

# Effects of intranasal insulin on hepatic fat accumulation and energy metabolism in humans

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## Abstract

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 provided conflicting results. In this randomized controlled cross-over trial we investigated effects of intranasal insulin on hepatic insulin sensitivity and energy metabolism in 10 patients with type 2 diabetes and 10 lean healthy participants (CON). Endogenous glucose production (EGP) was monitored with [6,6-<sup>2</sup>H<sub>2</sub>]glucose, hepatocellular lipids (HCL), adenosine triphosphate (ATP) and inorganic phosphate concentrations with <sup>31</sup>P/<sup>1</sup>H magnetic resonance spectroscopy. Intranasal insulin transiently increased serum insulin, followed by gradual lowering of blood glucose in CON only. Fasting hepatic insulin sensitivity index (HIS) was not affected by intranasal insulin in CON and patients. Only in CON, HCL decreased by 35%, whereas absolute hepatic ATP concentrations increased by 18% after three hours. 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 HCL, 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.

## Introduction

Evidence from rat models indicates that insulin signalling in the central nervous system (CNS) contributes to 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<sub>ATP</sub>) channels (2). However,

recent findings in dogs indicate that, in contrast to rodents, brain insulin action does not acutely regulate glucose production and gluconeogenesis in larger mammals (3).

As EGP regulation differs between species (4) and its impairment is a metabolic hallmark of type 2 diabetes, it is important to examine whether brain insulin signalling also modulates rates of EGP in humans. Interestingly, 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, by reporting either reduction of EGP (7) or no acute changes in glucose metabolism (8).

Intranasal insulin may also lower plasma free fatty acids (FFA) and rates of labelled 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). As insulin resistance tightly associates with hepatic fat accumulation (12) it is of interest to examine whether central insulin may improve hepatic glucose and lipid metabolism. So far, no studies have addressed the effects of intranasal insulin on hepatic energy metabolism in insulin resistant type 2 diabetes patients compared with healthy individuals under physiological fasting conditions.

Thus, we designed a randomized controlled crossover clinical study to investigate intranasal insulin effects on hepatic insulin sensitivity, lipid and energy homeostasis. In order to investigate extreme states of normal and impaired glucose metabolism, we enrolled young healthy lean controls (CON) and elderly overweight type 2 diabetes patients. 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.

## Methods

**Participants.** Ten insulin-naïve type 2 diabetes patients on oral glucose-lowering medication were enrolled in this randomized controlled single-blind cross-over monocenter trial. Patients taking thiazolidinediones were excluded from the study because of their possible long-term CNS effects (13). Patients withdrew their oral glucose-lowering medication for three days before the experiments to exclude possible effects on metabolic tests (14; 15). Two type 2 diabetes patients were on atorvastatin and one on gemfibrozil, both of which were not withdrawn throughout the study period. Ten young lean and healthy volunteers not taking any medications, without family history of diabetes and normal glucose tolerance based on a standard 75-grams oral glucose tolerance test were also enrolled. Before inclusion, all participants gave written informed consent to this registered trial (ClinicalTrials.gov registration no. NCT01479075), which was approved by the ethics board of Heinrich-Heine University Düsseldorf. They underwent screening including medical history, clinical exam and blood tests. None had clinical or lab signs of infection, hepatic, vascular, renal or endocrine diseases. All volunteers were sedentary and refrained from any exercising for three days before the study. Female participants were either postmenopausal or examined between day 5 and 8 of their regular menstrual cycle.

**Study design.** The volunteers arrived at 7:00 a.m. at the German Diabetes Center after 10 hours overnight fasting 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, the participants received a continuous infusion ( $0.036 \text{ mg} \cdot \text{min}^{-1} \cdot \text{kg body}$

weight<sup>-1</sup>) of D-[6,6-<sup>2</sup>H<sub>2</sub>]glucose (99% enriched in <sup>2</sup>H glucose; Cambridge Isotope Laboratories, Andover, MA) after a priming bolus of 3.6 mg·kg body weight<sup>-1</sup>·fasting plasma glucose [mg/dl]/90 [mg/dl] for 5 min (16). The tracer infusion lasted until +180 min in all participants and in order to study delayed insulin effects (17) - for an extended time period until +360 min in a subgroup of 12 participants (6 CON and 6 type 2 diabetes patients). Before and during the infusions, blood samples were drawn to measure tracer enrichments, metabolites and hormones. All participants were studied on two study days spaced by at least 7 days according to identical protocols, except for the intranasal administration of insulin or placebo at zero time (18). One puff of the 0.1 ml spray solution contained either 10 IU human insulin (100 IU/ml, Insulin 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 8 CON received 0.1 IU of human insulin (Actrapid) intravenously and otherwise underwent the identical study protocol.

**<sup>1</sup>H/<sup>31</sup>P magnetic resonance spectroscopy (MRS).** All measurements were performed in a 3-Tesla MR scanner (Achieva 3T Philips, Best, The Netherlands) using a 14-cm circular <sup>31</sup>P surface transmit-receive coil (Philips Healthcare, Best, The Netherlands) for <sup>31</sup>P-MRS and the built-in <sup>1</sup>H whole body coil for localization and proton spectroscopy. The participants were scanned in the supine position at baseline, +180 min and in the long-duration subgroup at +360 min. For the acquisition of <sup>31</sup>P spectra a volume of interest of 6x6x6 cm<sup>3</sup> was positioned within the liver and 3-D 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: 3000 Hz, data points: 2K). Absolute quantification of phosphorus metabolites (γ-ATP and Pi) was performed as described (20) using the AMARES algorithm (advanced method for

accurate, robust and efficient spectral fitting of MRS data) (21) in jMRUI (Java-based Magnetic Resonance User Interface) (22).

For assessment of liver fat content, a set of non-water suppressed and water suppressed  $^1\text{H}$  spectra were acquired using stimulated echo acquisition mode (repetition time/echo time/mixing time: =4000/10/16 ms, number of signal averages: =32, volume of interest: = $3 \times 3 \times 2 \text{ cm}^3$ ). Data from localized  $^1\text{H}$ -MRS was analyzed to assess fat content as described (23) and absolute concentrations were expressed as percent hepatocellular lipids relative to water content (HCL) 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 inter-observer variability in spectral processing of  $^{31}\text{P}$ -MRS was reported previously (20).

**Metabolites and hormones.** Blood samples were immediately chilled, centrifuged and supernatants were stored at  $-80 \text{ C}$  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 (TG) were enzymatically analyzed on a Roche/Cobas c 311 analyzer (Roche Diagnostics, Mannheim, Germany). FFA were quantified enzymatically (intra-assay coefficient of variation [CV]: 1%, inter-assay CV: 2.4%; Wako, Neuss, Germany) in samples, which contained orlistat to prevent ex vivo lipolysis (27). Serum C-peptide, serum insulin and plasma glucagon were measured by radioimmunoassay (intra-assay CV for all: 4-6%; inter-assay CV: 6-7, 5-9, and 5-10%, respectively; Millipore, St. Charles, Miss, USA).

**Gas chromatography-mass spectrometry.** After plasma deproteinization using  $\text{Ba}(\text{OH})_2\text{-ZnSO}_4$ , atom percent enrichments (APE) of  $^2\text{H}$  were measured on a Hewlett-Packard 6890 gas chromatograph equipped with a 25-m CPSil5CB capillary column (0.2-mm inner diameter,

0.12- $\mu\text{m}$  film thickness; Chrompack/Varian, Middelburg, The Netherlands), interfaced to a Hewlett Packard 5975 mass selective detector (Hewlett Packard) as described previously (28). APE of the fragments C3 to C6 with the average mass units 187 for the endogenous glucose and 189 for the  $[6,6\text{-}^2\text{H}_2]$  glucose were determined by using selected ion monitoring. APE was calculated as mass ratio, corresponding to the tracer enrichment in plasma glucose. Intra- and inter-assay CVs were 0.6 and 1.0%, respectively.

**Calculations.** Rates of EGP were calculated by dividing the tracer ( $[6,6\text{-}^2\text{H}_2]$ glucose) infusion rate times tracer enrichment by the tracer enrichment in plasma glucose and subtracting the tracer infusion rate (25). Fasting hepatic insulin sensitivity was estimated as HIS index by multiplying 100 times the inverse of the product of EGP and serum insulin (29). Homeostasis Model Assessment (HOMA) indices of fasting insulin resistance and  $\beta$ -cell function and the Quantitative Insulin Sensitivity Check Index (QUICKI) were calculated as described (30; 31).

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

## Results

### Participants' characteristics

As expected, type 2 diabetes patients had higher age, body mass index, waist circumference, HbA1c, fasting blood glucose, serum C-peptide and cholesterol levels than CON (Table 1). Mean fasting insulin levels trended to be higher, but were not significantly different from CON, likely due to larger within-group variability in type 2 diabetes patients. Indices of insulin resistance, insulin sensitivity and  $\beta$ -cell function were different between groups, whereas fasting plasma TG and FFA were comparable. Within each group, no differences in baseline EGP, hormones and metabolites between placebo, intranasal and intravenous insulin studies were noted.

### **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 x time) with a maximal decrease of about 5% at 30 to 40 min compared to placebo (Fig. 1A,B). Likewise, the  $AUC_{0-180min}$  for glucose was lower after insulin application only in CON (Fig. 2A). 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 x time) (Fig. 1I) and in a greater maximal decrease ( $138\pm 41$   $\mu\text{mol/l}$ ,  $33.3\pm 8.5\%$ ) at 60 min vs baseline than after placebo administration ( $23.4\pm 27.3$   $\mu\text{mol/l}$ ,  $-11.7\pm 9.7\%$ ). No changes were observed in type 2 diabetes patients ( $p=0.456_{ANOVA}$  time x treatment) (Fig. 1J). The  $AUC_{0-180min}$  and  $AUC_{0-60min}$  for FFA were not different between experimental conditions in both groups (Fig. 2B). TG and glycerol levels remained unchanged and the respective  $AUC_{0-180min}$  were similar after both interventions in CON and type 2 diabetes patients (Fig. 2C,D).

In CON, serum insulin levels transiently increased by  $3.7\pm 0.7$   $\mu\text{U/ml}$  and nearly doubled (92%) at 10 min after insulin vs. placebo ( $p<0.001_{ANOVA}$  time x treatment) (Fig. 1C). In type 2 diabetes patients, the trend towards an increase in serum insulin by 59% at 10 min was not

significant ( $4.9 \pm 2.1 \mu\text{U/ml}$ ,  $p=0.3_{\text{ANOVA}}$  time x treatment) (Fig. 1D). In subgroups studied for 360 min after intranasal insulin/placebo, similar changes in the time course were found at 10 min in CON and at 20 min in type 2 diabetes patients. Overall,  $\text{AUC}_{0-180\text{min}}$  for insulin was comparable after insulin vs placebo administration in both groups (Fig.2E). After intranasal insulin serum C-peptide levels decreased only in CON and remained lower compared to placebo from 30 to 180 min ( $p<0.01_{\text{ANOVA}}$  time x treatment) (Fig.1E,F). In CON,  $\text{AUC}_{0-180\text{min}}$  for C-peptide was lower after intranasal insulin vs placebo, but not in type 2 diabetes patients (Fig.2F). Lower C-peptide levels were found also at 40, 100, 120 and 140 min after insulin in the subgroup of CON studied for 360 min, but not in type 2 diabetes patients. Neither intranasal insulin nor placebo affected glucagon concentrations in CON and type 2 diabetes patients (Fig.1G,H).

### **Effects of intranasal insulin on endogenous glucose production and hepatic insulin sensitivity**

Baseline EGP was similar under insulin and placebo conditions in both groups ( $p=0.93$  CON insulin vs placebo,  $p=0.98$  type 2 diabetes patients 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 values of HIS index in CON than in type 2 diabetes patients at baseline. In CON, HIS increased by  $4.0 \pm 1.1$  and by  $3.7 \pm 1.4$   $\text{kg}\cdot\text{min}\cdot\text{dl}/(\text{mg}\cdot\mu\text{U})$  after both insulin and placebo application, respectively ( $p<0.05$  at 180 min vs baseline for insulin and placebo), but was not altered in type 2 diabetes patients (Fig.3A,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 type 2 diabetes patients, HCL did not change under both experimental conditions (Fig.4A,B).

Baseline hepatic ATP and Pi concentrations were not different between both conditions in CON and type 2 diabetes patients. In CON, ATP increased at 180 min after insulin by 18% ( $0.5 \pm 0.2$  mmol/l,  $p=0.03$  vs baseline), but was 23% lower in type 2 diabetes patients at 180 min after intranasal insulin than after placebo ( $2.8 \pm 0.2$  and  $3.4 \pm 0.2$  mmol/l, respectively,  $p=0.03$  vs placebo) (Fig.4C,D). Intranasal insulin did not affect hepatic Pi in both groups, while it increased only in type 2 diabetes patients by  $0.4 \pm 0.1$  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  $AUC_{0-20min}$  (Fig.5A) and a trend for higher insulin levels under both conditions compared with placebo was observed ( $p=0.110$  intranasal insulin vs placebo and  $p=0.088$  intravenous insulin vs placebo). Also the absolute maximal decrease in blood glucose (Fig.5B) and plasma FFA was comparable between intravenous and intranasal insulin studies and similarly differed from placebo ( $p=0.02$   $\Delta_{max}$  glucose intravenous insulin vs placebo,  $p<0.0001$   $\Delta_{max}$  FFA intravenous insulin vs placebo). No differences were observed in EGP after intravenous insulin administration. Relative percent increase in HCL was 34% ( $p=0.02$  baseline vs 180 min) (Fig.5C), whereas hepatic ATP concentrations remained unaltered (Fig.5D).

### **Discussion**

This study found that intranasal insulin administration does not affect glucose production over up to 6 hours in humans with or without type 2 diabetes. However, intranasal insulin resulted in increased absolute hepatic ATP and decreased hepatic triglyceride contents in the 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. Surprisingly, intravenous application of insulin leading to comparable changes in circulating insulin, glucose and FFA did not affect hepatic ATP, but increased hepatic triglyceride contents.

In CON, blood glucose levels transiently and slightly decreased after intranasal insulin administration. Comparable insulin doses were reported to lead to significant changes in blood glucose levels by some (32; 33), but not by all previous studies (18; 34). Of note, the fall in blood glucose was preceded by a temporary rise in serum insulin concentration along with prolonged reduction of C-peptide levels. Again, this increase in serum insulin is consistent with some (32-34), but not with other previous reports (18). The absence of detection of these ultra short-term changes in circulating insulin and glucose is likely due to lower frequency of measurements in the previous studies. Even the present 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  $AUC_{0-180}$  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/or improved insulin action. The transient decrease in glucose concentration in CON could be due to greater glucose disposal and/or hepatic insulin sensitivity (35). In

elderly type 2 diabetes patients, the response of peripheral insulin levels to intranasal insulin varied considerably, which might be due to altered intranasal mucosal function. This as well as the impaired  $\beta$ -cell function and ambient hyperglycemia likely contributed to the absence of changes in the measured metabolites and hormones.

We assessed EGP in fasting physiological state and related it to ambient insulinemia to provide a direct measure of fasting hepatic insulin sensitivity (29), which takes into account the sensitivity 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 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 non-physiologic 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 in part explain the increase in hepatic ATP of the present study.

While central regulation of glucose metabolism by insulin in humans cannot be excluded, hepatic insulin sensitivity is predominantly modulated by direct insulin action (38). In line, we

detected no changes in EGP for up to 6 hours after nasal insulin application suggesting that brain insulin signalling 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 hours 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 to 6 hours again during pancreatic clamps (7). These studies point to interesting effects of central insulin signalling 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 via the intranasal route on HIS. This seems to differ from recent findings suggesting that intranasal insulin might modulate insulin sensitivity of glucose metabolism via brain regions known to affect the regulation of the autonomous nervous system outflow (33). Nevertheless, the present 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 due to prolonged fasting state after placebo administration and to compensatory reduction of insulin secretion at 3 hours after the preceding insulin peak after intranasal insulin administration.

Interestingly, the present study demonstrated that HCL transiently decrease upon intranasal insulin exclusively in CON. These findings suggest novel peripheral metabolic effects of intranasal insulin, but require scrutiny and careful interpretation, as both brain insulin signaling and increased serum insulin could be responsible. To address this question, we

applied insulin intravenously to match the spill-over of intranasal insulin in the peripheral circulation in a subgroup of CON. Interestingly, intravenous insulin application resulted in increased HCL at 180 min, which is in line with insulin's known effect on lipogenesis and the observed increase in HCL 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, our control persons exhibited very low baseline HCL ( $0.5 \pm 0.1\%$ ) and the slight decrease in HCL observed at 180 min disappeared at 360 min. However, a previous study reported catabolic effects of chronic intranasal insulin administration in men (11), which could also reduce liver fat storage. Moreover, the contrasting increase in HCL observed with intravenous insulin rather suggests the intranasal route exerts unique HCL lowering effects. The moderate reduction of FFA levels after intranasal insulin in our CON is likely due to the known direct insulin effect on adipose tissue lipolysis, which is usually blunted in insulin resistant type 2 diabetes patients. Of note, previous studies described diminished lipolysis and enhanced lipogenesis by brain insulin signalling 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 HCL in CON of the present study. On the other hand, the lack of any effect of intranasal insulin on HCL in the type 2 diabetes patients group could result from 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 or adipose tissue insulin resistance. Of note, lipid lowering medication in three of ten patients with type 2 diabetes was not withdrawn prior to testing, which limits the interpretation of possible effects on lipogenesis in the present study.

Another novel finding of the present study was the rise in hepatic ATP after intranasal insulin administration to CON, but not to type 2 diabetes patients. 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 non-alcoholic fatty liver disease (25; 42). In skeletal muscle, insulin can increase flux through ATP synthase in healthy humans but not in type 2 diabetes patients (43), indicating impaired mitochondrial plasticity in insulin resistant states (42). We recently reported that lower muscle ATP synthase flux associates with higher HCL in humans (44). Impaired mitochondrial plasticity could therefore be present also in liver of patients with type 2 diabetes of the present study. However, we cannot exclude that brain insulin signalling contributes to regulation of liver energy homeostasis, as intranasal insulin can enhance energy levels in brain (45). It remains unclear whether the diminished response to intranasal insulin in the type 2 diabetes patients group is due to participants' age, insulin resistance or 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 physiologic fasting conditions as well as from using localized measures of intrahepatic energy metabolism independent of EGP for the first time. In addition, this study confirmed the spill-over 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). As the spill-over 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 spill-over on circulating insulin and metabolites. Nevertheless, this approach does not allow detecting the intracerebral mechanisms leading to the observed hepatic effects. Finally the present study cannot discriminate whether the

different response of the type 2 diabetes patients 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 hepatic insulin sensitivity 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.

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**Table****Table 1. Characteristics of participants**

<b>Parameters</b>	<b>Control</b>	<b>Type 2 diabetes</b>
	<b>participants</b>	<b>patients</b>
n (females)	10 (3)	10 (1)
Age [years]	25.7 ± 1.6	60.9 ± 2.0*
BMI [kg/m <sup>2</sup> ]	23.1 ± 0.9	29.0 ± 1.0*
Waist [cm]	78.2 ± 5.7	103.2 ± 3.0*
Plasma triglycerides [mg/dl]	61 ± 8	97 ± 19
Plasma free fatty acids [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*
HbA1c [%]	5.1 ± 0.1	7.5 ± 0.4*
HbA1c [mmol/mol]	31 ± 1	58 ± 4*
Alanine aminotransferase [U/l]	21 ± 2	30 ± 4
Aspartat aminotransferase [U/l]	19 ± 1	21 ± 2
HOMA-IR	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	1840 ± 468*
Liver fat [HCL%]	0.5 ± 0.1	7.7 ± 1.6*

Data presented as mean value ± SEM; \*p<0.05 vs CON

## Figure legends

**Fig. 1 Time course of blood glucose (A and B), serum insulin (C and D), C-peptide (E and F), s glucagon (G and H) and free fatty acid (I and J).** Intranasal insulin/placebo were applied at time point 0 min. Data are means  $\pm$  SEM; healthy participants (CON, n=10), type 2 diabetes patients (T2D, n=10); \*p<0.05 insulin vs placebo

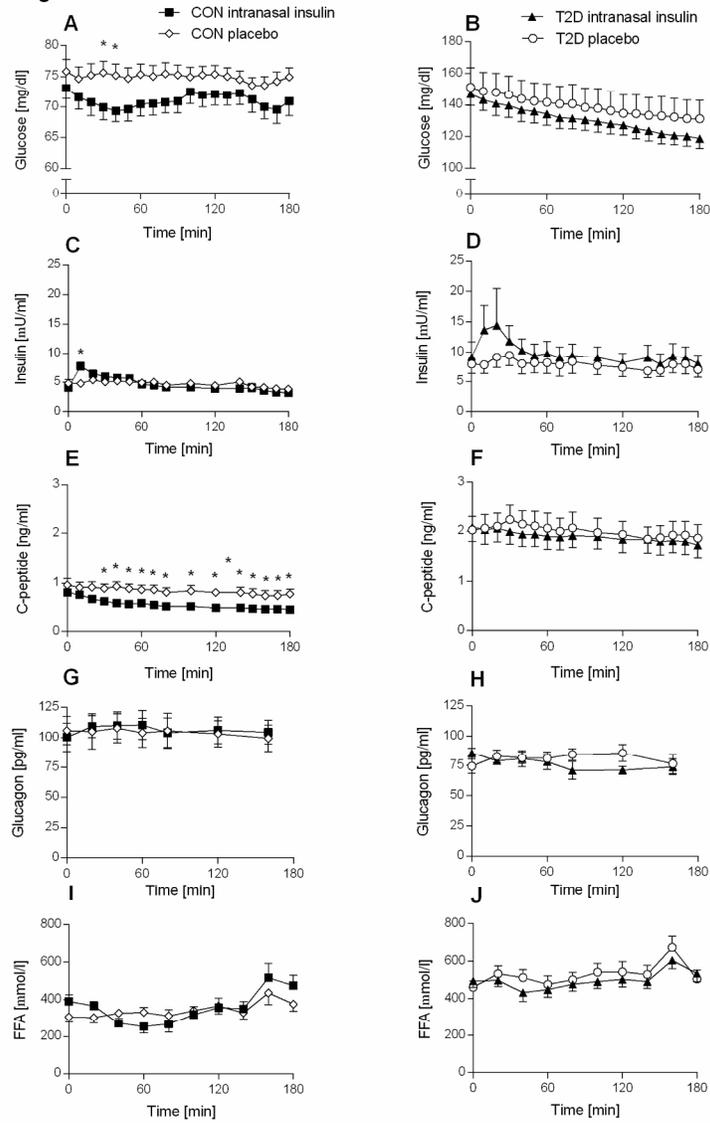
**Fig. 2 Comparison of AUC<sub>0-180min</sub> for metabolic parameters.** A – blood glucose, B – plasma free fatty acids, C – plasma triglycerides, D – plasma glycerol, E – serum insulin, F – serum C-peptide. Data are means  $\pm$  SEM; healthy participants (CON, n=10), type 2 diabetes patients (T2D, n=10); \*p<0.05 CON vs T2D; #p<0.05 CON insulin vs placebo

**Fig. 3 Hepatic insulin sensitivity index (HIS) in healthy participants (CON) (A) and type 2 diabetes patients (T2D) (B) after intranasal insulin/placebo (given at zero time).** Data are means  $\pm$  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

**Fig. 4 Absolute changes in liver fat content (hepatocellular lipids, HCL %) and hepatic ATP in healthy participants (CON) and type 2 diabetes patients (T2D) after intranasal insulin/placebo.** A and B – liver fat content, C and D - ATP concentrations. Data are means  $\pm$  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

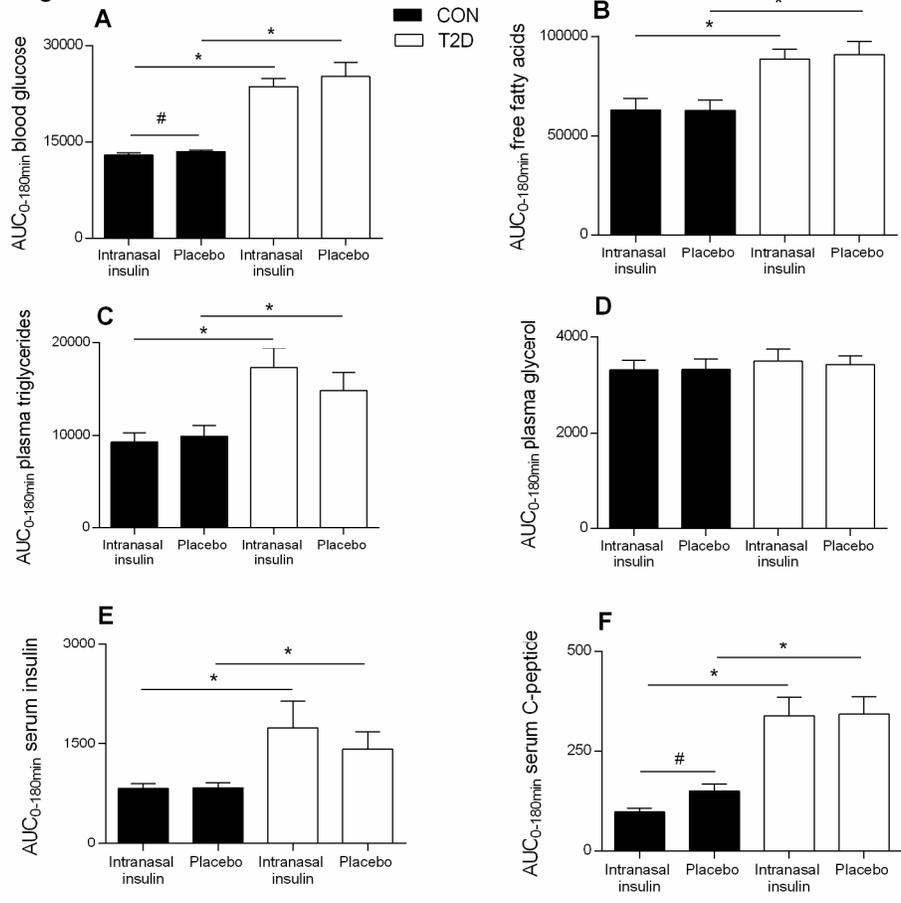
**Fig. 5 Absolute change in AUC<sub>0-20min</sub> for serum insulin (A), maximal decrease in blood glucose (B) ( $\Delta_{\max}$ =nadir value-baseline), absolute change in hepatocellular lipids (HCL, %) (C) and hepatic ATP concentrations (D) after intranasal insulin and intravenous (iv) insulin application in CON subgroup.** Data presented as mean  $\pm$  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

Fig.1

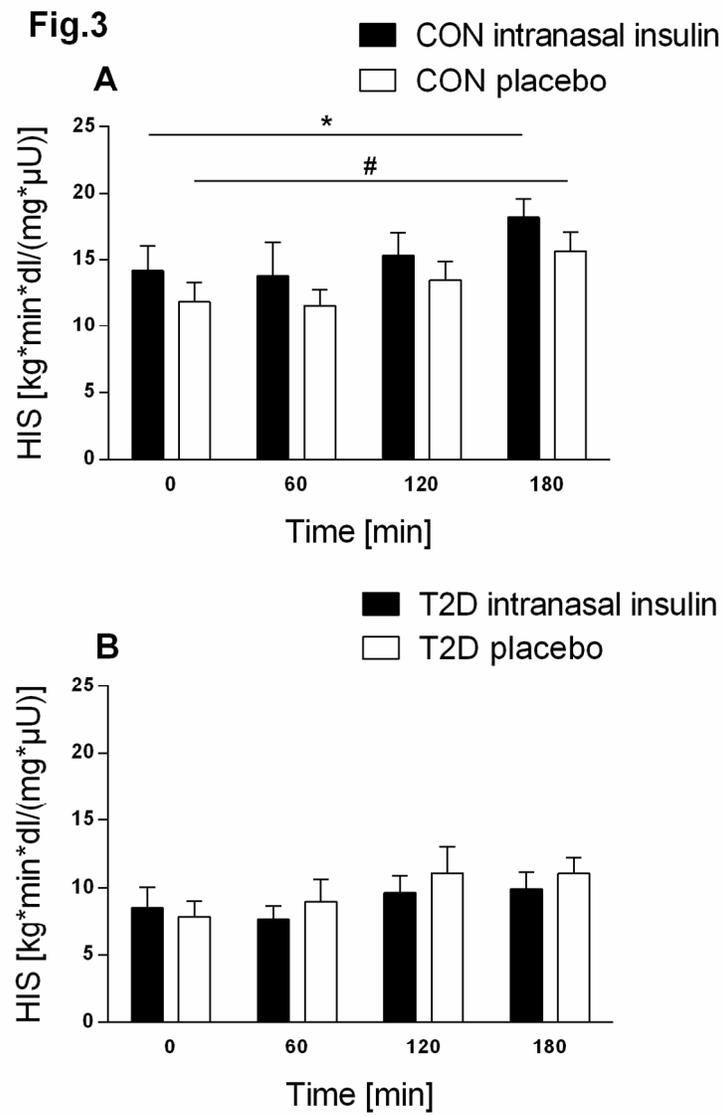


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Fig.2

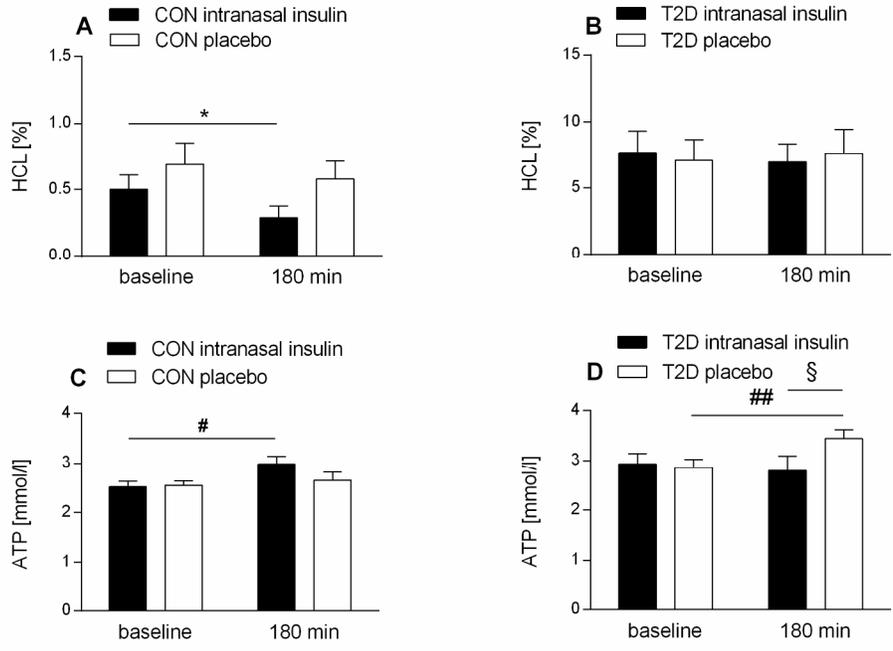


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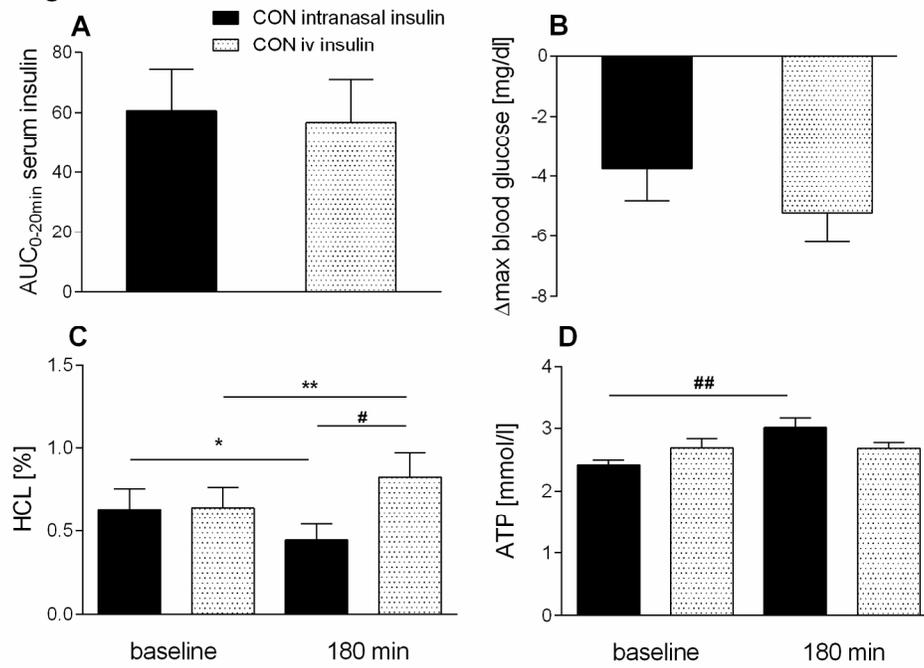
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Fig.4



203x149mm (300 x 300 DPI)

Fig.5



174x132mm (300 x 300 DPI)