATP synthase

FFA - free fatty acids

FOXO1 – forkhead box protein O1

HCL - hepatocellular lipid content

HIS – hepatic insulin sensitivity

HOMA-IR – homeostatic model assessment of insulin resistance

IRS – insulin receptor substrate

K_{ATP} – potassium-dependent ATP channels

MRS - magnetic resonance spectroscopy

NAFLD – non-alcoholic fatty liver disease

NASH – non-alcoholic steatohepatitis

OXPHOS – oxidative phosphorylation

Pi – inorganic phosphate

ROS – reactive oxygen species

STAT3 - signal transducers and activators of transcription 3

T2D - type 2 diabetes

VLDL – very low-density lipoproteins

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1. Introduction

The growing epidemic of obesity and type 2 diabetes (T2D) is one of the most serious and expensive biomedical challenges for the industrialized world [1]. It represents a major health problem and places an enormous financial burden on society for the care and treatment of patients. Additionally, the constant rise of obesity and T2D-associated co-morbidities such as cardiovascular disease, cancer and Alzheimer's disease demonstrate the necessity to define the molecular mechanisms underlying their onset, manifestation and progression, to ultimately combat this epidemic.

1.1. Type 2 diabetes

The T2D prevalence in the general German population is 9.9%, but raises steeply in age group 50-80 years [2]. Just like in other industrialized countries, the incidence of T2D is also increasing in Germany as well, with the number of affected individuals rising by 5% annually [3]. According to the CoDiM study, the financial burden of T2D in Germany has been estimated to 26 billion Euro in 2000 [4]. Globally, about 1 in 11 adults have diabetes mellitus and the number of diabetes patients has quadrupled in the past three decades [5]. There is an especially alarming trend for increase in the prevalence in younger individuals [6].

T2D is marked by chronic hyperglycemia and initial hyperinsulinemia, due to insulin resistance of insulin-sensitive target tissues. The diagnostic criteria for T2D according to the American Diabetes Association [7] include:

1. HbA1c ≥ 6.5%*
   OR
2. Fasting plasma glucose ≥ 126 mg/dl (7 mmol/l)**
   OR
3. Symptoms of hyperglycemia and plasma glucose ≥ 200 mg/dl (11.1 mmol/l)***
   OR
4. 2-h plasma glucose ≥ 200 mg/dl (11.1 mmol/l) during an oral glucose tolerance test****

* The test should be performed in a laboratory using a method that is certified by the NGSP (National Glycohemoglobin Standardization Program) and standardized by the assay according to DCCT (Diabetes Control and Complications Trial).

** Fasting is defined as no caloric intake for at last 8 hours.

*** The classic symptoms of hyperglycemia are defined as polyuria, polydipsia and unexplained weight loss.

**** The test should be performed as described by the World Health Organization, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.

Impaired insulin sensitivity represents an early abnormality and can be compensated by augmented β-cell function for a long time before the insulin-glucose feedback loop fails and T2D manifests clinically [8]. Insulin resistance might also occur independently of inadequate insulin secretion and be prerequisite for incident T2D [9-11]. The Whitehall II cohort study has demonstrated that a rapid deterioration of insulin sensitivity and beta-cell function precedes T2D diagnosis by 5 and 4 years, respectively [8]. Still, it has been suggested that gradual decrease in insulin sensitivity begins more than a decade before disease onset [12].

Genetic predisposition plays an important role for T2D development together with other important risk factors such as age, male sex and metabolic syndrome [13]. While age appears to be the most influential risk factor for T2D onset, genetic predisposition varies between ethnic groups with Hispanics, Carribeans and Asian Indians known to have higher risk in comparison to Caucasians [14]. In women, the medical history of gestational diabetes or the presence of polycystic ovary syndrome additionally increase the risk of T2D development [15]. In addition, infants with low birth weight are predicted to have diminished beta-cell function in adulthood and represent another T2D risk group [14]. Obesity is tightly related to insulin resistance and is therefore another major risk factor for T2D. It results from excessive energy intake in form of hyper-caloric diet accompanied by physical inactivity. Visceral obesity with waist circumference > 94 cm for European male individuals and > 80 cm for European females is considered as another major determinant for increased risk [16]. Approximately 90% of T2D patients are obese, while only 20-25% of obese individuals have overt diabetes [17]. In obese patients, increased free fatty acid (FFA) flux into the circulation is observed, inducing both

lipotoxicity and ectopic lipid storage in peripheral tissues, which in turn induce insulin resistance.

1.2. Insulin resistance

Insulin is a key regulator of glucose homeostasis and its action in target tissues plays an essential role in metabolic disease. The biological function of insulin is to stimulate glucose uptake in skeletal muscle and adipose tissue, to suppress endogenous glucose production (EGP) in the liver and to suppress lipolysis in adipose tissue which is termed insulin sensitivity [18, 19] (Figure 1). In the central nervous system insulin inhibits appetite and induces satiety [20]. Insulin resistance is defined by reduced glucose clearance in skeletal muscle, impaired suppression of glucose production by the liver and decreased suppression of lipolysis in adipose tissue. A number of perturbations in the target tissues such as abnormalities in mitochondrial function, defects in insulin receptor signalling and chronic subclinical inflammation have been suggested as responsible for the development of insulin resistance. Genetic predisposition as well as reduced physical activity, age, obesity and some nutritional factors are considered as main risk factors for insulin resistance.

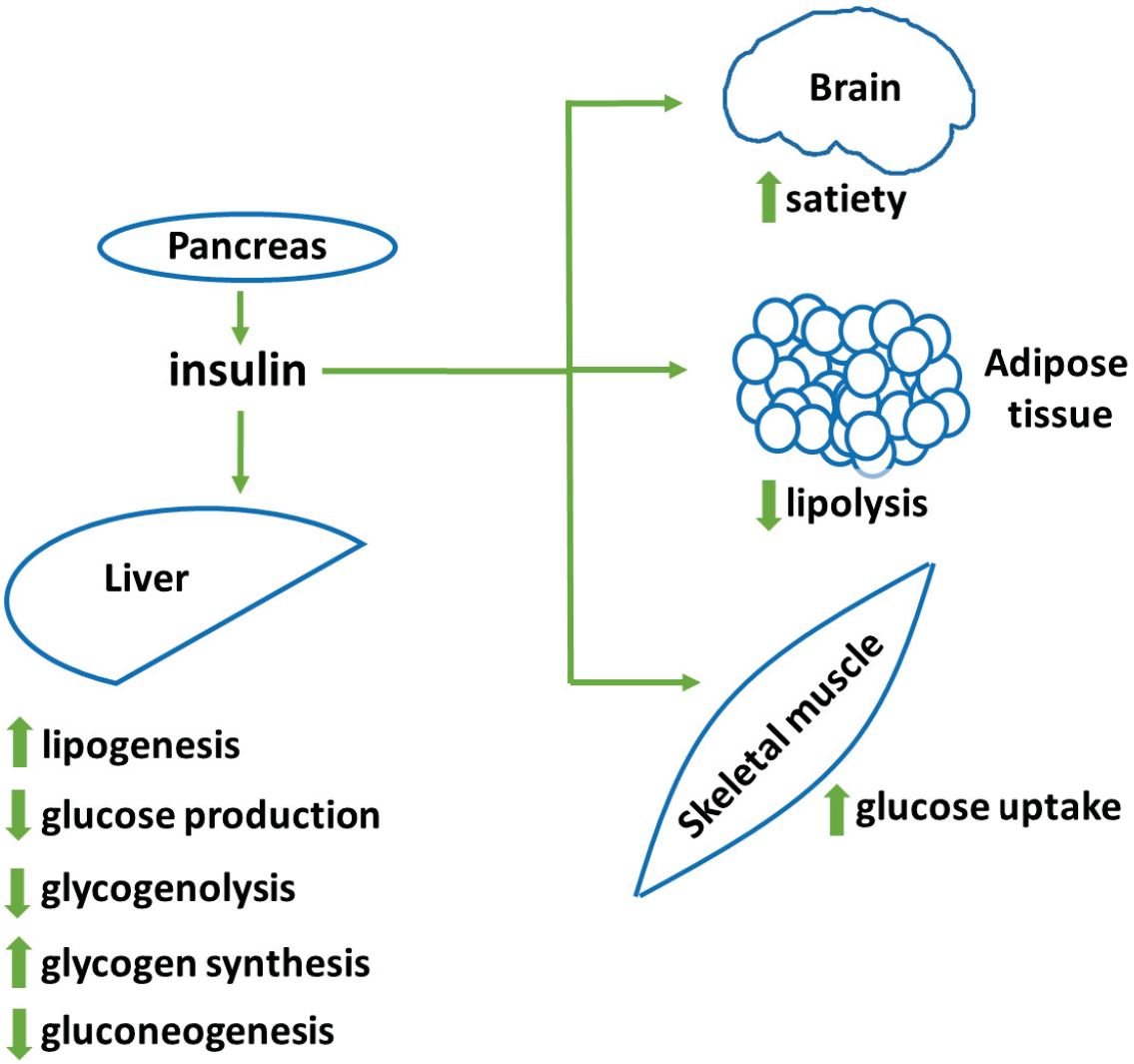

Figure 1. Insulin's direct action on target tissues. Insulin released from the endocrine pancreas upon elevation in blood glucose acts in distant target organs. In the hypothalamus insulin signals satiety after a meal. In adipose tissue insulin suppresses lipolysis to eventually reduce circulating free fatty acids. Insulin action in skeletal muscle increases glucose uptake, while in the liver glucose production is suppressed via inhibition of gluconeogenesis and glycogenolysis.

Interestingly, insulin resistance represents a physiological phenomenon in certain conditions such as pregnancy, puberty, infections or adaptation to acute stress situations with release of glucocorticoids and catecholamines. Prolonged stress reactions as seen in hypercortisolism lead to accumulation of visceral fat and insulin resistance and subsequently increased risk of T2D [21].

Insulin exerts its action on cellular level by binding to the insulin receptor. This leads to activation of insulin signalling pathway including insulin receptor substrate phosphorylation and subsequent activation of phosphoinositol-3-kinase (PI3K) and protein kinase B. As a result a plethora of further metabolic effects in target tissues are induced. As skeletal muscle is responsible for 80% of postprandial glucose uptake, impairment of muscle insulin action plays a central role for perturbations of glucose metabolism with postprandial hyperglycemia [22]. Patients with T2D exhibit a 60 % reduction in insulin stimulated muscle glycogen synthesis, which represents the main abnormality underlying their insulin resistance [23, 24]. Furthermore, increase in muscle glycogen synthesis after meal ingestion is ~30% lower in T2D patients in spite of doubled serum insulin concentrations [25]. Further defects such as diminished increases in glucose-6-phosphate and lower intramyocellular glucose concentrations in skeletal muscle of T2D patients during hyperinsulinemia have been described [26]. Impaired insulin-stimulated glucose transport via glucose transporter 4 (GLUT4) represents the key abnormality responsible for muscle insulin resistance in T2D, obesity and insulin resistant first degree relatives of T2D patients [27].

In insulin resistant individuals adipose tissue lipolysis rates remain high despite elevated systemic insulin levels. The resulting increased lipid availability and, in particular, raised free fatty acid concentrations exert detrimental effects on distant organs such as the liver and skeletal muscle [28]. Thereby lipid-mediated impairment of hepatic and muscle insulin sensitivity is induced. Free fatty acids taken up by the liver serve as gluconeogenesis precursors and thereby contribute to elevated hepatic glucose production rates. In addition, obesity and the metabolic syndrome have been related to subclinical inflammation arising from adipose tissue and resulting in imbalance between secretion of adipocytokines with anti-inflammatory and insulin sensitizing properties such as adiponectin and proinflammatory cytokines such as leptin, tumor necrosis factor α, interleukin – 6 and many more [29]. The mechanism of induction of insulin resistance by these adipokines involves higher serine phosphorylation of insulin receptor substrate 1 (IRS-1) by activation of c-Jun N-terminal kinase and activation of inhibitor of nuclear factor κ-B kinase subunit β.

The liver plays a central role in insulin resistance as it is key to the transition from fasted to the fed state by its ability to rapidly switch from a glucose-producing to a glucose-storing organ. Insulin action in the liver after a meal supresses glucose production and enhances

glucose uptake for storage in the form of glycogen [30]. In insulin resistant T2D patients excessive postprandial hyperglycemia results from impaired suppression of glucose production with ~45% decreased hepatic glycogen accumulation than in healthy individuals [31]. Insulin–mediated hepatic glycogen synthesis inversely correlates with ectopic lipid accumulation in the liver in diabetic and non-diabetic individuals, supporting the tight link between hepatic lipid content and hepatic insulin resistance [28].

1.3. Ectopic lipid storage in the liver

Excessive accumulation of hepatic triglycerides >5.5% defines non-alcoholic fatty liver disease (NAFLD), which is considered as the liver manifestation of the metabolic syndrome and associates tightly with obesity and T2D [32]. NAFLD encompasses a spectrum of liver diseases ranging from simple steatosis in the absence of inflammation to steatohepatitis (NASH), liver cirrhosis and hepatocellular carcinoma. The histological features of NAFLD resemble those found in alcoholic liver disease. Around 70-95% of obese patients with BMI>30 kg/m^2 and ca. 70% of patients with T2D have hepatic steatosis [33]. The prevalence of NAFLD in industrialized western countries is currently estimated to 20-25% and continues to rise [33]. The causal relationship between hyperlipidemia, ectopic lipid storage in the liver and insulin resistance has been studied intensively, but remains not completely understood. Hepatic insulin sensitivity strongly and negatively relates to hepatocellular lipid content (HCL) in healthy, prediabetic and diabetic populations [34]. NAFLD has been suggested to ensue in the setting of or secondary to prevailing insulin resistance [35], but increased lipid availability achieved by lipid infusion also induces hepatic lipid accumulation and insulin resistance [36]. A reduction in HCL improves whole body glucose metabolism and reduces glycaemia [37]. Therefore strategies to treat NAFLD are essential not only for preventing its progression to liver cirrhosis, but also for the treatment and prognosis of T2D. Current therapeutic options for NAFLD include lifestyle interventions with exercise and caloric restriction as well as pharmaceutical treatment with thiazolidinedions (glitazones), vitamin E, liraglutide and some new agents [32, 38]. Still, no established treatment for NAFLD currently exists and therefore new approaches need to be investigated.

Increased lipid availability from high circulating free fatty acids is a feature of insulin resistant states such as obesity and T2D [28]. Elevated FFA result from increased fat intake and/or

hypercaloric feeding, but may also be derived from unrestrained lipolysis in insulin resistant adipose tissue. The greater FFA flux to the liver may stimulate rates of EGP as already described in 1.2 via generation of nicotinamide-adenine-dinucleotide (NADH) from increased β-oxidation of FFA [39]. In addition to the higher FFA influx to the liver, elevated insulin and glucose concentrations further activate hepatic de novo lipogenesis. As a result, hepatic steatosis develops when lipid uptake and synthesis in the liver exceed the hepatic capacity for lipid oxidation and export.

1.4. Hepatic energy metabolism

Hepatic mitochondria are highly dynamic organelles engaged in the generation of adenosine triphosphate (ATP) by oxidative phosphorylation (OXPHOS), the production and detoxification of reactive oxygen species (ROS) thereby controlling cellular redox state and the regulation of cellular substrate metabolism and apoptosis [40, 41]. Mitochondrial function entails cellular respiration via the activities of the mitochondrial electron transport chain complexes (ETC), substrate oxidation through the tricarboxylic acid cycle, ketogenesis and ROS formation [42, 43]. Hepatic mitochondria are the sites of fatty acid oxidation and energy production in the form of ATP and thereby play a key role for liver substrate utilization. Because the glucose uptake system of hepatocytes is insulin independent [44], liver mitochondrial function is expected to be more prone to putative effects induced by glycemia and excessive glucose influx, redirected from insulin resistant skeletal muscle.

Insulin is key regulator of hepatic energy metabolism as it is responsible for the metabolic transition of the liver from the fasted to the fed state by switching from glucose producing organ to a glucose storing organ [30]. During fasting the liver is mainly responsible for gluconeogenesis and glycogenolysis so that glucose is released in the circulation in order to maintain euglycemia. In the postprandial state hepatic glucose production is supressed by insulin and glucose is stored in the form of glycogen. Both fasting and postprandial glycemic levels are elevated in insulin resistant humans with T2D due to upregulated gluconeogenesis [45] resulting in impaired suppression of glucose production along with 45% lower hepatic glycogen synthesis than in healthy humans [31].

Notably, insulin resistance might affect mitochondrial capacity in the liver, but impaired mitochondrial activity has also been found to promote insulin resistance by disrupting insulin

signalling [40]. The mutual relationship between hepatic mitochondrial function and insulin sensitivity in the liver has been proposed by evidence from mice model with genetic deletion of IRS-1 and IRS-2 [46]. The transcription factor forkhead box protein O1 (FOXO1) in the liver has been suggested as the factor integrating insulin signalling with mitochondrial function in the hepatocyte [46]. Thereby insulin signalling in the liver underpins mitochondrial ETC integrity and promotes ATP production [41], while also activating peroxisome proliferator-activated receptor γ coactivator 1α, the major transcription factor stimulating mitochondrial biogenesis [47]. In overall, most of the evidence for insulin control of hepatic mitochondrial function stems from research in animal models and *in vivo* studies in humans are missing.

1.5. Alterations in hepatic energy metabolism in metabolic diseases

Due to their pivotal role in cellular substrate utilization and metabolic processes, hepatic mitochondria exhibit distinct abnormalities in metabolic disease states [39]. Alterations in hepatic energy metabolism in T2D have first been suggested from gene expression analyses in the liver of patients with T2D which revealed upregulated expression of genes involved in OXPHOS, gluconeogenesis and ROS generation [48, 49]. In contrast, non-invasive *in vivo* $^{31}P/^{1}H$ magnetic resonance spectroscopy (MRS) studies of liver homeostasis in T2D patients demonstrated reduced hepatic energy metabolism [50, 51]. In particular, diabetic humans exhibited 23-26% lower hepatic ATP concentrations and 28-31% lower hepatic Pi concentrations when compared both to age- and body mass index (BMI)-matched and to young lean controls [50]. Furthermore, 42% reduced flux through hepatic ATP synthase has been reported in a similar type 2 diabetic patient group [51]. Whether hepatic energy metabolism can indirectly be modulated by interorgan crosstalk mechanisms has not been studied so far. In addition, it remains unclear whether altered hepatic energy metabolism in T2D could be affected by interventional treatments.

Lower hepatic ATP concentrations in T2D patients were shown to associate with hepatic insulin resistance independent of hepatocellular fat content and EGP was the only independent predictor of ATP levels explaining 57% of the variance of hepatic ATP concentrations [50]. Furthermore, the reduced hepatic flux through ATP synthase (fATP) related positively to both peripheral and hepatic insulin sensitivity (HIS) and negatively to waist circumference and BMI [51]. Another factor influencing hepatic fATP in T2D was short-

and long-term glycaemic control, reflected by fasting glycaemia and glycosylated haemoglobin. From these studies it became apparent that distinct metabolic and anthropometric factors affect hepatic energy status. The role of insulin action per se for hepatic ATP levels has not been investigated so far.

Hepatic energy metabolism in obesity and NAFLD also exhibits alterations as direct ex vivo quantification of mitochondrial respiration in liver biopsies demonstrated up to 5-fold higher mitochondrial oxidative capacity in obese humans with and without hepatic steatosis compared to lean persons, whereas respiratory rates were reduced in obese patients with NASH [52]. Elevated oxidative stress coupled to lower antioxidant capacity as well as mitochondrial proton leak and reduced coupling efficiency possibly underlie the loss of adaptation of hepatic mitochondria in NASH, which probably drives the progression of NAFLD and insulin resistance. In line with these findings, obese insulin resistant humans exhibit augmented increase in hepatic ATP concentrations after ingestion of a single mixed meal compared to lean insulin sensitive humans [53]. These results suggest a tight link between alterations of hepatic energy metabolism, ectopic lipid storage and hepatic insulin resistance in metabolic disease. The human liver can adapt its energy metabolism to lipid availability by increasing its mitochondrial oxidative capacity in an attempt to compensate the excess substrate influx. However, adaptation related hepatic oxidative stress can augment hepatic lipid accumulation and promote hepatic insulin resistance. These data raise the question as to what the relative contribution of hyperglycemia, hyperinsulinemia and hyperlipidemia to the alterations of hepatic mitochondrial function is. Strategies to modulate altered hepatic energy metabolism are needed to better understand its role in metabolic disease.

1.6. The brain as an insulin sensitive organ

Apart from its indisputable importance in peripheral target tissues, insulin signaling in the central nervous system (CNS) is essential for the regulation of energy and glucose homeostasis. Claude Bernard first described the relevance of the brain for glucose homeostasis in animals back in 1854 [54]. In his dog experiments, he demonstrated induction of glucosuria by puncturing the floor of the fourth ventricle. His findings initially received less attention as insulin's discovery and effects on blood glucose attracted great interest. As early as the 1970s the insulin receptor was discovered in many tissues, including the brain, with

marked regional variations in receptor density [55]. Subsequently, the fundamental work by Woods and Porte in baboons and rats established a role for brain insulin in the regulation of food intake and body weight. Intracerebroventricular insulin infusion reduced feeding which related to weight loss [56]. In 2000 the report of the brain specific knockout of the insulin receptor in a mouse model raised additional interest in the role of brain insulin action [57]. Inactivation of the insulin receptor in the brain increased food intake, body fat and leptin levels resulting in insulin resistance, dyslipidemia and impaired reproductive function. This was followed by the discovery of specific insulin-responsive cell populations in the hypothalamus - the proopiomelanocortin and agouti-related protein neurons – responsible for the feeding control [58, 59]. Intensive studies of the central insulin effects have revealed the individual contributions of various components of the principal pathways in the CNS [60]. Subsequently, central insulin's effects on peripheral metabolism were studied.

1.7. Brain insulin control of glucose metabolism in animal models

Pioneering work from Luciano Rossetti´s lab demonstrated that hypothalamic insulin singling is required for the inhibition of EGP [61, 62]. Insulin infusion into the 3rd cerebral ventricle of rats activates potassium-dependent ATP-channels (K_{ATP}) in the mediobasal hypothalamus via activation of the insulin receptor-PI3K pathway [61]. This leads to reduction in gluconeogenic gene expression in the liver and lowers hepatic glucose production. The effects were shown to be hypothalamic K_{ATP} channel-dependent [62]. These studies were conducted under controlled hormone conditions using pancreatic clamps with somatostatin infusion thereby creating systemic hypoinsulinemia. Although the experiments did not resemble physiological states, they unequivocally demonstrated brain insulin induced reduction of hepatic glucose output. Interestingly, this effect was abolished by hepatic vagotomy, suggesting that the brain-liver crosstalk is mediated by the vagus nerve. Furthermore, activation of the insulin receptor – extracellular signal–regulated kinase (ERK) pathway in the dorsal vagal complex has also been shown to modulate hepatic glucose production [63], which additionally supported the notion of the involvement of the vagal nerve.

However, these findings were not confirmed in larger mammals. In series of elegant experiments performed in conscious dogs, the Edgerton and Cherrington lab demonstrated that the canine brain can sense insulin [64] and thereby regulate hepatic glucoregulatory

enzyme expression [65]. Insulin delivery into the head arteries enhances hepatic glycogen synthesis and lowers mRNA expression of gluconeogenic enzymes. However, this does not result in changes in EGP. In this large animal model insulin's direct hepatic effects predominate the central effects of the hormone in the acute regulation of hepatic glucose metabolism [66]. These conflicting findings from different animal models raised even more the interest in the study of brain insulin action in humans and its potential to modulate peripheral glucose metabolism.

Animal studies also indicated brain insulin modulation of lipid metabolism as insulin infusion in the mediobasal hypothalamus was able to inactivate hormone–sensitive lipase and suppress lipolysis [67] and intracebroventricular insulin treatment promoted adipogenesis by inducing the expression of lipogenic proteins in adipose tissue [68]. Fatty acid retention in adipocytes was shown to be enhanced thereby promoting energy storage [69]. Of note, CNS insulin action stimulates fatty acid uptake in white adipose tissue of insulin sensitive mice only, while no effect is observed in overfed insulin resistant animals [69].

1.8. Brain insulin effects on glucose metabolism in humans

Intranasal insulin application in humans delivers insulin to the brain and serves as a tool to mimic enhancement of brain insulin signalling [70]. After intranasal administration insulin enters the nasal cavity and is transported to the CNS, bypassing the blood brain barrier. Transport and distribution of insulin in the brain might be explained by rapid bulk flow within the perivascular space of cerebral blood vessels, as described recently [71]. Alternatively, small lipophilic peptides such as insulin have been proposed to potentially enter the CNS directly by diffusing across the olfactory epithelia and intercellular spaces into the subarachnoid space [70, 72]. An increase in liquor insulin concentration after intranasal insulin has been demonstrated in humans, suggesting successful delivery of the peptide to the CNS [70].

Intranasal insulin has been shown to exert a plethora of brain, behavioural and metabolic effects in humans. The studies investigating the metabolic effects of intranasal insulin in humans are summarized in Table 1. Administration of 160 IU of intranasal insulin has been shown to modulate food-related activity in CNS [73], suppress food intake [74], but also reduce palatable food consumption and increase satiety [75]. Moreover, it improves cognitive

performance in women [76], enhances postprandial thermogenesis and reduces postprandial insulinemia [77]. Suppression of systemic lipolysis in healthy humans has been observed which is explained primarily by reduction of non-subcutaneous adipose tissue lipolysis, as gene expression of key lipolytic enzymes in subcutaneous fat biopsies were not altered after intranasal insulin [78]. Moreover, intranasal insulin acutely lowers plasma glucose and alters peripheral insulin sensitivity measured from homeostatic model assessment of insulin resistance (HOMA-IR) [79]. Further studies using 160 IU intranasal insulin administration revealed higher glucose infusion rate needed to maintain euglycemia indicating improved insulin sensitivity [80]. These effects were proposed to be mediated through parasympathetic outputs.

Table 1. Acute metabolic effects of intranasal insulin in humans

Citation	INI dose	Participants/ Conditions	IS	EGP	FFA/TG	Glucose	Insulin	C-peptide	Findings
[75]	160 IU	32 CON; fasted	-	-	-	↓	↔	↓	-
[73]	160 IU	9 CON; overnight fasted	-	-	-	↔	↑	↓	-
[77]	160 IU	19 CON; fasted and after a meal	-	-	↓FFA after INI	↓	↑after INI, ↓postprandial	↓postprandial	↑postprandial thermogenesis
[79]	160 IU	103 CON; fasted	↓HOMA-IR	-	-	↔	↑	↓	Hypothalamic insulin action relates to insulin sensitization
[80]	160 IU	10 CON and 5 OBE; HEC	↑GIR	-	-	↔	↔	↔	Insulin sensitization relates to parasympathetic output
[78]	160 IU	41 CON; fasted	-	-	↓FFA, ↔TG, ↔Glycerol	↔	↔	↔	↑suppression of lipolysis of non-subcutaneous adipose tissue

Citation	INI dose	Participants/ Conditions	IS	EGP	FFA/TG	Glucose	Insulin	C-peptide	Findings
[81]	40 IU	8 CON; pancreatic clamp	↑GIR, ↔Rd	↓EGP	↓FFA, ↔TG	↔	↔	-	-
[82]	210 IU	30 CON; fasted	-	-	-	↓	↑	↓	No impact of INI on glucose homeostasis beyond effects of concurrent systemic hyperinsulinemia
[83]	160 IU	10 CON and 10 T2D; fasted	-	↔EGP	↓FFA ↔TG ↔Glycerol	↓	↑	↓	↓liver fat content and ↑hepatic ATP in CON, but not in T2D
[84]	160 IU	11 CON and 10 OBE; HEC	↑GIR in CON	↓EGP in CON	-	↔	↔	↔	Hypothalamic and striatum insulin action relates to insulin sensitization

Citation	INI dose	Participants/ Conditions	IS	EGP	FFA/TG	Glucose	Insulin	C-peptide	Findings
[85]	40 IU	9 CON; fasted		-	↔ triglyceride rich lipoprotein apoB100 or B48	-	-	-	No effect on triglyceride rich lipoprotein apoB100 or B48
[86]	40 IU	7 OBE; pancreatic clamp	↔Rd	↔EGP	↔	-	↔	-	-
[87]	160 IU	16 CON; test breakfast buffet	-	-	-	↓	↔	↔	↓Breakfast carbohydrate intake
[88]	160 IU	14 elderly CON and 30 young CON; before bedtime	-	-	-	↓in young only	↑in young only	↔	↓Cortisol in elderly only, ↔adrenocorticotropic hormone

CON – lean healthy humans, EGP – endogenous glucose production, FFA – free fatty acids, GIR – glucose infusion rate, HEC – hyperinsulinemic euglycemic clamp, HOMA – IR – homeostatic index insulin resistance, INI – intranasal insulin, IS – insulin sensitivity, IU – international units, Rd – glucose disposal rate, T2D – type 2 diabetes, TG – triglycerides

Notably, human studies also investigated central insulin effects on EGP under pancreatic clamp conditions (Table 1). In these experiments glucoregulatory hormones were clamped at basal levels using somatostatin, growth hormone, insulin and glucagon infusions and brain insulin signalling was stimulated by intranasal insulin application [81] or mimicked by oral diazoxide administration [89] in lean healthy humans. Diazoxide is pharmacologic K_{ATP} channel activator and its application during pancreatic clamp conditions, when endogenous insulin secretion is blocked by somatostatin, allows investigation of the extrapancreatic diazoxide effects. In particular, the CNS K_{ATP} channels activation is examined, which is known as downstream target of CNS insulin action [61]. Both studies found lower EGP rates starting around 2.5h after intranasal insulin or diazoxide application which remained reduced for the subsequent 4h. However, whether intranasal insulin is able to control EGP under hyperinsulinemic conditions remains unknown. Moreover, these studies did not evaluate effects of intranasal insulin on fasting HIS, thereby leaving the role of brain insulin under physiological fasting conditions unclear.

A spillover of small amount of insulin in the circulation after intranasal insulin has been reported in some [73, 77, 79, 82, 88] but not all studies [75, 78, 80, 81, 86, 87] (Table 1). The increase in systemic insulin exposure has been observed using 160 IU insulin, which raises the question whether effects on peripheral glucose metabolism and insulin sensitivity are mediated by central (CNS) or peripheral (systemic) insulin action. In some reports even though insulin spillover in the circulation was suspected, observed effects in peripheral metabolism were found to be independent from serum insulin excursions by statistical test [78]. However, the reduction in plasma glucose [77, 79] after rise in serum insulin reported after nasal insulin application indicates that this spillover cannot be considered negligible and might indeed contribute to the observed acute effects on peripheral glucose metabolism. Intranasal insulin at lower dose of 40 IU increases cerebrospinal fluid insulin concentration with no significant change in serum insulin level [70]. However, sampling at 10 minute intervals for the first 40 minutes and 20 minute intervals for further 40 minutes might have missed to detect rapid excursions in insulin levels. Careful delineation between centrally-mediated and spillover effects of intranasal insulin is needed when studying effects on peripheral metabolism. However, this hasn't always been addressed by investigators and still needs to be established.

1.9. Brain insulin resistance

More than 30 years ago brain insulin resistance has been suggested to be responsible for altered effects of central insulin in obese rats [90]. Later, it has also been noted that obese humans respond differently to hyperinsulinemia from neuronal activity measured with magnetoencephalography [91]. A reduced or even absent action of intranasal insulin on brain activity has been associated with obesity [73, 92]. Excessive visceral fat accumulation has been related to brain insulin resistance in the cerebral cortex [93] and in the hypothalamus [94]. Furthermore, it has been suggested that brain insulin resistance might unfavourably influence body fat distribution as brain insulin sensitivity predicts the ability to reduce visceral fat during lifestyle intervention programme [95]. Saturated fatty acids (SFA) are increased in obesity and are associated with brain insulin resistance independent of body mass index [93]. Binding of SFA to Toll-like receptors is known to trigger inflammatory responses and contribute to deterioration of brain insulin action in animals [96-98]. Furthermore, the insulin sensitizing effect demonstrated in lean individuals is abrogated in obese individuals [80]. The importance of body weight for brain metabolism is further underlined by the discovery that insulin-induced brain activity in people with obesity normalizes when they lose weight after bariatric surgery [99]. Moreover, peripheral insulin resistance has been linked to brain insulin resistance in studies using both systemic insulin infusion [91, 100-102] and intranasal insulin administration [94, 103]. In addition to body weight and peripheral insulin resistance, age seems to be a strong determinant of brain insulin sensitivity, with reduced responsiveness described in older individuals [104]. Furthermore genetic background has been shown to influence brain insulin sensitivity as different polymorphisms in genes such as *IRS1*, *FTO* and *MC4R* have been suggested to interfere with brain insulin action [105]. Still, whether brain insulin resistance plays a role for intranasal insulin modulation of peripheral glucose metabolism remains unknown.

Study Nr: 3638, Ethic commission approval on 21.06.2011

Study Nr. 402/2014BO1, Ethic commission approval on 25.09.2014

2. Aims and hypotheses

Although rodent data strongly suggest a role of brain insulin for the regulation of HIS, evidence from human studies is missing. In particular, the acute effects of intranasal insulin on hepatic energy and glucose metabolism in humans remain unclear. As intranasal insulin application successfully delivers insulin to the human brain, this technique represents a reliable method to investigate brain insulin action in humans.

The present work tested the following hypotheses:

1. Intranasal insulin improves fasting HIS and hepatic energy metabolism in lean humans, but not in T2D patients
2. Intranasal insulin improves insulin-mediated suppression of endogenous glucose production during hyperinsulinemic-euglycemic clamp and increases skeletal muscle glucose uptake in lean, but not in overweight humans

To this end intranasal insulin effects on hepatic glucose homeostasis in fasting and hyperinsulinemic-euglycemic clamp conditions were investigated in placebo-controlled single-blinded randomized interventional studies in lean healthy humans, overweight individuals and T2D patients (Study 1 and Study 2). The acute effects of intranasal insulin on HIS and energy metabolism in fasting conditions were studied in lean healthy humans and T2D patients in Study 1 performed at the Institute for Clinical Diabetology at the German Diabetes Center (DDZ) Düsseldorf (Study Nr: 3638, Ethic commission approval on 21.06.2011) [83]. In cooperation with the research group of Hans-Ulrich Häring at Eberhard-Karls University Tübingen the acute effect of intranasal insulin on EGP was tested using hyperinsulinemic euglycemic clamps in lean and overweight humans (Study 2) (Study Nr. 402/2014BO1, Ethic commission approval on 25.09.2014) [84]. Evaluation of EGP and skeletal muscle glucose uptake from isotope dilution with [6,6-$^{2}H_{2}$]glucose during clamp was performed at German Diabetes Center Düsseldorf.

3. Publications

Sofiya Gancheva, Chrysi Koliaki, Alessandra Bierwagen, Peter Nowotny, Martin Heni, Andreas Fritsche, Hans-Ulrich Häring, Julia Szendroedi, Michael Roden. Effects of Intranasal Insulin on Hepatic Fat Accumulation and Energy Metabolism in Humans. **Diabetes** 2015;64(6):1966-1975

Martin Heni, Robert Wagner, Stephanie Kullmann, **Sofiya Gancheva**, Michael Roden, Andreas Peter, Norbert Stefan, Hubert Preissl, Hans Ulrich Häring, Andreas Fritsche. Hypothalamic and Striatal Insulin Action Suppresses EGP and May Stimulate Glucose Uptake During Hyperinsulinemia in Lean but not in Overweight Men. **Diabetes** 2017;66(7):1797-1806

Sofiya Gancheva,[1,2] Chrysi Koliaki,[1,2] Alessandra Bierwagen,[1,2] Peter Nowotny,[1,2] Martin Heni,[3,4,5] Andreas Fritsche,[3,4,5] Hans-Ulrich Häring,[3,4,5] Julia Szendroedi,[1,2,6] and Michael Roden[1,2,6]

Effects of Intranasal Insulin on Hepatic Fat Accumulation and Energy Metabolism in Humans

Diabetes 2015;64:1966–1975 | DOI: 10.2337/db14-0892

Studies in rodents suggest that insulin controls hepatic glucose metabolism through brain-liver crosstalk, but human studies using intranasal insulin to mimic central insulin delivery have provided conflicting results. In this randomized controlled crossover trial, we investigated the effects of intranasal insulin on hepatic insulin sensitivity (HIS) and energy metabolism in 10 patients with type 2 diabetes and 10 lean healthy participants (CON). Endogenous glucose production was monitored with [6,6-$^2H_2$]glucose, hepatocellular lipids (HCLs), ATP, and inorganic phosphate concentrations with $^1H/^{31}P$ magnetic resonance spectroscopy. Intranasal insulin transiently increased serum insulin levels followed by a gradual lowering of blood glucose in CON only. Fasting HIS index was not affected by intranasal insulin in CON and patients. HCLs decreased by 35% in CON only, whereas absolute hepatic ATP concentration increased by 18% after 3 h. A subgroup of CON received intravenous insulin to mimic the changes in serum insulin and blood glucose levels observed after intranasal insulin. This resulted in a 34% increase in HCLs without altering hepatic ATP concentrations. In conclusion, intranasal insulin does not affect HIS but rapidly improves hepatic energy metabolism in healthy humans, which is independent of peripheral insulinemia. These effects are blunted in patients with type 2 diabetes.

Evidence from rat models indicates that insulin signaling in the central nervous system (CNS) contributes to the regulation of hepatic glucose metabolism (1). Intracerebroventricular application of insulin has been shown to decrease endogenous glucose production (EGP) through activation of hypothalamic ATP-dependent potassium (K_{ATP}) channels (2). However, findings in dogs indicate that, in contrast to rodents, brain insulin action does not acutely regulate glucose production and gluconeogenesis in larger mammals (3).

Because EGP regulation differs among species (4) and its impairment is a metabolic hallmark of type 2 diabetes, examining whether brain insulin signaling also modulates rates of EGP in humans is important. Of note, activation of K_{ATP} channels with diazoxide may suppress EGP in healthy humans (5), supporting an insulin-mediated brain periphery crosstalk in humans. Intranasal insulin administration raises cerebrospinal fluid insulin levels (6) and could therefore mimic brain insulin delivery in humans. Most recently, two studies using intranasal insulin revealed conflicting results, reporting either a reduction of EGP (7) or no acute changes in glucose metabolism (8).

Intranasal insulin may also lower plasma free fatty acids (FFAs) and rates of labeled glycerol appearance (9), suggesting central insulin regulation of lipolysis in humans as demonstrated before in rodents (10). Intranasally administered insulin may further affect body weight and fat content (11). Because insulin resistance tightly associates with hepatic fat accumulation (12), examining whether central insulin may improve hepatic glucose and

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Received 6 June 2014 and accepted 7 January 2015.
Clinical trial reg. no. NCT01479075, clinicaltrials.gov.

lipid metabolism is of interest. So far, no studies have addressed the effects of intranasal insulin on hepatic energy metabolism in patients with insulin resistant type 2 diabetes compared with healthy individuals under physiological fasting conditions.

This randomized controlled crossover clinical study investigated intranasal insulin effects on hepatic insulin sensitivity (HIS), lipids, and energy homeostasis. To examine extreme states of normal and impaired glucose metabolism, we enrolled young healthy lean control subjects (CON) and elderly overweight patients with type 2 diabetes. In a subgroup of CON, we further studied the metabolic effects of an intravenous insulin injection mimicking the transient increase in serum insulin levels observed upon intranasal insulin application.

RESEARCH DESIGN AND METHODS

Participants

Ten insulin-naive patients with type 2 diabetes taking oral glucose-lowering medication were enrolled in this randomized controlled single-blind crossover single-center trial. Patients taking thiazolidinediones were excluded from the study because of the drug's possible long-term CNS effects (13). Patients withdrew their oral glucose-lowering medication for 3 days before the experiments to exclude possible effects on metabolic tests (14,15). Two patients with type 2 diabetes were taking atorvastatin and one gemfibrozil; neither patient was withdrawn from the study. Ten young lean and healthy volunteers not taking any medications, without a family history of diabetes, and with normal glucose tolerance based on a standard 75-g oral glucose tolerance test were also enrolled. Before inclusion, all participants gave written informed consent. This trial was approved by the ethics board of Heinrich Heine University Düsseldorf. The participants underwent screening, including medical history, clinical examination, and blood tests. None had clinical or laboratory signs of infection or hepatic, vascular, renal, or endocrine diseases. All participants were sedentary and refrained from any exercising for 3 days before the study. Female participants were either postmenopausal or examined between days 5 and 8 of their regular menstrual cycle.

Study Design

The participants arrived at 7:00 A.M. at the German Diabetes Center after a 10-h overnight fast and remained fasted until the end of the study day. Intravenous catheters were inserted in both antecubital veins for blood sampling and infusions. At time point −180 min, participants received a continuous infusion (0.036 mg · min^{-1} · kg body $weight^{-1}$) of D-[6,6-$^2H_2$]glucose (99% enriched in ^2H glucose; Cambridge Isotope Laboratories, Andover, MA) after a priming bolus of 3.6 mg · kg body $weight^{-1}$ · fasting plasma glucose [mg/dL]/90 [mg/dL] for 5 min (16). The tracer infusion lasted until 180 min in all participants and to study delayed insulin effects (17) for an extended period until 360 min in a subgroup of 12 participants (6 CON and 6 patients with type 2 diabetes). Before and during the infusions, blood samples were drawn to measure tracer enrichments, metabolites, and hormones. All participants were studied using identical protocols on 2 study days spaced by at least 7 days, except for the intranasal administration of insulin or placebo at time 0 (18). One puff of the 0.1-mL spray solution contained either 10 IU human insulin (100 IU/mL Actrapid; Novo Nordisk, Copenhagen, Denmark) or 0.1 mL vehicle as placebo. Eight puffs were administered in each nostril, resulting in a total dose of 160 IU insulin or 1.6 mL vehicle on the respective study days. The participants were blinded to the order of spray application. On a third day, a subgroup of eight CON received 0.1 IU human insulin (Actrapid) i.v. and otherwise underwent the identical study protocol.

$^1H/^{31}P$ Magnetic Resonance Spectroscopy

All measurements were performed in a 3-T magnetic resonance scanner (Achieva 3T; Philips Healthcare, Best, the Netherlands) using a 14-cm circular ^{31}P surface transmit-receive coil (Philips Healthcare) for ^{31}P magnetic resonance spectroscopy (MRS) and the built-in ^1H whole-body coil for localization and proton spectroscopy. Participants were scanned in the supine position at baseline and at 180 min, and the long-duration subgroup was scanned at 360 min. For the acquisition of ^{31}P spectra, a volume of interest of 6 × 6 × 6 cm^3 was positioned within the liver, and three-dimensional localized spectra were obtained using image-selected MRS (19) (time of repetition 4 s, number of signal averages 192, acquisition time 13 min, spectral width 3,000 Hz, data points 2,048). Absolute quantification of phosphorus metabolites (γ-ATP and Pi) was performed as previously described (20) using the AMARES (advanced method for accurate, robust, and efficient spectral fitting of MRS data) algorithm (21) in jMRUI (Java-based magnetic resonance user interface) software (22).

For assessment of liver fat content, a set of nonwater-suppressed and water-suppressed ^1H spectra were acquired using stimulated echo acquisition mode (repetition time/echo time/mixing time 4,000/10/16 ms, number of signal averages 32, volume of interest 3 × 3 × 2 cm^3). Data from localized ^1H-MRS were analyzed to assess fat content as previously described (23), and absolute concentrations were expressed as percent hepatocellular lipids (HCLs) relative to water content using the equations by Longo et al. (24). Concentrations of phosphorus metabolites were corrected for the volume captured by lipid droplets within hepatocytes (25). Reproducibility in acquisition and intra- and interobserver variability in spectral processing of ^{31}P-MRS was reported previously (20).

Metabolites and Hormones

Blood samples were immediately chilled and centrifuged, and supernatants were stored at −80°C until analysis. Venous blood glucose concentrations were measured by

a glucose oxidase method (EKF Biosen C-Line glucose analyzer; EKF Diagnostic GmbH, Barleben, Germany) (26). Serum triglycerides (TGs) were enzymatically analyzed on a Roche cobas c 311 analyzer (Roche Diagnostics, Mannheim, Germany). FFAs were quantified enzymatically (intra-assay coefficient of variation [CV] 1%, interassay CV 2.4%) (Wako, Neuss, Germany) in samples containing orlistat to prevent ex vivo lipolysis (27). Serum C-peptide, serum insulin, and plasma glucagon levels were measured by radioimmunoassay (intra-assay CV for all 4–6%; interassay CV 6–7, 5–9, and 5–10%, respectively) (Millipore, St. Charles, MS).

Gas Chromatography–Mass Spectrometry

After plasma deproteinization using $Ba(OH)_2$-$ZnSO_4$, atom percent enrichments (APEs) of ^2H were measured on a Hewlett-Packard 6890 gas chromatograph equipped with a 25-m CP-Sil 5 CB capillary column (inner diameter 0.2 mm, film thickness 0.12 μm; Chrompack/Varian, Middelburg, the Netherlands), interfaced with a Hewlett-Packard 5975 mass selective detector as previously described (28). APEs of the fragments C3–C6 with average mass units of 187 for endogenous glucose and 189 for [6,6-$^2H_2$]glucose were determined by using selected ion monitoring. APE was calculated as mass ratio corresponding to the tracer enrichment in plasma glucose. Intra- and interassay CVs were 0.6 and 1.0%, respectively.

Calculations

Rates of EGP were calculated by dividing the tracer ([6,6-$^2H_2$]glucose) infusion rate times the tracer enrichment by the tracer enrichment in plasma glucose and subtracting the tracer infusion rate (25). Fasting HIS was estimated as the HIS index by multiplying 100 times the inverse of the product of EGP and serum insulin (29). HOMA indices of fasting insulin resistance and β-cell function and QUICKI were calculated as previously described (30,31).

Statistical Analysis

Data are presented as mean ± SEM and were subjected to two-way ANOVA with repeated-measures factors time and treatment. Areas under concentration-time curves (AUCs) were calculated according to the trapezoidal rule and compared with two-sided paired and unpaired *t* tests for within- and between-group comparisons, respectively. Comparisons at baseline between CON and patients with type 2 diabetes were performed with the two-sided unpaired *t* test. $P < 0.05$ indicates significant differences.

RESULTS

Participant Characteristics

As expected, patients with type 2 diabetes were older and had greater BMI; waist circumference; and HbA_{1c}, fasting blood glucose, serum C-peptide, and cholesterol levels than CON (Table 1). Mean fasting insulin levels trended higher but were not significantly different from CON likely due to larger within-group variability in patients with type 2 diabetes. Indices of insulin resistance, insulin sensitivity, and β-cell function were different between groups, whereas fasting plasma TG and FFA levels were comparable. Within each group, no differences in baseline EGP, hormones, and metabolites among placebo, intranasal, and intravenous insulin studies were noted.

Table 1—Participant characteristics

Parameters	CON	Patients with type 2 diabetes
n (females)	10 (3)	10 (1)
Age (years)	25.7 ± 1.6	60.9 ± 2.0*
BMI (kg/m^2)	23.1 ± 0.9	29.0 ± 1.0*
Waist (cm)	78.2 ± 5.7	103.2 ± 3.0*
Plasma TGs (mg/dL)	61 ± 8	97 ± 19
Plasma FFAs (μmol/L)	418 ± 73	650 ± 92
Serum cholesterol (mg/dL)	147 ± 12	176 ± 13*
Blood glucose (mg/dL)	75 ± 2	175 ± 13*
Serum insulin (μU/mL)	4.4 ± 1.0	10.7 ± 2.8
Serum C-peptide (ng/mL)	1.1 ± 0.2	2.2 ± 0.3*
HbA_{1c} (%)	5.1 ± 0.1	7.5 ± 0.4*
HbA_{1c} (mmol/mol)	31 ± 1	58 ± 4*
Alanine aminotransferase (units/L)	21 ± 2	30 ± 4
Aspartate aminotransferase (units/L)	19 ± 1	21 ± 2
HOMA insulin resistance	0.8 ± 0.2	4.5 ± 1.2*
HOMA-β	148 ± 38	38.3 ± 10.0*
QUICKI	0.41 ± 0.01	0.32 ± 0.01*
Hepatic insulin resistance	329 ± 59	1,840 ± 468*
Liver fat (HCL %)	0.5 ± 0.1	7.7 ± 1.6*

Data are mean value ± SEM unless otherwise indicated. *$P <$ 0.05 vs. CON.

Effects of Intranasal Insulin on Circulating Metabolites and Hormones

After intranasal insulin application, there was a trend for a decrease in blood glucose only in CON ($P = 0.060_{ANOVA}$ treatment × time), with a maximal decrease of ~5% at 30–40 min compared with placebo (Fig. 1*A* and *B*). Likewise, the $AUC_{0–180\ min}$ for glucose was lower after insulin application only in CON (Fig. 2*A*). Similar differences were noted at 40 min after insulin in the subgroups studied for 360 min.

Intranasal insulin resulted in lower FFA concentrations in CON ($P < 0.01_{ANOVA}$ treatment × time) (Fig. 1*I*) and a greater maximal decrease (138 ± 41 μmol/L, 33.3 ± 8.5%) at 60 min versus baseline than after placebo administration (23.4 ± 27.3 μmol/L, −11.7 ± 9.7%). No changes were observed in patients with type 2 diabetes ($P = 0.456_{ANOVA}$ time × treatment) (Fig. 1*J*). The $AUC_{0–180\ min}$ and $AUC_{0–60\ min}$ for FFA were not different between experimental conditions in both groups (Fig. 2*B*). TG and glycerol levels remained unchanged,

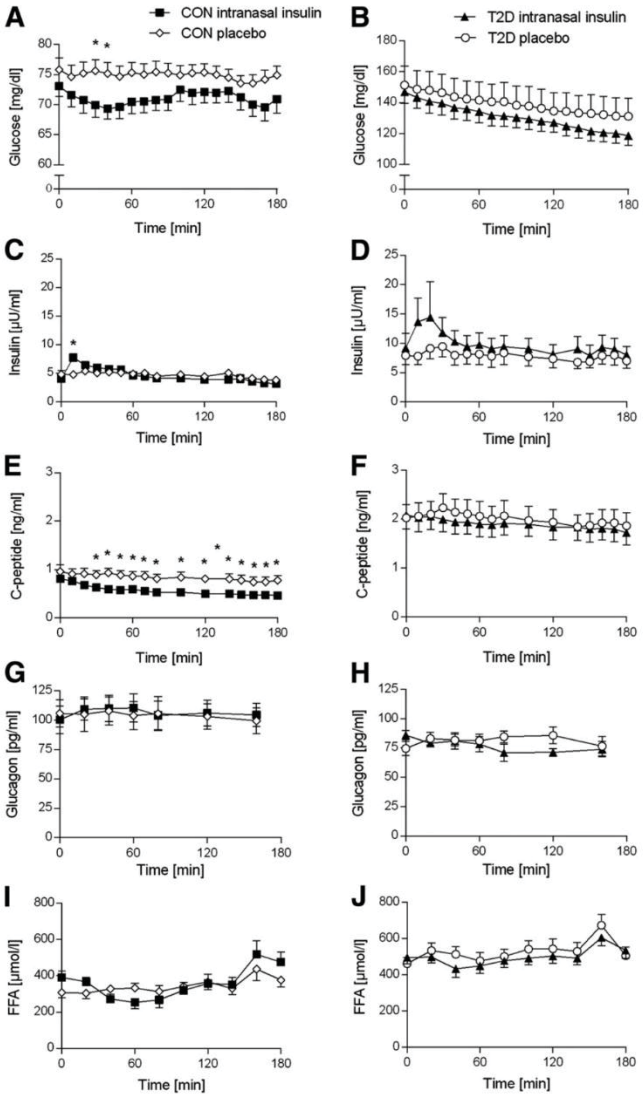

Figure 1—Time course of blood glucose (*A* and *B*), serum insulin (*C* and *D*), C-peptide (*E* and *F*), glucagon (*G* and *H*), and FFA (*I* and *J*). Intranasal insulin/placebo were applied at time point 0 min. Data are mean ± SEM; CON (n = 10), patients with type 2 diabetes (T2D) (n = 10). *P < 0.05 insulin vs. placebo.

and their respective $AUC_{0-180\ min}$ were similar after both interventions in CON and patients with type 2 diabetes (Fig. 2*C* and *D*).

In CON, serum insulin levels transiently increased by 3.7 ± 0.7 µU/mL and nearly doubled (92%) at 10 min after insulin versus placebo ($P < 0.001_{ANOVA}$ time × treatment) (Fig. 1*C*). In patients with type 2 diabetes, the trend toward an increase in serum insulin by 59% at 10 min was not significant (4.9 ± 2.1 µU/mL, $P = 0.3_{ANOVA}$ time × treatment) (Fig. 1*D*). In subgroups studied for 360 min after intranasal insulin/placebo, similar changes in the time course were found at 10 min in CON

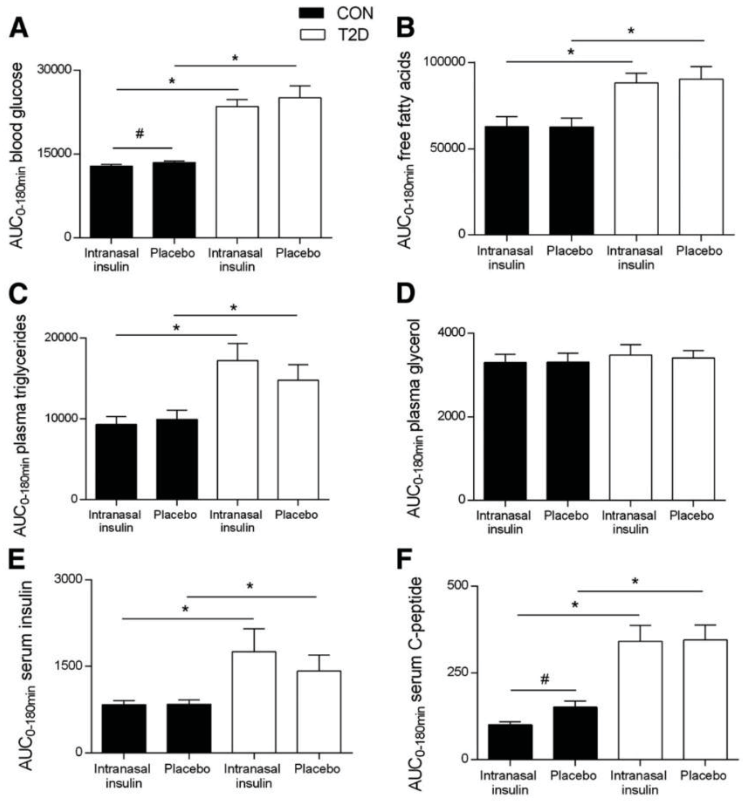

Figure 2—Comparison of $AUC_{0–180\ min}$ for metabolic parameters. *A*: Blood glucose. *B*: Plasma FFAs. *C*: Plasma TGs. *D*: Plasma glycerol. *E*: Serum insulin. *F*: Serum C-peptide. Data are mean ± SEM; CON (n = 10), patients with type 2 diabetes (T2D) (n = 10). *P < 0.05 CON vs. T2D; #P < 0.05 CON insulin vs. placebo.

and at 20 min in patients with type 2 diabetes. Overall, $AUC_{0–180\ min}$ for insulin was comparable after insulin versus placebo administration in both groups (Fig. 2*E*). After intranasal insulin, serum C-peptide levels decreased only in CON and remained lower compared with placebo from 30 to 180 min ($P < 0.01_{ANOVA}$ time × treatment) (Fig. 1*E* and *F*). In CON, $AUC_{0–180\ min}$ for C-peptide was lower after intranasal insulin versus placebo but not in patients with type 2 diabetes (Fig. 2*F*). Lower C-peptide levels were also found at 40, 100, 120, and 140 min after insulin in the subgroup of CON studied for 360 min but not in patients with type 2 diabetes. Neither intranasal insulin nor placebo affected glucagon concentrations in CON and patients with type 2 diabetes (Fig. 1*G* and *H*).

Effects of Intranasal Insulin on EGP and HIS

Baseline EGP was similar under insulin and placebo conditions in both groups (P = 0.93 CON insulin vs. placebo, P = 0.98 patients with type 2 diabetes insulin vs. placebo). Neither insulin nor placebo application affected rates of EGP, which remained comparable over 180 min and 360 min. Between-group analysis revealed higher HIS index values in CON than in patients with type 2 diabetes at baseline. In CON, the HIS increased by 4.0 ± 1.1 and 3.7 ± 1.4 kg · min · dL/(mg · μU) after both insulin and placebo applications, respectively ($P < 0.05$ at 180 min vs. baseline for insulin and placebo) but was not altered in patients with type 2 diabetes (Fig. 3*A* and *B*).

Effect of Intranasal Insulin on Hepatic Lipid and Energy Metabolism

At baseline, HCL was lower in CON and did not differ between the experimental conditions (Table 1). In CON, HCL decreased by 35% at 180 min only after insulin (P = 0.04 at 180 min vs. baseline) but not after placebo. In patients with type 2 diabetes, HCL did not change under either experimental condition (Fig. 4*A* and *B*).

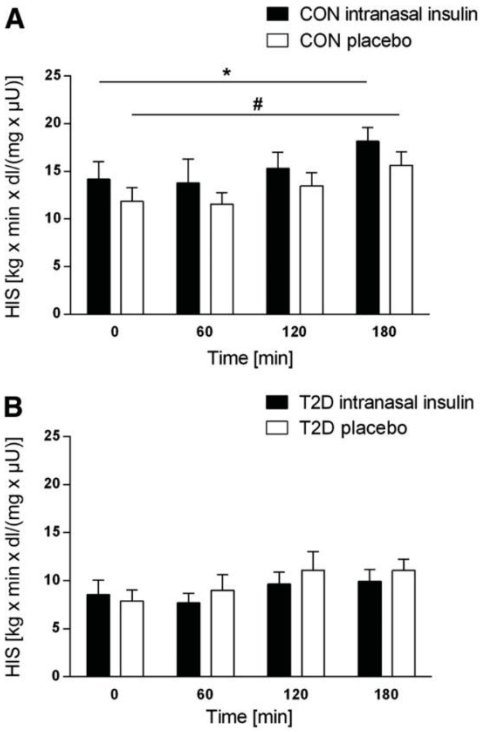

Figure 3—HIS index in CON (*A*) and patients with type 2 diabetes (T2D) (*B*) after intranasal insulin/placebo (given at time 0). Data are mean ± SEM; CON (n = 10), T2D (n = 10); HIS index = 100/(EGP × insulin). *P < 0.05 CON intranasal insulin 180 min vs. baseline; #P < 0.05 CON placebo 180 min vs. baseline.

Baseline hepatic ATP and Pi concentrations were not different between conditions in CON and patients with type 2 diabetes. In CON, ATP increased at 180 min after insulin by 18% (0.5 ± 0.2 mmol/L, P = 0.03 vs. baseline) but was 23% lower in patients with type 2 diabetes at 180 min after intranasal insulin than after placebo (2.8 ± 0.2 and 3.4 ± 0.2 mmol/L, respectively; P = 0.03 vs. placebo) (Fig. 4*C* and *D*). Intranasal insulin did not affect hepatic Pi in either group, whereas it increased only in patients with type 2 diabetes by 0.4 ± 0.1 mmol/L after placebo administration (P = 0.02 at 180 min vs. baseline).

Effects of Intravenous Insulin on Metabolic Parameters

Insulinemia was comparable between intravenous and intranasal insulin studies as demonstrated by similar $AUC_{0-20\ min}$ (Fig. 5*A*), and a trend for higher insulin levels under both conditions compared with placebo was observed (P = 0.110 intranasal insulin vs. placebo, P = 0.088 intravenous insulin vs. placebo). Additionally, the absolute maximal decrease in blood glucose (Fig. 5*B*) and plasma FFA levels was comparable between intravenous and intranasal insulin studies and similarly differed from placebo (P = 0.02 Δmax glucose intravenous insulin vs. placebo, P < 0.0001 Δmax FFA intravenous insulin vs. placebo). No differences were observed in EGP after intravenous insulin administration. The relative percent increase in HCL was 34% (P = 0.02 baseline vs. 180 min) (Fig. 5*C*), whereas hepatic ATP concentrations remained unaltered (Fig. 5*D*).

DISCUSSION

This study shows that intranasal insulin administration does not affect glucose production over up to 6 h in humans with or without type 2 diabetes. However, intranasal insulin resulted in increased absolute hepatic ATP and decreased hepatic TG concentrations in glucose-tolerant CON but not in patients with type 2 diabetes. This was observed in the presence of a transient increase in serum insulin along with a minor transient decline in circulating glucose and FFA levels. Intravenous application of insulin unexpectedly led to comparable changes in circulating insulin, glucose, and FFA and did not affect hepatic ATP but increased hepatic TG concentrations.

In CON, blood glucose levels transiently and slightly decreased after intranasal insulin administration. Comparable insulin doses have been reported to lead to significant changes in blood glucose levels in some (32,33) but not all previous studies (18,34). Of note, the fall in blood glucose levels was preceded by a temporary rise in serum insulin concentration along with a prolonged reduction of C-peptide levels. Again, this increase in serum insulin is consistent with some (32–34) but not all previous reports (18). The absence of detection of these ultra-short-term changes in circulating insulin and glucose concentrations is likely due to a lower frequency of measurements in previous studies. Even the current study with its 10-min blood sampling interval might still underestimate the possible maximal changes in circulating insulin and subsequent peripheral metabolites and hormones. Nevertheless, short-term doubling of peripheral insulin levels followed by lower levels and $AUC_{0-180\ min}$ for glucose and C-peptide indicates that some amounts of intranasal insulin can be absorbed and suppress endogenous insulin secretion. However, the sustained reduction of C-peptide in CON after application of single-dose intranasal insulin suggests the operation of other mechanisms inhibiting insulin secretion, such as neural mechanisms initiated by cerebral insulin, lower FFA levels, and improved insulin action. The transient decrease in glucose concentration in CON could be due to greater glucose disposal and/or HIS (35). In elderly patients with type 2 diabetes, the response of peripheral insulin levels to intranasal insulin varied considerably, which might be a result of altered intranasal mucosal function. This as well as the impaired β-cell function and ambient hyperglycemia likely contributed to the absence of changes in the measured metabolites and hormones.

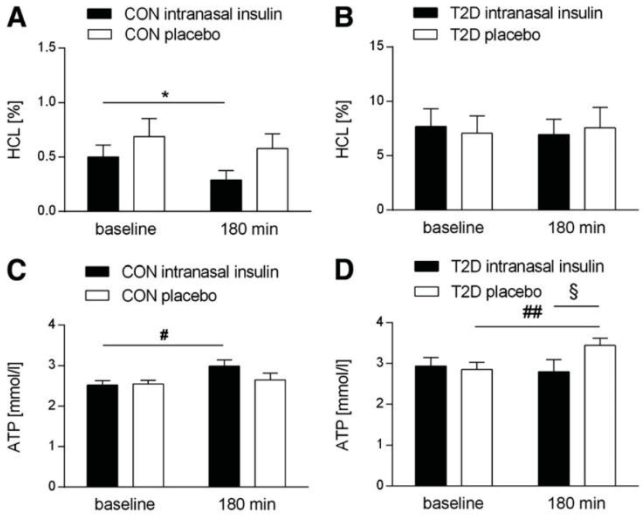

Figure 4—Absolute changes in liver fat content (HCL %) and hepatic ATP in CON and patients with type 2 diabetes (T2D) after intranasal insulin/placebo. *A* and *B*: Liver fat content. *C* and *D*: ATP concentrations. Data are mean ± SEM; CON (n = 10), T2D (n = 10). *P < 0.05 CON intranasal insulin after 180 min vs. baseline; #P < 0.05 CON intranasal insulin after 180 min vs. baseline; ##P < 0.05 T2D placebo after 180 min vs. baseline; §P < 0.05 T2D after 180 min intranasal insulin vs. placebo.

We assessed EGP in the fasting physiological state and related it to ambient insulinemia to provide a direct measure of fasting HIS (29), which takes into account the sensitivity of the liver to small changes in serum insulin levels (36). This approach, therefore, reflects physiological basal conditions of overnight fasting, whereas the hyperinsulinemic-euglycemic clamp creates a state of continuous submaximal stimulation by insulin. The finding of

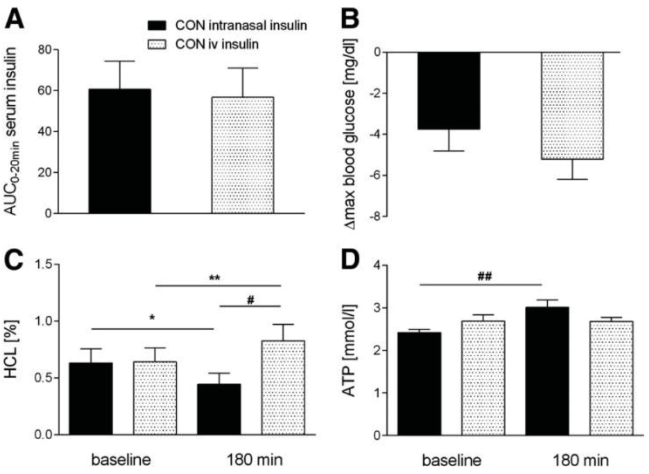

Figure 5—Absolute change in $AUC_{0-20\ min}$ for serum insulin (*A*), maximal decrease in blood glucose (*B*) (Δmax = nadir value − baseline), absolute change in HCLs (%) (*C*), and hepatic ATP concentrations (*D*) after intranasal insulin and intravenous (iv) insulin application in CON subgroup. Data are mean ± SEM; CON (n = 8). *P < 0.05 nasal insulin baseline vs. 3 h; **P < 0.05 iv insulin baseline vs. 180 min; #P < 0.01 180 min intranasal insulin vs. iv insulin; ##P < 0.05 intranasal insulin baseline vs. 180 min.

comparable EGP and HIS after intranasal insulin contributes human data to the ongoing discussion on effects of brain insulin on peripheral, mainly hepatocyte and adipocyte metabolism observed in mice (1,10) but not in dogs (37). Differences in the experimental settings may explain variable results obtained in other species. In mouse models, effects of brain insulin on EGP were demonstrated under hyperinsulinemic clamp conditions during peripheral and CNS insulin infusion (1,2). Additionally, stimulation of hypothalamic insulin signaling was initiated in advance and continued throughout the clamps. Moreover, the nonphysiological high-dose cerebrospinal fluid insulin infusion, hepatic hypoinsulinemia, and hypoglucagonemia in rodents could be the reason for the observed suppression of EGP. When brain and portal hormone levels were adequately adjusted to correspond to physiological ratios of glucoregulatory hormones, no changes in EGP were detected (37). Nevertheless, brain hyperinsulinemia decreased glycogen synthase phosphorylation in dogs (37), which may partly explain the increase in hepatic ATP in the current study.

Although central regulation of glucose metabolism by insulin in humans cannot be excluded, HIS is predominantly modulated by direct insulin action (38). Furthermore, we detected no changes in EGP for up to 6 h after nasal insulin application, suggesting that brain insulin signaling is not primarily responsible for acute effects on liver glucose metabolism. On the other hand, elegant experiments in humans demonstrated suppression of hepatic glucose output appearing 6 h after diazoxide consumption (5), an intervention that activates hypothalamic K_{ATP} channels, thereby mimicking brain insulin action. Most recently, intranasal application of insulin lispro also decreased EGP at 3–6 h, again during pancreatic clamps (7). These studies point to interesting effects of central insulin signaling observed in the somatostatin-mediated absence of physiological endocrine counterregulation. In addition, not only diazoxide but also several nutritional and hormonal signals can activate hypothalamic channels (17), and insulin lispro seems to act more potently in the brain than human insulin (39).

We also found no effect of insulin applied through the intranasal route on HIS. This seems to differ from recent findings suggesting that intranasal insulin might modulate insulin sensitivity of glucose metabolism through brain regions known to affect the regulation of the autonomous nervous system outflow (33). Nevertheless, the current study primarily monitored fasting glucose production but not peripheral insulin sensitivity during hyperinsulinemic clamps. The observed increase in the HIS index during both placebo and insulin exposure in CON only probably relates to a decline in serum insulin levels resulting from a prolonged fasting state after placebo administration and a compensatory reduction of insulin secretion at 3 h after the preceding insulin peak after intranasal insulin administration.

Of note, the current study demonstrated that HCLs transiently decrease upon intranasal insulin exclusively in CON. These findings suggest novel peripheral metabolic effects of intranasal insulin but require scrutiny and careful interpretation because both brain insulin signaling and increased serum insulin could be responsible. To address this question, we applied insulin intravenously to match the spillover of intranasal insulin in the peripheral circulation in a subgroup of CON. Of note, intravenous insulin application resulted in increased HCLs at 180 min, which is in line with the known effect of insulin on lipogenesis and the observed increase in HCLs by short-term insulin infusion (14). Thus, the contrasting results observed with intranasal and intravenous insulin administration under otherwise matched metabolic conditions suggest the operation of a central mechanism of insulin action for lowering liver fat content. Of note, CON exhibited very low baseline HCL levels (0.5 ± 0.1%), and the slight decrease in HCLs observed at 180 min disappeared at 360 min. However, a previous study reported catabolic effects of long-term intranasal insulin administration in men (11), which could also reduce liver fat storage. Moreover, the contrasting increase in HCLs observed with intravenous insulin rather suggests that the intranasal route exerts unique HCL-lowering effects. The moderate reduction of FFA levels after intranasal insulin in CON is likely a result of the known direct insulin effect on adipose tissue lipolysis, which is usually blunted in patients with insulin resistant type 2 diabetes. Of note, previous studies described diminished lipolysis and enhanced lipogenesis by brain insulin signaling in rodents (10) and by intranasal insulin in humans (9). Thus, it cannot be excluded that reduced adipose tissue lipolysis contributed to the reduction of HCLs in CON. On the other hand, the lack of any effect of intranasal insulin on HCLs in the current patients with type 2 diabetes could result from not only alterations of brain insulin signaling and central insulin resistance in obesity and type 2 diabetes (40,41) but also other factors such as age, sex, visceral fat distribution, and adipose tissue insulin resistance. Of note, lipid-lowering medication in 3 of 10 patients with type 2 diabetes was not withdrawn before testing, which limits the interpretation of possible effects on lipogenesis in the current study.

Another novel finding of the current study was the rise in hepatic ATP after intranasal insulin administration to CON but not to patients with type 2 diabetes. The intravenous insulin administration failed to affect hepatic ATP, again suggesting a central effect of insulin independent of peripheral insulinemia. Reduced mitochondrial activity has been described in insulin resistant skeletal muscle and liver and plays an important role in the development of nonalcoholic fatty liver disease (25,42). In skeletal muscle, insulin can increase flux through ATP synthase in healthy humans but not in patients with type 2 diabetes (43), indicating impaired mitochondrial plasticity in insulin resistant states (42). We recently reported that

lower muscle ATP synthase flux associates with higher HCLs in humans (44). Impaired mitochondrial plasticity, therefore, could have also been present in the liver of the current patients with type 2 diabetes. However, we cannot exclude that brain insulin signaling contributes to the regulation of liver energy homeostasis because intranasal insulin can enhance energy levels in brain (45). It remains unclear whether the diminished response to intranasal insulin in the current patients with type 2 diabetes is due to participants' age, insulin resistance, or being overweight, but evidence from human studies suggests that peripheral insulin sensitivity and body weight are important factors for adequate insulin action in the brain (41,46).

This study benefits from the close monitoring of key metabolites and EGP under physiological fasting conditions as well as from use of localized measures of intrahepatic energy metabolism independent of EGP for the first time. In addition, this study confirmed the spillover of intranasal insulin into the systemic circulation (32,33). The established dose of 160 IU insulin administered intranasally in previous studies has been clearly shown to influence CNS functions (33,34,47). Because the spillover of insulin might limit the use of this approach when assessing peripheral metabolism, we established a protocol for intravenous insulin application to match the effects of systemic spillover on circulating insulin and metabolites. Nevertheless, this approach does not allow detection of the intracerebral mechanisms leading to the observed hepatic effects. Finally, the current study cannot discriminate whether the different response of the patients with type 2 diabetes to intranasal insulin was due to other factors than insulin resistance known to influence brain insulin action, such as age, sex, or obesity.

In conclusion, intranasal insulin application does not affect fasting HIS but can stimulate hepatic energy metabolism and reduce lipid storage in healthy humans. The changes proved to be independent of concurrent transient increases in serum insulin levels and unique for the intranasal route of administration. Intranasal insulin effects are blunted in patients with type 2 diabetes, which may result from lower ambient insulinemia and/or impairment of the indirect effects of insulin on peripheral metabolism.

Acknowledgments. The authors thank Andrea Nagel, Nicole Achterath, Kai Tinnes, and Myrko Esser (Institute for Clinical Diabetology, German Diabetes Center, Düsseldorf, Germany) for excellent help with the experiments.

Funding. This work was supported by the Ministry of Science and Research of the State of North Rhine-Westphalia (MIWF NRW) and the German Federal Ministry of Health (BMG). This study was supported in part by a grant of the Federal Ministry for Research (BMBF) to the German Center for Diabetes Research (DZD), by a grant of the Helmholtz Alliance Imaging and Curing Environmental Metabolic Diseases (ICEMED), and by the Schmutzler-Stiftung.

The funding sources had no input in the design and conduct of this study; in the collection, analysis, and interpretation of the data; or in the preparation, review, or approval of the article.

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

Author Contributions. S.G. contributed to the research and collection of data and writing of the manuscript. C.K. and A.B. contributed to the data collection and manuscript. P.N. contributed to the data collection. M.H., A.F., and H.-U.H. contributed to the manuscript. J.S. and M.R. contributed to the study design, research of data, and manuscript. M.R. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Prior Presentation. Parts of this study were presented in abstract form at the 74th Scientific Sessions of the American Diabetes Association, San Francisco, CA, 13–17 June 2014.

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Hypothalamic and Striatal Insulin Action Suppresses Endogenous Glucose Production and May Stimulate Glucose Uptake During Hyperinsulinemia in Lean but Not in Overweight Men

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Diabetes 2017;66:1797–1806 | https://doi.org/10.2337/db16-1380

Intranasal spray application facilitates insulin delivery to the human brain. Although brain insulin modulates peripheral metabolism, the mechanisms involved remain elusive. Twenty-one men underwent two hyperinsulinemic-euglycemic clamps with D-[6,6-$^2H_2$]glucose infusion to measure endogenous glucose production and glucose disappearance. On two separate days, participants received intranasal insulin or placebo. Insulin spillover into circulation after intranasal insulin application was mimicked by an intravenous insulin bolus on placebo day. On a different day, brain insulin sensitivity was assessed by functional MRI. Glucose infusion rates (GIRs) had to be increased more after nasal insulin than after placebo to maintain euglycemia in lean but not in overweight people. The increase in GIRs was associated with regional brain insulin action in hypothalamus and striatum. Suppression of endogenous glucose production by circulating insulin was more pronounced after administration of nasal insulin than after placebo. Furthermore, glucose uptake into tissue tended to be higher after nasal insulin application. No such effects were detected in overweight participants. By increasing insulin-mediated suppression of endogenous glucose production and stimulating peripheral glucose uptake, brain insulin may improve glucose metabolism during systemic hyperinsulinemia. Obese people appear to lack these mechanisms. Therefore, brain insulin resistance in obesity may have unfavorable consequences for whole-body glucose homeostasis.

Over the past years, the human brain has been variously identified as an insulin-sensitive organ (1). Whereas insulin influences activity in specific brain areas in some individuals, others experience attenuated or even absent responses, with the result that they are considered to be brain insulin resistant (1,2). This phenomenon was first observed in overweight people, who appeared to be resistant to brain insulin action not only in terms of regional brain activity but also with regard to many functional consequences: whereas insulin in the brain influences food intake and body weight (1,3) in lean people, no such effects have been observed in overweight and obese individuals (1,4). However, insulin resistance of the brain does not appear to

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Received 9 November 2016 and accepted 31 January 2017.
Clinical trial reg. no. NCT02468999, clinicaltrials.gov.
This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db16-1380/-/DC1.

negatively affect all brain functions in overweight subjects since the hormone improves memory consolidation regardless of body weight (4). This might be due to the fact that insulin resistance differentially affects certain brain areas (5).

Many of the more recent studies in this field used intranasal insulin administration to induce brain insulin action in humans. Shortly after nasal application, insulin can be found in the cerebrospinal fluid, where it can be detected for a considerable period of time (6). It probably reaches the brain equally quickly, since effects of nasal insulin have been detected as early as 15 min after spray administration (5). In line with this, research in animals suggests rapid uptake of insulin from the nasal cavity into the brain by mechanisms different from insulin transport at the blood-brain barrier (7).

Animal studies show that insulin action in the brain regulates peripheral metabolism (8). In rodents, brain insulin suppresses hepatic glucose production (9–12). Most (but not all) (11) of these experiments were conducted under systemic hypoinsulinemia. Furthermore, some studies in rodents reported brain insulin action to inhibit lipolysis in adipose tissue (13,14) and to stimulate glucose uptake into skeletal muscle (9,15), and especially the effect on muscle was not seen in all studies (10,11). Furthermore, not all of these findings could be replicated in dogs (16,17). Nonetheless, experimental evidence from animals suggests that the brain orchestrates insulin action in various organs throughout the body to regulate energy fluxes and whole-body metabolism (8).

A number of studies followed up these animal data on the brain's role for whole-body metabolism in humans by combining nasal insulin administration with assessment of peripheral glucose regulation (18–22). While under physiological circumstances brain insulin action can be stimulated only when insulin enters the brain via the bloodstream, i.e., during systemic hyperinsulinemia, some of these studies were conducted under fasting systemic insulin levels (18,21,22). The first of these studies estimated peripheral insulin sensitivity from fasting insulin and glucose levels (18). The results suggested that nasal administration of insulin might indeed improve peripheral insulin sensitivity in humans due to the influence on specific brain areas (18). A further study showed that intranasal application of the insulin analog lispro suppresses endogenous glucose production, albeit only after a substantial delay of approximately 3 h (21). A third study demonstrated an increase in hepatic adenosine triphosphate concentrations and concomitant reduction in liver fat content (22). The latter two studies aimed to experimentally mimic the spillover of small amounts of the nasal insulin into the circulation by administering insulin intravenously coordinated with placebo spray application (21,22).

So far, we are the only group to have investigated the effects of nasal insulin application under systemic hyperinsulinemia (19). After intranasal insulin, higher glucose infusion rates (GIRs) were necessary to maintain euglycemia during a hyperinsulinemic-euglycemic glucose clamp. This is suggestive of improved insulin sensitivity. This response was limited to lean men; insulin sensitivity did not improve in overweight men (19). However, without using a tracer dilution technique to measure endogenous glucose production and glucose disposal to peripheral tissues, the mechanism responsible for the increase in GIR in the previous study remained obscure. Furthermore, one drawback of nasal insulin application was not experimentally taken into account in this study: small amounts of nasally administered insulin are absorbed into the bloodstream (1). This spillover of nasal insulin into the circulation can be measured shortly after insulin spray administration of larger doses of the peptide (21,22). However, the exact kinetics of this phenomenon have not yet been systematically addressed in humans. Although this spillover probably does not present a major obstacle when studying the nasal insulin effects on the brain itself, it might nevertheless interfere in studies that focus on peripheral metabolism.

Methodological differences and difficulties in study design therefore caused controversies over the interpretation of previous results regarding the role of brain insulin action in whole-body metabolism (1,17,20,23,24). To clarify these issues, we now conducted a placebo- and spillover-controlled randomized study to address the importance of brain insulin action for peripheral glucose metabolism in different tissues under systemic hyperinsulinemia.

RESEARCH DESIGN AND METHODS

Participants

We studied 21 healthy volunteers. The initial intention was to study 20 subjects (10 with BMI <25 kg/m^2 and 10 with BMI >25 kg/m^2); however, since one lean participant showed up only for one clamp experiment, we recruited an additional lean subject. Clinical characteristics are presented in Table 1. All participants underwent a screening visit with medical history, clinical examination, and blood tests to ensure that they were healthy. Written informed consent was provided, and the local ethics committee approved the protocol.

Table 1—Clinical characteristics

	Lean (BMI <25 kg/m^2)	Overweight (BMI >25 kg/m^2)	*P*
Age (years)	26.4 ± 3.4	26.6 ± 2.9	0.9
BMI (kg/m^2)	23.3 ± 1.8	28.3 ± 4.6	0.0050
Body fat content (%)	17 ± 4	23 ± 4	0.0041
Fasting glucose (mmol/L)	4.7 ± 0.4	5.2 ± 0.5	0.0246
Fasting insulin (pmol/L)	58 ± 21	77 ± 34	0.2
HbA_{1c} (%)	5.1 ± 0.4	5.3 ± 0.5	0.4

Data are given as mean ± SD. HbA_{1c}, hemoglobin A_{1c}.

Hyperinsulinemic-Euglycemic Clamp

A summary of the experiments is shown in Supplementary Fig. 1. Experiments commenced at 7:00 A.M. after overnight fast. Participants underwent two hyperinsulinemic-euglycemic clamp experiments in randomized order. A cannula was placed into the dorsal hand vein for blood sampling. The arm was warmed to facilitate arterialized blood sampling. Another cannula was placed into the contralateral antecubital vein for infusions. A primed-continuous intravenous infusion of D-[6,6-$^2H_2$]glucose (98% enriched; Cambridge Isotope Laboratories) with 0.036 mg $\times$ min^{-1} $\times$ kg^{-1} was administered 2 h before initiation of insulin infusion (22). At -90 min, an intravenous insulin bolus of 6.25 mU $\times$ kg^{-1} was administered, after which insulin was infused intravenously at 0.25 mU $\times$ kg^{-1} $\times$ min^{-1} for the rest of the experiment. Nasal spray was administered 1.5 h after initiation of insulin infusion (time point 0 min). On one day, subjects received 160 units of insulin (eight puffs in each nostril over 4 min, 10 units per puff) and vehicle as placebo on the other day, as in our previous study (5). On the placebo day, insulin infusion was increased by 0.17 mU $\times$ kg^{-1} $\times$ min^{-1} for 15 min after the first placebo spray puff (resulting in an intravenous insulin bolus of 2.5 mU $\times$ kg^{-1} over 15 min). The participants were not informed as to whether they had received insulin or placebo spray.

During the experiment, blood samples was taken every 5 min to measure blood glucose, and the GIR of 20% dextrose (2% enriched with D-[6,6-$^2H_2$]glucose) was adjusted to maintain euglycemia with a target glucose of 5 mmol/L. Additional blood samples were taken to determine tracer enrichment, metabolites, and hormones.

In 4 of the 41 hyperinsulinemic-euglycemic clamp experiments, steady GIR could not be reached in the designated time before spray administration (coefficient of variation in GIR for the 20 min before spray >15%). We therefore excluded these experiments from further analyses.

Analytic Procedures

Blood glucose was measured by the glucose oxidase method (EKF Diagnostic, Barleben, Germany). Insulin, C-peptide, and prolactin concentrations were determined by chemiluminescence assays (ADVIA Centaur XPT; Siemens, Eschborn, Germany). On insulin day, the prolactin concentration was more than two SD above the mean in one lean participant. This measurement was therefore excluded from the analysis involving prolactin. Plasma concentrations of nonesterified fatty acid were determined using an enzymatic (acyl-coA synthetase, acyl-coA oxidase) colorimetric method (Wako Chemicals, Neuss, Germany) adapted on the ADVIA 1800 XPT clinical chemistry analyzer. Glucagon was determined as described previously (25).

Gas Chromatography–Mass Spectrometry

The determination of atom percent enrichment of ^2H in blood glucose was performed after deproteinization using $Ba(OH)_2$-$ZnSO_4$. Measurements were performed on a Hewlett-Packard 6890 gas chromatograph equipped with a 25-m CPSil5CB capillary column (0.2 mm inner diameter, 70.12 μm film thickness; Chrompack/Varian, Middelburg, the Netherlands), interfaced to a Hewlett Packard 5975 mass selective detector. Selected ion monitoring was used to determine enrichments of the fragments C3 to C6, with the average mass units 187 for the endogenous glucose and 189 for the D-[6,6-$^2H_2$]glucose. Intra- and interassay coefficients of variation were 0.6 and 1.0%. Tracer enrichment data are presented in Supplementary Fig. 6.

Functional MRI

On a separate day, participants underwent a pulsed arterial spin labeling measurement to determine cerebral blood flow (CBF). After the first measurement, 160 units of nasal insulin was applied. A second measurement was performed 30 min after administration of the spray.

Functional MRI Data Acquisition

Scanning was performed on a 3T scanner with a 12-channel trans-receiver head coil (Magnetom Prisma; Siemens). Pulsed arterial spin labeling images were obtained with a PICORE-Q2TIPS (proximal inversion with control for off-resonance effects–quantitative imaging of perfusion by using a single subtraction) sequence. Each measurement consisted of 78 alternating tag and control images with the following imaging parameters: inversion time (TI), TI1 = 700 ms, TI2 = 1,800 ms, repetition time (TR) = 3,000 ms, echo time (TE) = 13 ms, inplane resolution = 3 $\times$ 3 mm^2, field of view = 192 mm, matrix size = 64 $\times$ 64, and flip angle = 90°. The same sequence was used to estimate the equilibrium magnetization of the blood (M0B) for absolute CBF quantification. In addition, a high-resolution T1-weighted anatomical image was acquired.

Functional MRI Data Processing

Image preprocessing was performed with the ASLtbx (26) with SPM8 (Wellcome Trust Centre for Neuroimaging). As previously reported (5), we used the general kinetic model for absolute perfusion quantification. Perfusion images were generated by calculating the control-tag differences by surround subtraction. We determined the perfusion on each voxel with an equilibrium magnetization (M0) map to obtain accurate CBF quantification (mL $\times$ 100 g^{-1} $\times$ min^{-1}). Functional images were coregistered to the individual anatomical image and smoothed (full width at half maximum: 6 mm). A brain mask was used to exclude extracranial voxels in the normalized CBF images. Baseline-corrected CBF maps were computed to quantify the CBF change 30 min after intranasal insulin administration. Change in CBF was extracted from the hypothalamic region of interest based on our recent finding (5). Multiple regression analyses were performed to evaluate the relationship between the increase in GIR and hypothalamic brain insulin action. Furthermore, multiple regression analysis was performed

on a whole-brain level evaluating the relationship between increase in GIR post–insulin spray and change in CBF after intranasal insulin.

Calculations

Two periods of time were prespecified for analysis of spray-induced changes in both GIR and tracer enrichment (from 20 to 0 min before spray administration to 100–120 min and 190–210 min postspray; clinicaltrials.gov NCT02468999). These changes were calculated by dividing the average value of the latter period by the average value of the former period. The result was then multiplied by 100 and is therefore expressed as a change in percent.

Rates of endogenous glucose production were determined from the tracer infusion rate of D-[6,6-$^2H_2$]glucose and its enrichment to the hydrogens bound to carbon 6 divided by the mean percent enrichment of plasma D-[6,6-$^2H_2$]glucose. Steel's single-pool steady-state equations were used to calculate insulin-stimulated glucose rate of disappearance (27).

Statistical Analyses

Pairwise two-tailed Student *t* tests were used to compare conditions. Correlations and adjustments were addressed by multiple linear regression analyses. *P* values ≤ 0.1 were considered as a trend and <0.05 were considered to be significant. The statistical software package JMP (SAS Institute, Cary, NC) was used for statistical analysis.

RESULTS

Hyperinsulinemic-Euglycemic Clamps in the Whole Group

In the whole cohort, onset of the clamp with intravenous insulin infusion resulted in comparable plasma insulin levels on both study days in the first designated time period for analysis, i.e., in the 20 min before spray administration (insulin day: 198 ± 39 pmol/L, placebo day: 204 ± 51 pmol/L, $P = 1.0$). During this period, comparable glucose levels were reached (insulin day: 5.1 ± 0.4 mmol/L, placebo day: 4.9 ± 0.2 mmol/L, $P = 0.08$) by comparable GIRs (insulin day: 2.8 ± 1.2 mg × kg^{-1} × min^{-1}, placebo day: 3.2 ± 1.3 mg × kg^{-1} × min^{-1}, $P = 0.2$).

After spray administration, plasma insulin levels were determined in 5-min intervals for 30 min. After nasal insulin administration, plasma insulin levels increased and peaked 15 min postspray (mean increase 51 ± 53 pmol/L), returning to baseline afterward (Fig. 1*B*). On placebo spray day, this was mimicked by infusion of an intravenous insulin bolus over 15 min. The insulin course over the 30 min after spray administration was comparable between study days ($P_{AUC0-30} = 0.6$) (Fig. 1*B*). For the rest of the experiment, insulin levels between study days were comparable (Fig. 1*B*).

However, to maintain euglycemia, glucose infusion had to be more strongly increased after intranasal insulin than after placebo (Fig. 1*A*). The increase in GIR was higher after insulin spray than after placebo in the first designated period of analysis (100–120 min postspray, difference 0.63 ± 0.26 mg × kg^{-1} × min^{-1}, $P = 0.0277$). Whereas glucose levels had been comparable up until this point in time ($P = 0.5$), they were slightly higher thereafter on the insulin day (see Fig. 1*D*). Despite a higher increase in GIR during the second designated period of analysis, i.e., 190–210 min postspray ($P = 0.0408$), glucose levels were again comparable between study days for the whole cohort ($P = 0.1$). In both designated periods of time, C-peptide, glucagon, and free fatty acid concentrations were comparable (all $P > 0.1$) (Fig. 1*C* and Supplementary Table 1).

Analysis of tracer enrichment within the whole group (lean and overweight combined) showed no significant difference between the two sprays in the suppression of endogenous glucose production ($P \geq 0.4$ for both periods). However, the increase in the rate of glucose disappearance was greater after nasal insulin than after placebo spray application ($P = 0.0318$ for the first period and $P = 0.1$ for the second).

The magnitude of suppression of endogenous glucose production was associated with serum prolactin levels only after insulin spray ($P = 0.0204$) (Supplementary Fig. 4*A*) but not after placebo spray ($P = 0.3$) (Supplementary Fig. 4*B*). The increase in glucose disappearance rate was neither after insulin nor after placebo spray associated with prolactin concentrations (both $P > 0.6$) (Supplementary Fig. 4*C* and *D*).

Comparison of Lean and Overweight Participants During Hyperinsulinemic-Euglycemic Clamps

On the basis of our previous results (19), we stratified the group into lean and overweight participants. The absolute increase in GIR after nasal insulin was different between the two weight groups ($P = 0.0092$ for the first period and $P = 0.08$ for the second). In the lean group, the increase in GIR was greater after insulin than after placebo spray application for the two designated periods ($P = 0.0298$ and $P = 0.0413$) (Fig. 2*A* and *B* and Supplementary Fig. 2*A*). In overweight participants, no comparable differences between study days were found ($P \geq 0.3$) (Fig. 2*A* and *B* and Supplementary Fig. 2*B*).

For the lean participants, analysis of tracer enrichment revealed that endogenous glucose production was more strongly suppressed from before to after nasal insulin than after placebo spray for the first designated time period ($P = 0.0015$) (Fig. 2*C*) but not for the second time period ($P = 0.9$) (Fig. 2*D*). However, the rate of glucose disappearance did not differ significantly in the first period ($P = 0.1$) (Fig. 2*E*) but tended to be higher in the second ($P = 0.05$) (Fig. 2*F*).

In the overweight participants, no comparable differences could be detected either for endogenous glucose production or for rate of glucose disappearance (all $P \geq 0.09$) (Fig. 2*C*–*F*).

Functional MRI

On the basis of our recent findings (5), we assessed the insulin-induced decrease of hypothalamic regional blood

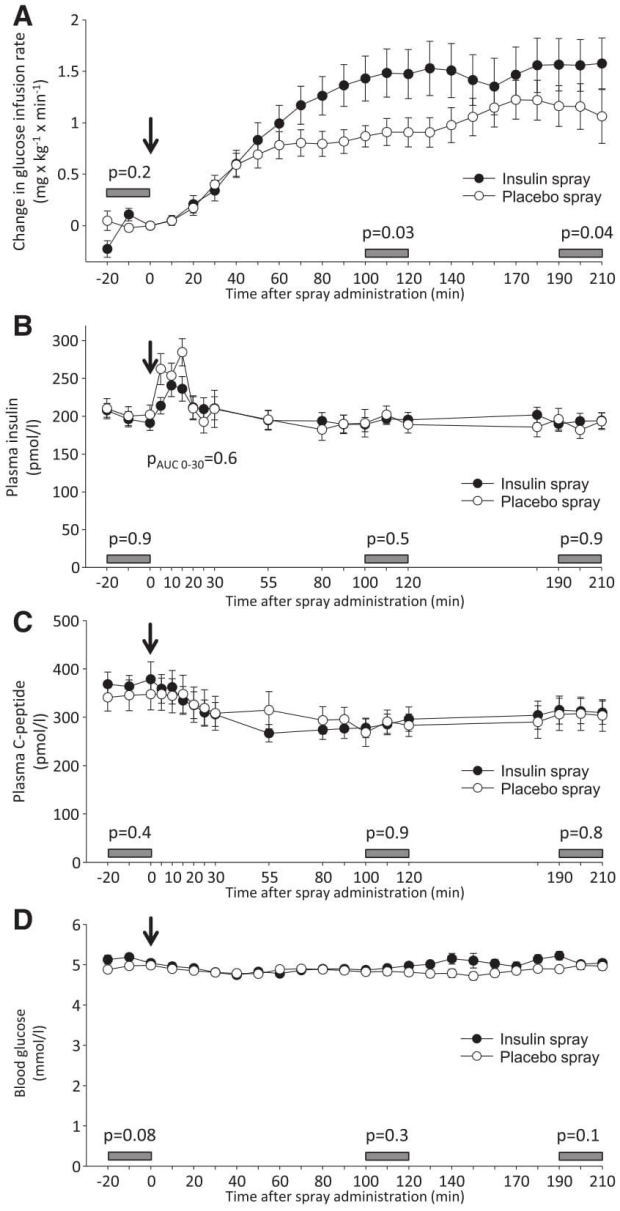

Figure 1—Hyperinsulinemic-euglycemic clamp results in the whole cohort (lean and overweight participants combined). The absolute change in GIR after spray administration at t = 0 min is presented here (*A*). Time courses of plasma insulin (*B*), plasma C-peptide (*C*), and blood glucose (*D*) also begin with the first time period for calculation 20 min before spray application at t = 0 min. Means ± SEM for the whole group (lean and overweight combined) are given. Periods for further assessments (see Fig. 2) are indicated as gray boxes. The black arrows indicate the time of spray application. Differences in means between insulin and placebo spray during these designated periods were tested by pairwise two-tailed Student t tests.

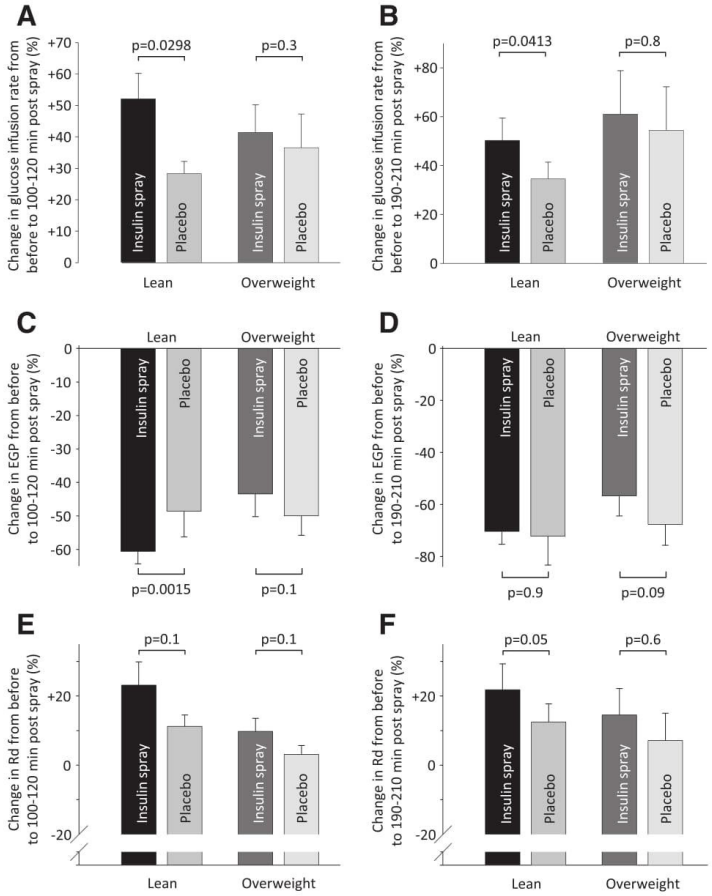

Figure 2—Effects on GIR, endogenous glucose production (EGP), and glucose disappearance rate (Rd) in lean and overweight participants. Changes from the 20 min before spray application to the first predefined time period after spray, i.e., 100–120 min postspray (*A*, *C*, and *E*), and to the second predefined time period after spray, i.e., 190–210 min postspray (*B*, *D*, and *F*), in lean (left two bars) and overweight (right two bars) participants are indicated. *A* and *B* represent changes in GIR, *C* and *D* show changes in EGP, and *E* and *F* are changes in Rd. Means ± SEM. Differences between insulin and placebo spray were tested by pairwise two-tailed Student *t* tests.

flow as a readout for hypothalamic insulin action. After adjustment for BMI, a significant correlation was observed between hypothalamic insulin sensitivity and the increase in GIR from before insulin nasal spray to the first designated period after spray ($P = 0.0314$, $r^2 = 0.29$). This association remained significant after additional adjustment for age ($P = 0.0249$, $r^2 = 0.33$).

To enlarge the sample size, we next pooled the data of our current study with those of our earlier study (19). For two subjects who participated in both studies, only the functional MRI recordings of the current study were included. In the resulting 28 subjects, we analyzed associations between the increase in GIR from before to 100–120 min after insulin spray and insulin-induced changes in regional brain activity on the whole-brain level, i.e., in a hypothesis-free approach ($P < 0.001$, uncorrected). We found a significant association with the caudate nucleus (MNI coordinates $x = -9$, $y = 20$, and $z = 4$), part of the striatum (Fig. 3, left panel). The change in striatal regional blood flow after intranasal insulin correlated with the increase in GIR from before to 100–120 min after insulin nasal spray ($P = 0.0026$, $r^2 = 0.30$) (Fig. 3). This association remained significant after adjustment for age and BMI ($P = 0.0101$, $r^2 = 0.26$) and after limiting the analysis to the subjects of the current study ($P = 0.0311$, $r^2 = 0.29$).

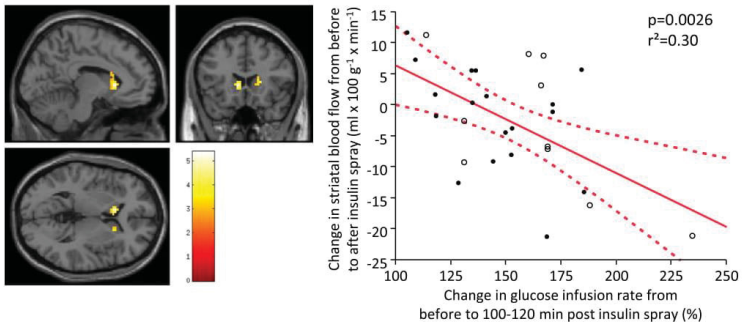

Figure 3—Change in GIR from before to 100–120 min after insulin spray application is associated with insulin effects on the striatum. The left panel shows the striatal regions for which significant associations were detected on a sagittal (left), coronal (right), and axial section plane (bottom). In the right panel, change in GIR is plotted against absolute change in striatal CBF after insulin spray application. Filled dots represent participants from the current study, and open dots are participants from the previous study. Lines represent fit line ± CI from an unadjusted model.

DISCUSSION

In this study, glucose infusion had to be increased more to keep blood glucose stable when intranasal insulin instead of a placebo was administered to the brain of our subjects during a hyperinsulinemic-euglycemic clamp. However, this effect was restricted to lean participants. The higher amount of glucose required in this group was due to improved suppression of endogenous glucose production, and partly by higher glucose uptake into peripheral tissues. In overweight participants, who are known to be brain insulin resistant (1,28), these effects were absent.

We had predefined two periods of time for the assessment of changes in peripheral metabolism (100–120 min and 190–210 min postspray). In both time intervals, considerably more glucose had to be infused to maintain euglycemia after nasal insulin application, indicating improved peripheral insulin sensitivity. This effect was more pronounced during the first but was also detectable in the later period. Thus, we hypothesize that the brain-derived modulation of peripheral metabolism occurs around 60 min after spray administration and persists for a considerable length of time, probably up to 3.5 h postspray, when our experiments ended.

However, the underlying mechanisms appear not to be the same for the rapid and the delayed effects. During the earlier period, i.e., 100–120 min postspray, glucose infusion had to be increased more after insulin spray since endogenous glucose production was more strongly reduced. This suppression by brain insulin is well in line with studies in rodents in which the hormone's actions in specific brain areas are shown to regulate hepatic glucose production (9–12). Since the present results were obtained at systemic hyperinsulinemia, the mechanism detected in our study might involve insulin sensitization of the liver itself. By contrast, no immediate effects on endogenous glucose production could be detected in two earlier studies under fasting systemic insulin levels (21,22). In line with rodent data at fasting insulin levels (12), the study by Dash et al. (21) found such effects with marked delay, i.e., 3–6 h after spray application. After this length of time, a comparable reaction could no longer be detected in our study. Of note, research in rodents with systemic hyperinsulinemia detected effects of brain insulin action on endogenous glucose production in a time frame comparable to that of our study (11). Brain insulin action might thus regulate hepatic glucose output more rapidly and without major delayed effects when occurring simultaneously in the presence of systemic hyperinsulinemia, as it takes place under physiological circumstances, i.e., after a meal. To unravel underlying mechanisms, more experiments in animals are needed that directly assess insulin signaling in the liver in the context of brain insulin action.

Another major difference between our present study and the work by Dash et al. (21) is that they used somatostatin. Since this substance is known to affect the central nervous system (29), this may complicate the interpretation of their results (24). Furthermore, the type of insulin used as nasal spray seems to be critical for the interpretation of the data. Whereas Dash et al. (21) used the rapid-acting insulin analog lispro, we applied regular human insulin. Previous results derived with other insulin analogs showed that when administered as a nasal spray, these analogs appear to act in a different way than regular human insulin (30,31), which might eventually also be relevant for insulin lispro. However, up to now, no information about a direct comparison with human insulin is available for this analog insulin.

In addition to the effects on endogenous glucose production, an increased rate of glucose disappearance was detected after nasal insulin application. Central insulin action might therefore also improve insulin sensitivity in peripheral tissues in addition to the liver. This action may

involve an elevation of glucose uptake. Findings from rodent studies suggest that skeletal muscle in particular could play a part in this respect (9,15). However, not all animal studies showed effects on glucose uptake into tissue (10,11). Besides species differences, a differential time course of brain insulin action on endogenous glucose production and glucose uptake into tissue may have contributed.

In sum, our current results indicate that brain insulin action may improve peripheral insulin sensitivity by suppressing endogenous glucose production and stimulating glucose uptake into tissue. This reaction might help to control whole-body metabolism after food intake when insulin levels rise quickly.

Earlier work has already demonstrated that higher body weight associates with insulin resistance of the human brain (1,28). Although this phenomenon does not affect all brain areas equally (5) or all insulin-regulated brain functions (4), our results indicate that it may be of importance for systemic metabolism. In line with previous work (19), insulin administration to the brain did not alter peripheral metabolism in the overweight participants of our study. In this group, neither endogenous glucose production nor glucose uptake was modified by nasal insulin spray. A lack of brain-derived modulation of peripheral metabolism could thus contribute to the pathogenesis of whole-body insulin resistance, which is often found in obesity, thereby increasing the risk for type 2 diabetes. Hence, the development of strategies that improve brain insulin resistance in humans will be one major goal for further research.

Our study indicates that speculations about a relevant major metabolic effect of the spillover of intranasal insulin into the circulation (17,20,24) are unwarranted. Since circulating insulin levels were similar on the two study days, the current study clearly demonstrates that brain insulin has additional effects to modulate peripheral glucose metabolism in humans.

Although the kinetics of spillover of nasally administered rapid-acting insulin analog have been reported before (21), our present study is the first to provide a precise description of the kinetics of this spillover of nasal human insulin application into the systemic circulation. Therefore, we used a specific protocol to accurately mimic this phenomenon by intravenous insulin infusion. It is worth mentioning that the spillover of intranasal human insulin into the circulation appears to differ from the insulin analog lispro in both magnitude and duration. Whereas intravenous administration of 2.5 mU $\times$ kg^{-1} human insulin was sufficient to mimic spillover after 160 units of intranasal human insulin, twice as much insulin lispro was necessary to mimic spillover after 40 units of intranasal lispro (21). Furthermore, plasma insulin peaked around 15 min post–human insulin spray and returned to prespray levels 15 min later, as observed in some (19,22), but not all (18), previous studies. This is well in line with insulin's half-life, which is very short in the blood circulation (32) but seems to prevail considerably longer in the brain (6,33). In contrast to human insulin, the peak venous insulin lispro concentration was not reached until 30 min after spray administration (21). Furthermore, the amount of intranasal insulin lispro absorbed into the bloodstream seems not to be strictly dose dependent (21). Such a dose dependency has not yet been tested for human insulin. Due to their increased and prolonged reabsorption into circulation, intranasal insulin analogs such as lispro might have stronger systemic side effects than intranasal human insulin. However, an appropriate randomized study with a direct comparison of human insulin and insulin analogs (including lispro) administered as nasal spray has not been reported yet.

To gain a better comprehension of the underlying physiology, it is important to investigate the brain areas in which the efferent outputs originate. Using an improved functional MRI approach (5), we now verified the involvement of the hypothalamus that had already been hypothesized by earlier studies in humans (18,19) and animals (14,34,35). This brain area plays a key role in the control of whole-body homeostasis. Reduced expression of insulin receptors in the hypothalamus causes peripheral insulin resistance (34,35). This well-known hypothalamic response to food intake (36,37) might contribute to the modulation of peripheral metabolism detected in our study.

The larger sample size, resulting from inclusion of comparable data from our previous study (19), enabled us to use a hypothesis-free approach to investigate any brain areas that might additionally be involved in this process. After rigorous correction for multiple comparisons, a specific brain area (the striatum) was detected. This part of the basal ganglia is a critical component of the reward system. It appears to respond to the postprandial rise in endogenous insulin concentrations (37,38) as well as to nasal insulin application (39). It is worth bearing in mind that the functional connection between the striatum and the hypothalamus was enhanced after glucose ingestion (38), suggesting that the interplay between these two crucial brain areas is regulated postprandially. Insulin action in this specific area was already suspected to contribute to the modulation of peripheral metabolism (18,40), as is supported by our current study. One major striatal neurotransmitter is dopamine. The striatal dopamine receptor availability is associated with whole-body insulin sensitivity (41). Central dopaminergic tone (as assessed by blood prolactin concentrations) is age-dependently associated with peripheral insulin sensitivity (42). In our current study, serum prolactin concentrations were associated with the magnitude of suppression in endogenous glucose production after nasal insulin. Since no such correlation was detected for glucose uptake into tissue, it is tempting to speculate that distinct mechanisms underlie these two effects of nasal insulin, with striatal dopamine being important especially for endogenous glucose production. However, in line with experimental evidence

for a molecular interaction between insulin and dopamine transporter in the striatum (43), insulin action in the human brain might modulate striatal dopamine signaling, which might, in turn, affect outflows that control endogenous glucose production. However, additional neurotransmitters in the striatum may contribute as well.

The effects of nasal insulin during a hyperinsulinemic-euglycemic clamp appear to be consistent in young men (see also Supplementary Fig. 5). Further studies are needed, though, to elucidate this effect in other groups, especially women, older people, and participants with another ethnic background. Furthermore, it is still not sufficiently studied how much of the nasally administered insulin reaches the brain. Further research should also assess what insulin concentrations are reached at the neuronal level after nasal insulin spray administration. Despite comparable C-peptide and glucagon concentrations, we cannot exclude that subtle alterations in portal insulin or glucagon concentrations may have potentially been present in our study. Whether distinct central processes underlie the differential time course of brain insulin action on endogenous glucose production versus glucose uptake into tissue will also be an important question for further research.

In sum, we provide novel evidence in support of the theory that brain insulin action may improve whole-body glucose metabolism during systemic hyperinsulinemia by enhancing insulin-mediated suppression of endogenous glucose production as well as by possibly stimulating glucose uptake into tissue. Besides the hypothalamus, the striatum might be an important brain area involved in this response. In overweight subjects, these mechanisms are not detectable. Brain insulin resistance might therefore contribute to the pathogenesis of whole-body insulin resistance in obesity.

Acknowledgments. The authors thank all study participants for their cooperation in this project. The authors also thank the excellent technical assistance of Maike Borutta, Torsten Cerny, Anja Dessecker, Dr. Louise Fritsche, Dr. Christoph Gassenmaier, Ellen Kollmar, and Andreas Vosseler (all University of Tübingen) and of Dr. Daniel Markgraf and Peter Nowotny (both German Diabetes Center).

Funding. This study was supported in part by a grant from the Federal Ministry of Education and Research (BMBF) (01GI0925) to the German Center for Diabetes Research (DZD), by the Helmholtz-Gemeinschaft (Helmholtz Alliance ICEMED-Imaging and Curing Environmental Metabolic Diseases), and by the European Foundation for the Study of Diabetes (Rising Star award supported by Novo Nordisk to M.H.).

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

Author Contributions. M.H. performed the study, analyzed data, and wrote the manuscript. R.W. and S.K. performed the study, analyzed data, and contributed to the discussion. S.G. and M.R. analyzed tracer dilution data and contributed to the discussion. A.P. and N.S. contributed to the discussion. H.P., H.-U.H., and A.F. designed the study and contributed to the discussion. M.R. is the guarantor for the tracer dilution data and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis, and A.F. is the guarantor for the clamp experiments and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Prior Presentation. Parts of this study were presented in abstract form at the 76th Scientific Sessions of the American Diabetes Association, New Orleans, LA, 10–14 June 2016.

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4. Discussion

The studies of this thesis contribute to the understanding of the role of intranasal insulin for glucose homeostasis. Insulin is a key regulator of hepatic glucose and energy metabolism and the studies here elucidate its direct and indirect effect on liver energy status and HIS. First, insulin administered via the nasal route, but not intravenously, increases hepatic ATP concentration and lowers liver fat content [83]. Second, reduction of EGP by intranasal insulin is found only in the settings of hyperinsulinemic clamp [84], but not during fasting [83], suggesting feeding status-dependent brain insulin action. Intranasal insulin also increases skeletal muscle insulin sensitivity. Finally, intranasal insulin effects are found in lean healthy individuals, but not in obese and T2D patients, which is possibly explained by brain insulin resistance in these patients. The precise mechanism underlying this brain-liver crosstalk in healthy humans remains unknown, but the parasympathetic nervous system has been suggested to be involved [80].

4.1. Spillover of intranasal insulin and direct versus indirect effects

Intranasal insulin has been used by several groups in the past few years to mimic delivery of insulin to the human brain. This technique allowed demonstrating *in vivo* CNS effects of intranasal insulin in the insulin sensitive occipital and prefrontal cortical regions, hypothalamus, striatal regions, insula cortex and the cingulate cortices [106]. However, scrutiny and careful interpretation are needed when assessing intranasal insulin effects on peripheral metabolism because of spillover of intranasal insulin into the circulation. Within 10-20 minutes after insulin spray application a rise in serum insulin has been shown by several investigators [73, 77, 79] including the present studies [83, 84]. While the spillover probably does not present a major obstacle when investigating central effects of intranasal insulin, it might affect peripheral metabolism and interfere with observations when studying distant organ homeostasis.

Study 1 found transient reduction of blood glucose levels due to increase in serum insulin shortly after insulin spray application [83]. This was in line with some previous reports detecting the spillover in systemic circulation [73, 77, 79, 82], but in contrast with other showing no changes in blood glucose and insulin levels after intranasal insulin application [74, 75, 78, 86, 87] (Table 1). These different observations might be explained by the different

insulin doses applied in the different studies (160 IU versus 40 IU). Dose dependent increases in serum insulin have been suggested after administration of ascending insulin doses with the lowest dose of 10 IU intranasal insulin also being able to elevate insulin in the circulation [81]. Therefore placebo spray application is not sufficient as control condition in intranasal insulin studies and the spillover of small amounts of the nasal insulin into the circulation has to be experimentally mimicked by administering insulin intravenously.

The first study to use intravenous insulin administration in parallel to the placebo spray application ensured similar venous insulin concentration between treatments by infusing 5 $mU*kg^{-1}$ over 30 min starting at the time of placebo administration [81]. The intranasal insulin studies in this thesis also successfully mimicked the peripheral spillover by insulin infusion achieving comparable changes in insulin and glucose concentrations shortly after the intervention [83, 84]. In Study 1 100 mU insulin were administered as an intravenous bolus on a separate test day and lead to reductions in circulating glucose and free fatty acids and rises in serum insulin similar to the substrate and hormone excursions after nasal insulin application. In Study 2 an intravenous bolus of 2.5 $mU*kg^{-1}$ over 15 minutes was applied which increased serum insulin to comparable levels with the nasal insulin treatment. Of note, the studies here applied 160 IU of regular human insulin intranasally, whereas in the study by Dash et al. 40 IU of intranasal insulin analogue lispro were used. Another human study with intranasal application of insulin analogue aspart aimed at discriminating exogenous from endogenous insulin kinetics and effects on fasting glycemia using intravenous insulin administration of 0.12 $mU*kg^{-1}*24h$ as control condition [82]. Despite intranasal application of different preparations and amounts of the hormone were used, all of the above mentioned intravenous insulin doses resulted in comparable serum insulin excursions between conditions and ensured that brain specific effects of insulin are delineated from changes induced by systemic insulinemia. This is of key importance when studying intranasal insulin effects on peripheral metabolism, as even small fluctuations in serum insulin might affect glucose and lipid fluxes in insulin sensitive organs such as liver, skeletal muscle and adipose tissue in lean healthy individuals.

Study 1 of this thesis also investigated the effect of intranasal insulin on circulating free fatty acids in the fasted state and found that in similarity to the Iwen et al. study [78] free fatty acid suppression is induced after intranasal insulin application in lean healthy humans. Intravenous

insulin application on a separate study day in Study 1 allowed for delineation of direct versus indirect effects of intranasal insulin on circulating lipids. Similar decrease in free fatty acids was found both after intranasal and intravenous insulin administration, suggesting that the potential effect on lipolysis is mediated by the spillover of intranasal insulin in the circulation and not via central mechanisms [83]. No changes were found in plasma triglycerides and glycerol concentrations after intranasal insulin, which further speaks for lack of effect on lipid metabolism.

4.2. Intranasal insulin effects on hepatic energy metabolism

In Study 1 intranasal insulin was shown to acutely improve hepatic energy metabolism in lean healthy humans. The study also allowed discrimination between the direct and indirect effects of intranasal insulin as placebo and intravenous insulin bolus were applied on separate experimental days. The effect on hepatic energy metabolism was specific to the intranasal route of insulin application, as neither placebo, nor intravenous bolus of insulin were able to elicit any changes in hepatic ATP content. These results suggest that insulin-mediated brain-liver crosstalk is able to acutely modulate hepatic energy status and the observed elevation in hepatic ATP concentrations after intranasal insulin application is not due to increased systemic insulin levels. The underlying mechanism of this crosstalk remains not completely understood. Animal studies point at the parasympathetic nervous system as possible mediator of brain insulin effects on hepatic glucose metabolism [62, 63]. In these studies surgical resection of the hepatic branch of the vagus nerve negates the effect of central insulin on hepatic glucose production. Although the effects of brain insulin on hepatic energy status from ATP levels have not been studied in animal model so far, it is possible that they might as well be mediated by parasympathetic outputs to the liver. In line with data in rodents, intranasal insulin induced improvement of peripheral insulin sensitivity is directly related to parasympathetic tone measured from heart rate variability in humans [80]. Still, non-invasive auricular vagus nerve stimulation did not elicit any changes in parasympathetic tone and did not have any effects on hepatic ATP levels or hepatic glucose production in healthy humans [107]. These findings suggest that non-invasive vagus stimulation is unable to mimick intranasal insulin effects on hepatic energy metabolism in healthy humans as found in Study 1. However, different and/or invasive vagus stimulation techniques might hold promise to demonstrate modulation of liver glucose and energy metabolism in humans in the future.

Acute elevations in lipid availability might modulate hepatic energy metabolism in humans. An increase in hepatic ATP concentrations of 16% has been observed after acute dietary fat intake, along with stimulated lipid oxidation and higher insulin resistance in lean healthy individuals [108]. These findings suggest acute adaptation of hepatic energy metabolism in states of increased lipid influx, which is comparable to the magnitude of the ATP elevation after intranasal insulin application in healthy humans in Study 1 [83]. These data support the notion that hepatic ATP content is sensitive marker of liver energy status which can be modulated by direct and indirect signals. As absolute hepatocellular ATP concentrations reflect resting mitochondrial activity, changes in ATP levels upon intranasal insulin as well as upon elevated substrate availability are possibly linked to altered balance between energy producing and energy consuming processes. Both after insulin spray application and oral lipid load lipid oxidation in the liver is suggested to be increased, which might result in the elevated energy status found in Study 1 and in [108].

Of note, intranasal insulin was shown acutely improves hepatic energy metabolism in lean healthy humans but not in patients with T2D [83]. These intriguing findings suggest central insulin modulation of hepatic energy metabolism, which is not operational in insulin resistant humans with T2D. Impaired brain insulin sensitivity in obesity, as suggested by previous works [91, 92, 94], might be responsible for the observed blunted effect of intranasal insulin in these patients. An alternative explanation might involve the alterations of hepatic mitochondrial function that have been previously described in T2D [50, 51]. As patients with T2D have lower absolute hepatic ATP concentrations and decreased hepatic ATP flux [50, 51], intranasal insulin application might not have been able to overcome abnormal hepatic energy metabolism and stimulate hepatic ATP levels in the T2D patient group. Of note, T2D individuals exhibit 26% lower hepatic ATP concentration which exceeds the increase of 18 % in lean healthy humans observed after intranasal insulin in Study 1. Thereby profoundly altered hepatic energy metabolism in T2D might prevent the central insulin mediated action to improve liver energy status. Interestingly, despite lower ATP concentrations hepatic energy metabolism does not seem to be inert in T2D, as postprandial increases of 21 % in hepatic ATP have been demonstrated in T2D patients [53]. Furthermore, postprandial maximum increase in hepatic ATP tended to be higher in T2D than in lean healthy humans suggesting a supply-driven change in ATP in response to elevated postprandial lipid and glucose availability in T2D [53]. Thereby unaltered hepatic ATP concentrations after intranasal insulin suggest that

insulin-mediated brain-liver crosstalk is rather impaired on central, but not on peripheral level in T2D.

4.3. Intranasal insulin effects on hepatic fat content

Hepatic steatosis is a feature of obesity and insulin resistance, which is defined by increased fat storage in the liver. Key mechanism controlling the lipid retention in the liver is the hepatic secretion of triglycerides packaged as very low-density lipoproteins (VLDL) [109]. Studies in Sprague Dawley rats demonstrated that intracerebroventricular insulin infusion increases hepatic triglyceride secretion thereby lowering liver fat content [110]. Interestingly, Study 1 found reduction of liver fat content after intranasal insulin application in the lean healthy humans [83], indicating that a central mechanism controlling hepatic lipid handling might be operational in humans as well. Of note, the reduction in hepatic triglycerides in this study was specific to the intranasal route of insulin administration, as intravenous insulin bolus even increased liver fat accumulation, in line with the known direct insulin effect on hepatic lipogenesis [111]. However, no differences in circulating triglycerides were found after intranasal insulin application [83], while VLDL were not measured, so that it cannot be concluded from this study whether enhanced lipid export from the liver is responsible for the observed decrease in hepatic triglycerides. Lower free fatty acids after intranasal insulin rather imply reduced lipid influx to the liver, but free fatty acids were suppressed also with intravenous insulin application, so that their specific contribution here is unlikely [83]. Increased hepatic lipid utilization via elevated β-oxidation might explain the reduction in hepatic lipid content and is in concert with the observed higher hepatic ATP concentrations after intranasal insulin. Increased hepatic β-oxidation would result in generation TCA-cycle substrates and NADH stimulating mitochondrial oxidative phosphorylation which would eventually result in elevated hepatic energy status with higher ATP levels. In overall, these findings suggest contrasting central and peripheral actions of insulin to lipid metabolism of the liver and coupling of changes in hepatic lipid handling to hepatic energy metabolism. This important observation from an interventional study finely complements previous cross-sectional data on the tight link between hepatic lipid and energy metabolism in humans [112].

Chronic elevation in substrate availability leading to ectopic lipid storage and insulin resistance in the liver in T2D is linked to altered hepatic mitochondrial function with reduced hepatic ATP

concentrations and lower ATP synthase flux [50, 51]. While these chronic changes might relate to glucolipotoxicity and increased oxidative stress in the liver [113, 114], in the acute settings of stimulated brain insulin action after intranasal insulin the decrease in hepatic lipid content possibly results from transient changes in liver lipid transport and/or utilisation, as shown in rodents [110]. The findings in rodents that brain insulin promotes hepatic triglyceride secretion [110] and data from Study 1 pointing to its potential role for the regulation of liver fat accumulation in healthy humans granted the further investigation of the effects of intranasal insulin on liver lipid content in T2D patients. Study 1 examined hepatic triglyceride content before and after intranasal insulin application in T2D patients and detected no differences [83], suggesting that the effects observed in healthy humans are blunted in elderly overweight diabetic volunteers. This might be attributed to brain insulin resistance of the T2D humans, as CNS insulin effects on lipid metabolism have previously been shown to be abrogated in states of insulin resistance [115]. Still, the relative contribution of age, obesity and T2D for the diminished response to intranasal insulin in the diabetic group could not be delineated by this study and remains a question for future investigation. Of note, not only acute effects of intranasal insulin are lost in diabetic humans, but the existence of chronic effect is also questionable, as 4 week intranasal insulin treatments induced no change in liver fat content in healthy males [116]. Still, brain insulin sensitization might represent a possible target to modulate metabolic disease and ectopic lipid storage.

4.4. Intranasal insulin effects on glucose metabolism

In Study 1 the effects of intranasal insulin on HIS were examined in the fasting state when glucose regulatory and counter-regulatory hormones are not fixed and might respond to central signals [83]. Serum insulin increased transiently after intranasal insulin application due to spillover in the circulation and C-peptide levels were reduced, indicating suppression of endogenous insulin secretion. This finding is in line with most [73, 77, 79], but not all [78] intranasal insulin studies performed under fasting conditions. Sustained reduction of C-peptide levels suggests increased peripheral insulin sensitivity with improved insulin action in skeletal muscle, which has been demonstrated in subsequent studies [79, 80, 84]. As the parasympathetic nervous system is known to regulate pancreatic endocrine function, prolonged reduction in C-peptide levels might also result from decreased vagal tone due to brain insulin-vagal nerve interaction. However, vagal nerve stimulation has been shown to

reduce postprandial C-peptide levels in rheumatoid arthritis patients [117], suggesting that the regulation of the parasympathetic control of pancreatic hormone secretion is more complex [107]. Although an interaction with the peripheral nervous system could not be excluded, suppressed endogenous insulin production after intranasal insulin likely results from transient hyperinsulinemia after insulin spray application which inhibits endogenous insulin release in a negative feedback mechanism manner.

Glucagon concentrations remain unaltered after intranasal insulin in healthy humans, suggesting lack of activation of counter-regulatory hormone response. This is surprising having in mind that glucose levels decrease slightly upon serum insulin excursion and glucose is known as the major regulator of glucagon secretion. Despite the raise in serum insulin followed by slight reduction in blood glucose, no difference in HIS was observed over 6 hours after intranasal insulin application both in healthy and diabetic humans [83]. The lack of effect of intranasal insulin on fasting HIS in Study 1 is in line with results from another human intranasal insulin study performed also in fasting conditions which demonstrated no acute changes in systemic glucose homeostasis due to central insulin effect [82]. This might be due to the fact that insulin's direct regulatory effects in the liver under fasting conditions are dominant. Still, hepatic glucose fluxes might have been affected by subtle changes in endogenous insulin and glucagon secretion, as shown in healthy humans previously [118], induced by fluctuations of blood insulin and glucose. The rates of gluconeogenesis and glycogen turnover have not been measured in this study so that possible changes in the contribution of glycogenolysis and gluconeogenesis to the EGP rates could not be determined. Increased hepatic glucose uptake and glycogen synthesis without changes in hepatic glucose production have been demonstrated upon brain insulin action in dogs [65], suggesting that altered glycogen synthase flux after intranasal insulin in humans could not be excluded. Future studies should elucidate in detail whether hepatic gluconeogenesis and glycogen fluxes are modulated following central insulin action in humans.

Study 2 aimed at delineating the effect of intranasal insulin on insulin sensitivity by measuring simultaneously rates of EGP and skeletal muscle glucose uptake after intranasal insulin application. Insulin sensitivity is improved as shown by higher glucose infusion rates and a trend for higher skeletal muscle glucose uptake after intranasal insulin application in healthy humans [84]. This confirms data from previous studies showing reduced HOMA-IR [79] and

again higher clamp glucose infusion rates [80] after intranasal insulin application. While HOMA-IR is an unprecise estimate of whole body insulin resistance and reflects fasting insulinemia, glucose infusion rates during hyperinsulinemic euglycemic clamp are primarily determined by skeletal muscle glucose uptake and may be used as a reliable estimate. Thereby Study 2 finely complements these earlier findings and distinctly points at skeletal muscle glucose uptake as feature of peripheral glucose metabolism modulated by intranasal insulin.

Furthermore, intranasal insulin is able to reduce EGP in Study 2 [84], in similarity to a previously reported pancreatic clamp study [81]. This confirms the notion that the brain-liver axis functions to regulate hepatic glucose metabolism in the postprandial period, possibly when CNS insulin action is maximal. During this period brain insulin is permitted to dominate and reduce EGP, whereas during fasting insulin's direct effect possibly prevails. Still, the relative contributions of gluconeogenesis and glycogenolysis not only during fasting, but also in state of hyperinsulinemia were not measured here so that brain insulin effect on these processes remains a question for future investigation.

It is important to note that similar effects on EGP were seen in the pancreatic clamp study by Dash et al. [81] and in Study 2 [84] despite differences in experimental procedures. In the pancreatic clamp study insulin concentrations were kept at basal and somatostatin was infused, whereas in Study 2 insulin levels were elevated to postprandial levels without somatostatin infusion. As somatostatin is well known for its neuroendocrine and metabolic effects [119], one might expect that its infusion in the pancreatic clamps might have interfered with the observed effects. However, the analogous finding of EGP suppression after intranasal insulin in Study 2 where no somatostatin was infused indicates that intranasal insulin control of hepatic glucose metabolism cannot be attributed to CNS somatostatin effects.

Of note, a delayed lowering of EGP in response to intranasal insulin was observed in Study 2 [84], as well as in a previous pancreatic clamp study [81]. The maximum effect on HIS was not found immediately after spray application, but 90 to 120 minutes later. Slow transport of insulin to the CNS is unlikely to explain the described delayed effects as liquor insulin concentrations raises within 10 minutes of intranasal insulin application [70] and in humans neuronal activity changes within 30 minutes of administration [80]. The delayed response is rather attributable to the relatively slow process of changes in STAT3 phosphorylation and

hepatic gluconeogenic gene transcription and translation, which have been suggested as potential underlying mechanisms for the suppression of EGP rates in rodents [62, 120].

Interestingly, diazoxide effect onset is 120 min after administration and lasts for up to 240 min [89]. In pancreatic clamp experiments intranasal insulin reduces EGP rates as late as 180 min after spray application and the effect is maximal at 240 min. Study 1 examined in a subgroup of participants whether delayed effect of intranasal insulin on hepatic glucose and energy metabolism up to 360 min after spray administration in the fasting state are detectable. Prolonged monitoring both in healthy and type 2 diabetic individuals revealed no delayed effect on hepatic glucose production or hepatic energy status, suggesting that modulation of hepatic energy metabolism in the fasting state is transient within the first three hours after intranasal insulin administration. This might be explained by rapid dynamic changes in hepatic energy metabolism after spray application which quickly return to basal state. Similar transient changes in hepatic lipid and energy metabolism measured form MRS have also been described upon nutritional interventions such as oral lipid challenge and mixed meal test [53, 108]. The lack of changes in hepatic glucose production for up to 6 hours found in Study 1 confirms the notion that intranasal insulin application does not acutely modulate fasting HIS and no delayed effects are present in contrast to clamp studies [81, 84].

Reduced circulating free fatty acids after intranasal insulin as seen in Study 1 and other works [78, 81] might contribute to changes in hepatic glucose production as they act as precursors for gluconeogenesis. Suppression of lipolysis is seen in both fasting and pancreatic clamp conditions suggesting this effect might be independent from prevailing insulinemia and spillover of intranasal insulin. However, recent findings from mouse model study suggest that lipolysis suppression mediated reduction in hepatic glucose production occurs within minutes and is linked to lower hepatic acetyl CoA levels and pyruvate carboxylase flux [121]. Thereby the observed delayed effect in hepatic glucose production found in intranasal insulin studies in clamp settings most likely does not relate to changes in lipid availability but possibly results from different neural and non-neural pathways.

Previous works have suggested that the vagal nerve is responsible for the mediation of central insulin effects to the periphery [61, 62]. In rodents, central insulin action supresses the activity of the hepatic branches of the vagal nerve which leads to activation of hepatic IL-6/STAT3

signalling and decreases gluconeogenic gene expression [122]. As a result, hepatic glucose output is diminished. Additionally, insulin signalling in the dorsal vagal complex has been shown to inhibit hepatic glucose production [63]. Lower gluconeogenic rates might spare ATP utilization and contribute to the higher hepatic ATP levels found here in the healthy humans after intranasal insulin. Moreover, human studies with intranasal insulin also suggest involvement of the vagal nerve. Intranasal insulin induced increase in peripheral insulin sensitivity correlates positively with parasympathetic tone [80]. However, in the latter human study parasympathetic activation, and not inhibition of vagal tone, as suggested from rodent data [122], was associated with insulin sensitization, so that the direction of changes remains controversial. Although the link between brain insulin action and vegetative nervous system has been proposed, the precise mechanism still remains to be clarified. The studies in this thesis did not assess parasympathetic system tone and therefore its involvement in the mediation of the brain-liver crosstalk here remains unclear. Future investigations should address the mechanism of brain insulin control of peripheral metabolism in humans in detail and elucidate whether vagal tone modulation *per se* might exert the beneficial effects of CNS insulin on glucose metabolism seen in animal models and in human studies with intranasal insulin.

4.5. Blunted intranasal insulin effects in obese and type 2 diabetic humans

It remains of high interest whether hepatic glucose fluxes can be modulated in insulin resistant humans and patients with T2D. Intranasal insulin holds promise as a treatment for human metabolic disease, as it exerts an acute anorexigenic effect [75] and reduces body weight after 8 weeks treatment [123]. The studies in this thesis tested whether EGP and HIS can be modulated by intranasal insulin in obese and T2D patients. Unfortunately, no effect of intranasal insulin was found in these patients as HIS remained unaltered in diabetic humans [83] and the insulin sensitizing effect of intranasal insulin was not found in obese individuals [84] (Figure 2). Further study examined intranasal insulin application effects on HIS in pancreatic clamp settings in insulin resistant participants and found no changes in hepatic glucose production [86]. Another follow-up study with diazoxide administration in patients with T2D [124] investigated whether diazoxide-induced improvement of HIS seen in lean humans [89] could be translated to diabetic participants. The results showed no lowering of EGP after diazoxide administration, which was confirmed in complementary mechanistic

studies in Zucker Diabetic Fatty rats. This is also in line with previous mouse model studies showing that high-fat feeding and streptozozin-induced diabetes impair brain insulin regulation of hepatic glucose metabolism [115, 125, 126]. In overall, the studies in this thesis help translate animal model data to humans and provide further support to the notion that metabolic disturbances including those present in obesity and T2D disrupt central regulation of glucose homeostasis. Given that unrestrained EGP is a major source of hyperglycemia in T2D, restoring the brain's sensitivity to insulin would be a promising future target for T2D treatment.

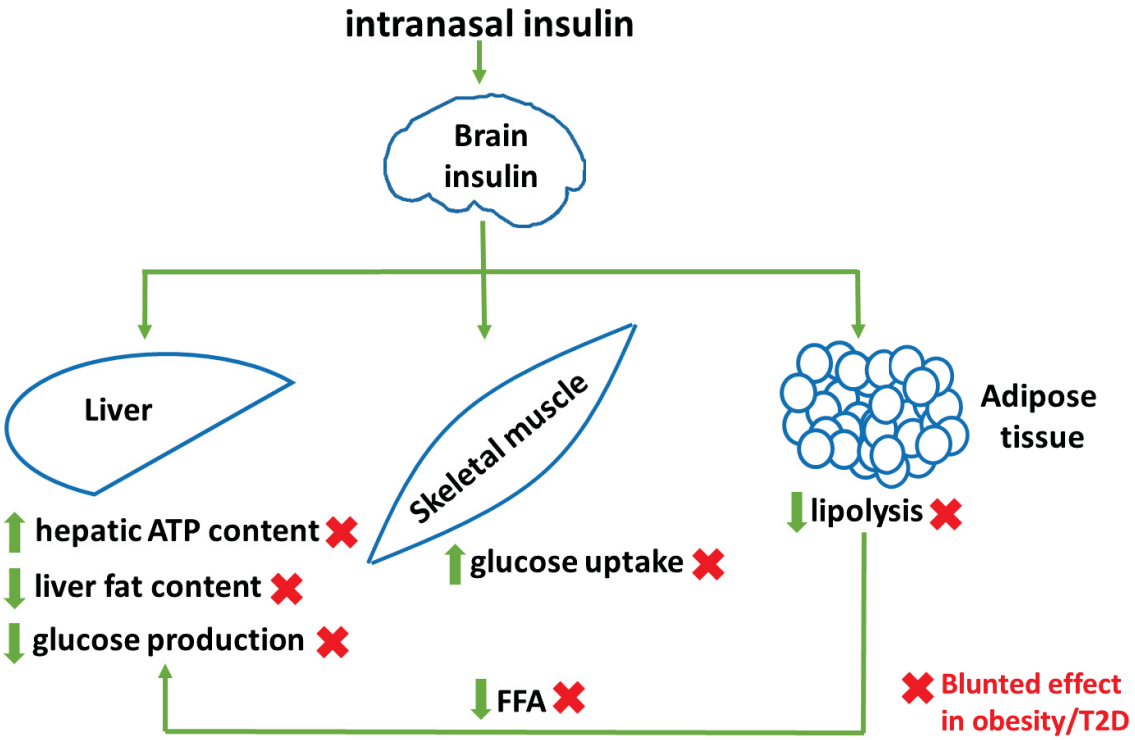

Figure 2. Intranasal insulin effects on glucose and energy metabolism in healthy humans and obese/type 2 diabetic (T2D) individuals. In lean healthy humans intranasal insulin acutely increases hepatic ATP concentrations, while reducing liver fat content. Hepatic glucose production is supressed in the settings of hyperinsulinemic-euglycemic clamp after intransal insulin. Skeletal muscle glucose uptake increases, while adipose tissue lipolysis is reduced. These effects of intranasal insulin are blunted in obese patients and/or patients with T2D.

Study 1 also tested whether intranasal insulin modulation of adipose tissue lipid metabolism is operational in patients with T2D. No difference in circulating free fatty acids was found after intranasal insulin application [83] pointing to lack of central insulin regulation of lipolysis in

human T2D. This result is in line with mouse model data showing blunted CNS insulin control of adipose tissue fatty acid uptake in insulin resistant and overfed mice [69, 115]. Effects of intranasal insulin on hepatic lipid and energy metabolism are not seen in T2D patients from Study 1. Of note, participants with T2D in Study 1 exhibit substantially higher hepatic triglyceride concentrations compared to lean individuals. Concomitant non-alcoholic fatty liver disease with perturbations in hepatic mitochondrial function as shown previously [52] might have as well interfered with intranasal insulin effects. However, impaired brain insulin resistance as repeatedly shown from magnetic resonance imaging studies in diabetic and insulin resistant individuals [91, 92, 94, 100] likely has major contribution for the blunted effect in this patient group. Overcoming brain insulin resistance in obesity and diabetes thereby represents an interesting treatment target for the future.

5. Conclusions

The studies in this thesis improve our understanding of the role of direct (systemic) and indirect (CNS) insulin effect on hepatic energy metabolism. Using gold standard techniques for measurement of tissue-specific insulin sensitivity and non-invasive *in vivo* assessment of hepatic triglyceride and ATP concentrations, the work here demonstrates fine modulation of liver lipid and energy metabolism by brain insulin action. This finding adds on to the discussion of the brain as possible therapeutic target to treat metabolic disease. Importantly, the question as to whether brain insulin action might be able to modulate hepatic glucose and energy metabolism is addressed here not only in lean healthy human, but also in obese individuals and patients with T2D. Thereby the reported observations serve not only to describe novel operational metabolic mechanisms in human physiology, but additionally contribute to our knowledge on the alterations in disease state and thus to the possible treatment targets. The studies included in this thesis consider the complexity of the factors affecting hepatic glucose and energy metabolism and aim at detailed examination of already known, but also of new players. Deeper look into possible mechanisms responsible for the observed effects is always challenging when performing studies in humans. However, here humans underwent experimental procedures involving simultaneous assessment of multiple metabolic processes using different modalities (stable isotope dilution technique*, in vivo* liver MRS measurements, repeated blood sampling for close monitoring of circulating substrate and hormone levels) in order to obtain mechanistic insights into the observed effects.

The intranasal insulin induced decrease in liver fat content paralleled by an elevation of hepatic ATP concentrations demonstrate directly the coupling of hepatic lipid and energy metabolism in humans, which has previously been suggested by cross-sectional studies. The intriguing observation that these effects are blunted in T2D patients complements our understanding of the metabolic crosstalk perturbations in diabetes.

The studies of this thesis provide novel insights into the intranasal insulin regulation of HIS in two different metabolic conditions and reveal complementing results. The fact that intranasal insulin does not influence HIS in the fasted state, but reduces EGP under hyperinsulinemic euglycemic clamp conditions points to the notion, that brain control of hepatic glucose metabolism prevails in the postprandial state, while in the settings of fasting the direct insulin

action predominates. Thereby CNS insulin action might be seen as physiological instrument to finely adjust glucose fluxes only in specific endocrine conditions.

The high relevance of the findings of this work resides in the precise and detailed assessment of substrate levels and fluxes which are carefully interpreted with scrutiny towards previous and new results. Novel insights into the role of direct and indirect insulin action for the regulation of hepatic glucose and energy metabolism are provided which contributes to our understanding of metabolic liver disease. The described findings point at the brain as promising future treatment target to combat metabolic disease.

6. References

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7. Acknowledgements

First and above all, I would like to express my deepest gratitude to Prof. Dr. Michael Roden for giving me the opportunity to perform these interesting studies and to write this thesis. Thank you for your exceptional guidance, patience, valuable input and sharing your knowledge. This work would not have been possible without your continuous support and encouragement.

My special gratitude goes to Dr. Chrysi Koliaki and Dr. Julia Szendroedi. Because of your guidance, I was able to stay focused, structured and motivated.

I would like to thank the members of the Energy metabolism group – thank you for your help, team work, professionalism and for providing me with an excellent atmosphere for doing research.

To all volunteers, who took part in these studies – thank you for participating.

At last, I want to thank my wonderful family and my partner. Thank you for your unconditional support, your inspiration and motivating words.