Treatment of diabetic rats with insulin or a synthetic insulin receptor agonist peptide leads to divergent metabolic responses

Running title:
Divergent metabolic response to insulin agonist

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Abstract

In addition to lowering of blood glucose, treatment with insulin also induces lipid synthesis and storage. Patients with type 2 diabetes often suffer from lipid-related co-morbidities including dyslipidaemia, obesity, and fatty liver disease. We examined here in two separate studies changes in lipid dynamics in Zucker Diabetic Fatty (ZDF) rats, in response to 7 days of treatment with either insulin or the insulin receptor agonist peptide S597. In concert with blood glucose normalization, the treated rats displayed large increases in hepatic *de novo* lipid synthesis and deposition of newly synthesized lipids in adipose tissue depots, accompanied by weight gain and expansion of adipose depots. In both treatment groups, heavy water labeling revealed that after 2 hours (study A), *de novo* lipogenesis was responsible for 80% of newly stored hepatic TG-palmitate, and after 5 days (study B), ~60% of newly deposited TG-palmitate in adipose tissues originated from this pathway. Interestingly, in both studies, treatment with the insulin mimetic peptide resulted in significantly lower blood TG levels, plasma TG production rates and hepatic *de novo* synthesized fatty acid in plasma TG compared to insulin. There were no differences in plasma TG turnover (clearance rate) in response to either treatment, consistent with differential actions on the liver. These results show that in ZDF rats, treatment with a synthetic insulin receptor-activating peptide or with insulin to lower blood glucose is accompanied by different effects on hepatic lipid anabolism and blood TG profiles.
A major target of interest when treating diabetic patients with insulin is lowering of blood glucose levels through activation of distinct signaling pathways downstream of the insulin receptor (1;2) in tissues including muscle and adipose (1;2). Insulin-regulated metabolic signaling also results in alterations of lipid homeostasis. Expansion of adipose depots is known to occur in animals as well as in diabetic patients during insulin treatment (3;4). In insulin resistant animals, there is evidence that the hepatic insulin receptor signaling pathways show differential responsiveness, such that the glucose related pathways are more resistant than the ones orchestrating lipid responses (5). This results in the so-called insulin resistance triad where two of the elements (hyperglycemia and hyperinsulinemia) reflect the reduced responsiveness to insulin on glucose metabolism, whereas the third element (hypertriglyceridemia) is believed to arise from the lipid-related pathways being less resistant and therefore excessively stimulated by the hyperinsulinemia (5). The hepatic metabolic response to insulin includes several processes: inhibition of endogenous glucose production (6), augmented glucose uptake (7), stimulation of de novo lipogenesis (DNL) (8), and inhibition of VLDL export (9). Insulin also induces fatty acid synthesis (10) and adipogenesis (11) in adipose tissues.

The main purpose of our experiments here was to assess lipid synthesis and storage in ZDF rats in response to insulin treatment. We used heavy water labeling to quantify in situ to what extent these processes are affected by treatment with insulin or the synthetic insulin receptor agonist peptide S597.

Previous tissue culture experiments have shown that S597 differentially affected the mitogenic signaling downstream of the insulin receptor and the metabolic response (12;13). Since the
classic metabolic responses such as inhibition of glucose production and induction of lipid synthesis diverge downstream from the insulin receptor and are differently affected in hepatic insulin resistance, our aim was to investigate if the intrinsic capability of S597 to differentially stimulate signaling also differently affected lipid and glucose metabolism in the whole animal.

Research Design and Methods:

Two independent studies were carried out, which we will call study A and B. In study A, heavy water labeling took place for 2 hours to measure acute effects on hepatic lipid synthesis. A group of lean heterozygous ZDF rats (Lean Vehicle group) was also included to reference if normoglycemia was reached with treatment. In study B, the labeling time was increased to 5 days to allow measurement of effects on lipid synthesis in adipose depots. In both studies, osmotic minipump treatment was for 7 days.

Animals:

All animal experiments were approved by the University of California Berkeley Animal Care and Use Committee (study A) or the Danish Animal Experiment Inspectorate (study B). Twelve-week-old male ZDF or heterozygous lean control (Lean Vehicle) rats (Charles River Breeding Laboratories, Portage, MI (study A) or Sulzfeld, GE (study B)) were fed the standard ZDF diet (Formulab 5008, LabDiet®) ad libitum. They were stratified by blood glucose, body weight, and glycated hemoglobin (HbA1c) values prior to randomization. In study B, the rats were additionally stratified for plasma TG levels.

Treatment with insulin or S597
At the age of 12 weeks, the rats had osmotic minipumps (Alzet®, Model 2ML2, Cupertino, CA) inserted subcutaneously under isoflurane anaesthesia. The pumps contained either vehicle (phosphate buffer) (Lean Vehicle and ZDF Vehicle), insulin Actrapid® (0.2mM) (Novo Nordisk, Bagsvaerd, DK) (Insulin), or S597 (0.4mM) (S597). Analgesia was provided by i.m. injection of 2mg/kg meloxicam (Metacam®, Boehringer Ingelheim, Ridgefield, CT) immediately prior to and every day for 3 days after implantation (study A) or by s.c. injection of 5mg/kg carprofen (Rimadyl®, Pfizer, Dundee, UK) (study B). In study B animals also received a single s.c. injection of enrofloxacin (Baytril®, Bayer, Leverkusen, DE). Throughout the treatment period all rats were weighed daily. The pumps remained inserted for 7 days, after which the rats were euthanized by cardiac puncture under isoflurane anaesthesia.

$^2$H$_2$O labelling protocol

Each rat received an i.p. injection (35ml pr kg body weight) of 100% $^2$H$_2$O (Cambridge isotopes, Andover, MA, USA) with 0.9% NaCl added for isotonicity. In study A, this was done 2 hours prior to euthanisation in non-fasted animals whereas in study B, the injection was given 5 days prior to euthanisation (i.e., 2 days after pump implantation). Sustained labeling took place by replacing drinking water with 8% $^2$H$_2$O for a sustained body water enrichment of ~5% (14).

Blood glucose and HbA1c quantification

Blood glucose was measured daily (Study A: Contour® Blood Glucose Meter, Bayer, Mishawaka, IN; study B: Biosen, EKF Diagnostic GmBH, Barleben, DE).
HbA1c was quantified in full blood on the day prior to pump implantation by tail vein bleed and at euthanisation by cardiac puncture. In study A kits from Chrystal Chem were used (catalog# 80300, Chrystal Chem, Downers Grove, IL, US). In study B HbA1c was measured by the use of standard laboratory procedures (Hitachi 912 automatic analyzer, Boehringer Mannheim).

**Body composition measurement**

In study B, body composition was measured in unanaesthetized animals using quantitative magnetic resonance (QMR) to determine the body water content, from which relative body fat content is calculated (EchoMRI, Echo Medical Systems, Houston, TX).

**Lipid quantification in liver and plasma**

All plasma lipids except free fatty acids (FFA) were quantified in EDTA-stabilized plasma. FFA was measured in NaF-stabilized plasma.

Plasma TG levels in study A were quantified by Cayman Chemicals Triglyceride Colorimetric Kit (Cayman Chemicals, Ann Arbor, MI, USA). Plasma TG levels in study B, hepatic TG levels in both studies, and cholesterol levels in both studies, were all measured by standard laboratory procedures (Hitachi 912 automatic analyzer, Boehringer Mannheim). Serum-free palmitate levels were quantified by gas chromatography-flame ionization detection as previously described (15) with heptadecanoic acid used as an internal standard. For the plasma samples that underwent thin layer chromatography (TLC) prior to derivatisation, heptadecanoic acid was
replaced by tripentadecaonin to ensure that this internal standard followed the TG fraction on the
tLC plates.

*Extraction, derivatization and analysis of TG-glycerol, palmitate, cholesterol, and body water*

For analysis of tissue approximately 100mg of tissue (the exact weight was known) or 150 µl of
plasma was placed in 2:1 methanol:chloroform for overnight lipid extraction. Subsequently the
fatty acids (FA) were separated from glycerol by methylation with 5% methanolic acid by
heating at 50°C for 1 h (16).

The FA methyl esters were extracted with hexane after the addition of water, whereas the phase
containing free glycerol was dried down and converted to glycerol triacetate by incubation with
2:1 acetic anhydride:pyridine. The derivatized compounds were then run on GC-MS, as
described elsewhere (15).

Body water was isolated by overnight distillation of whole blood and processed for analysis of
deuterium enrichment, as described previously (17).

*Adipogenesis in situ*

Adipocytes from the distinct depots were isolated by collagenase digestion of the fresh tissue, as
described elsewhere (18).

Isotopic enrichment of DNA was performed as previously described (19;20). Briefly DNA was
extracted following the protocol for samples with a low number of cells (21).

*Calculations*
Fractional synthesis:

The fraction of newly synthesized TG-glycerol and -palmitate formed during the $^2$H$_2$O labeling period was assessed using a combinatorial model of polymerization biosynthesis, as described previously (14;17).

Corrected fractional synthesis

Measurement of fractional palmitate synthesis includes dilution by all unlabelled palmitate that was present in the adipose depots before the labelling experiment began. This causes an underestimation of the fractional contribution from *de novo* palmitate to the newly assembled and deposited TG. To correct for this underestimation, the ratio of fractional palmitate synthesis to fractional TG-glycerol synthesis is calculated, representing the fractional contribution from newly synthesized palmitates to the newly assembled and deposited TGs (22).

Absolute rates of palmitate and TG-glycerol synthesis

The absolute synthesis rate of palmitate/TG-glycerol in the tissues was calculated by multiplying the fractional synthesis rate by the amount of palmitate-TG present within each sample.

Fractional glyceroneogenesis

From the enrichment of the glycerol moiety of TGs, the average number of deuterium incorporation sites within each molecule was calculated. As synthesis of glycerol via glyceroneogenesis and glycolysis, respectively, results in a different number of available sites for deuterium from deuterated water to become covalently incorporated, it is possible to calculate the fractional contributions from each pathway. A complete description of these calculations was presented elsewhere (23).
Statistical analysis

Statistical significance was tested by t-test, one-way ANOVA, or two-way ANOVA as appropriate (the two latter followed by Bonferroni pairwise comparisons). The type of test used is given with each figure. Prior to statistical analysis the ROUT outlier test with Q=1% was applied to all datasets to exclude potential outliers. Datasets that did not meet the requirements for equal variances were logarithm or square root transformed prior to analysis. p < 0.05 was considered significant for all tests. All statistical analysis were performed in Prism (version 5, GraphPad Software, Inc., La Jolla, CA).

Results

Two similar but independent studies were carried out in order to follow lipid dynamics: study A and study B. In study A, heavy water labeling took place for 2 hours to follow hepatic lipid synthesis and a lean control (Lean Vehicle) group was included. In study B, the labeling time was increased to 5 days to allow measurement of lipid synthesis in the adipose depots.

Blood glucose lowering by insulin and S597

In both studies, diabetic ZDF rats had osmotic minipumps implanted releasing either phosphate buffer (Lean Vehicle and ZDF Vehicle), insulin, or S597. The doses chosen resulted in blood
glucose levels that ended close to the levels in the non-diabetic Lean Vehicle group (fig 1A). The blood glucose lowering happened slightly more slowly with S597 than with insulin. During the heavy water labeling period, however, the response was similar (fig 1A and 1B). These findings were supported by the glucose AUC as well as the HbA1c values (data not shown) both of which were significantly lower when compared to the ZDF vehicle group but did not differ between insulin and S597 treatments.

**Body and tissue weight**

In both studies, the insulin or S597 treated animals gained more body weight than the vehicle treated ZDFs (Suppl. fig 1A and suppl. fig. 1B). Food intake, however, was not significantly different between groups (data not shown). qMR scans in study B showed that the insulin or S597 treated rats gained significantly more fat mass than the ZDF Vehicle group (table 1) with a non-significant trend for the S597 group to gain less fat than the insulin group. The same pattern was observed for the liver weights (fig 2A) where the untreated rats had significantly smaller livers than the treated groups. Additionally, S597 treatment resulted in slightly less expansion of liver mass than regular insulin. The subcutaneous adipose depot was highly responsive to treatment with a 25% increase in mass when comparing to the ZDF Vehicle group (fig 2B). Of the remaining adipose depots the epididymal and perirenal fat pads both increased slightly with insulin treatment whereas the mesenteric depot was unaffected (Suppl. fig 2A-C).

**Lipid levels in plasma, liver, and adipose tissues**
Total plasma and liver TG and palmitate levels were assessed at the end of both studies (Table 2). TG and palmitate both increased in plasma with insulin treatment albeit this was significant only in study B. A very prominent reduction in plasma TG levels, however, was observed with S597 treatment in both studies. The levels were more than halved as compared to Insulin and were decreased even below the levels found in the vehicle treated ZDF rats. The same pattern from S597 treatment was observed with plasma palmitate. In contrast, plasma FFAs were reduced with both insulin and S597 treatment and in study A slight increase in plasma cholesterol was found. The estimated TG content of the subcutaneous adipose depot reflected increased adipose mass and was highly responsive to treatment. The palmitate levels also showed a non-significant trend towards increasing with insulin or S597 treatment. Of the remaining adipose depots (supplemental table 1) the epididymal and perirenal fat pads both increased in mass with insulin and S597 treatment, whereas the mesenteric content of palmitate and TG did not change.

*Hepatic and adipose DNL*

In all tissues studied, fractional palmitate synthesis was greatly induced by insulin or S597 treatment, with approximately 3-fold increases in fractional synthesis in liver and plasma and a 10-fold increase in the subcutaneous adipose depot (figure 3A-C). No differences between the insulin and S597 groups were observed for fractional palmitate synthesis in any tissue studied. The corrected fractional synthesis (figure 3D-F) showed that in tissues from the vehicle treated ZDF rats only ca. 20% of the palmitate present in new TG molecules was *de novo* synthesized, whereas with treatment, this fraction increased to about 80% (liver) and even higher (plasma),
reflecting almost exclusively a DNL contribution to palmitate for new TG assembly. Interestingly, when compared to insulin, S597 gave rise to slightly but significantly higher corrected fractional synthesis contribution in plasma, yet when accounting for the total amounts of TG-palmitate present in plasma, the net result was a halving of newly synthesized palmitate (figure 3H). The massive increase in fractional synthesis in the subcutaneous depot was also observed in the remaining adipose depots (see supplemental figure 3), although fractional synthesis, corrected fractional synthesis and absolute palmitate synthesis were all non-significantly lower with S597 treatment.

**Hepatic and adipose TG-glycerol synthesis (all-source TG synthesis)**

TG-glycerol synthesis rate measures new TG formation, irrespective of the source of the fatty acids. In contrast to what was observed with palmitate synthesis, the fractional synthesis values for TG-glycerol in the liver and plasma over 2 hours (fig 4A-B) were affected only slightly by insulin or S597 treatment. This parameter reflects replacement rate or half-life of TG in the compartment and remained relatively constant at around 20% in the liver and 80% in plasma. There was only a small, though significant, increase with insulin treatment as compared to ZDF vehicle in the liver. In the adipose depots, in contrast, there was a significant increase in fractional TG-glycerol synthesis over 5 days with insulin or S597 treatment. Interestingly, fractional TG-glycerol synthesis was slightly but consistently and significantly higher in the S597 treated group as compared to the insulin group in all adipose depots (fig 4C for subcutaneous depot; supplemental figure 4A-C for the epididymal, perirenal, and mesenteric depots). Calculation of the absolute amounts of newly assembled TG (figure 4D-E and table 2)
revealed that the amounts of new TG in plasma were more than halved with S597 treatment as compared to both the insulin and vehicle groups. In all adipose depots, significantly more lipids were accumulated in the form of TG in response to insulin or S597 treatment (Figure 4I for the subcutaneous depot and supplemental figure 4D-F for the epididymal, perirenal, and mesenteric depots).

**Metabolic source of hepatic and adipose TG-glycerol synthesis**

The glycerol used for TG assembly can originate from two distinct biosynthetic pathways: glyceroneogenesis or glycolysis. The fraction of new glycerol being synthesized via the glyceroneogenic pathways is given in figure 4G-I. In liver and plasma, more than 70% of the new glycerol for TG came from glyceroneogenesis, whereas only up to 30% originated from this pathway in the adipose tissue (the same was observed in the remaining adipose depots; data not shown). Treatment affected the tissue specific biosynthetic origin differently. In liver and plasma, slightly more TG-glycerol originated from glycolysis in the insulin and S597 treated groups as compared to the vehicle treated ZDF group, whereas the percentage contribution from this pathway decreased slightly but significantly in the subcutaneous adipose depot after treatment.

In summary, these TG-glycerol synthesis results indicate that storage of newly assembled TG took place in the adipose depots upon insulin treatment, whereas TG synthesis in liver and plasma remained relatively unaffected. With S597 treatment, however, the amount of new TG was greatly reduced in plasma, and in all adipose depots the fractional TG-glycerol synthesis
rates were slightly but significantly increased. This could indicate a small improvement in TG uptake in the adipose tissues that was more sensitive than net TG accumulation, which did not reflect any difference between treatments.

*Adipogenesis in situ*

*In situ* adipogenesis was investigated by quantifying the fractional amount of newly replicated DNA in freshly isolated adipocytes (Figure 5A-B). In the subcutaneous depot, insulin treatment caused a significant up-regulation of DNA synthesis in the adipocytes, whereas S597 did not cause any significant changes in fractional DNA synthesis. In the epididymal depot, however, the adipogenic response to S597 treatment was more pronounced, with a doubling of fractional DNA synthesis as compared to the ZDF vehicle group, while insulin treatment had no significant effect.

**Discussion**

In the current studies, we set out to investigate the effects of insulin on lipid dynamics in the diabetic, insulin resistant ZDF rat model. Additionally, we tested whether the intrinsic capability of S597 to stimulate signaling downstream of the insulin receptor was differentiated from the effects of insulin at equal glucose-reducing doses in these animals.

Treatment of ZDF rats with the insulin receptor agonist peptide S597 by mini-osmotic pump resulted in significantly lower plasma TG levels than treatment with insulin, despite no significant differences in glycemic control, in both of the 7-day treatment studies that we carried out. In one of the studies, insulin treatment significantly increased the already elevated plasma
TG levels in ZDF rats, while S597 had no effect on TGs. In the other study, S597 significantly lowered plasma TGs while insulin did not have a significant effect. The differential plasma TG response was associated with a significantly lower plasma TG production rate and significantly less stimulation of hepatic de novo synthesized fatty acid input into plasma TG in response to S597. Importantly, there were no differences in plasma TG replacement (clearance rate) in response to either treatment, consistent with the differential effect acting through liver metabolism rather than peripheral clearance of TG.

The biosynthetic signature of blood lipids reflected that in liver, supporting the source of blood lipids sampled as deriving primarily from liver rather than adipose tissue or diet. Contribution from the glyceroneogenic pathway to TG-glycerol was lower in blood after treatment by either agent compared to vehicle treatment with very similar values and effects of treatment in the liver (vehicle-treated exhibited ~80% from glyceroneogenesis and treatment with either agent exhibited ~60% in both compartments). In contrast, adipose tissue TG-glycerol was derived only ~20% from glyceroneogenesis and was modestly increased by treatment with insulin or S597. The fraction of newly assembled TG-palmitate in plasma that derived from de novo synthesis was 80-100% after treatment with insulin or S597, compared to ~20% in vehicle-treated animals, again closely paralleling values in liver (80% and 20%, respectively), but different from adipose depots (~50% and 20%, respectively). Plasma TG clearly reflected, and likely derived from, lipids in the liver.

Moreover, liver weight was increased less by S597 than by insulin and these results indicate less stimulation of hepatic anabolic pathways by S597 compared to insulin.
Taken together, these findings strongly support the conclusion that both S597 and insulin treatment have potent effects on lipid biosynthesis in the liver of diabetic rats, but that there are significant differences in the rate of TG release into the bloodstream. Biosynthetic origins of the glycerol and fatty acid moieties of liver TG were altered by treatment with both insulin and S597, and these alterations were observed in plasma TG, but the rate of blood TG production was dramatically lower with S597 treatment. The lower production rate explained the more than 50% reduced blood TG concentrations with S597 treatment, as there was no effect of either agent on clearance (half-life) of plasma TG. These observations are all consistent with S597 treatment resulting in less secretory input from hepatic lipogenic pathways into plasma TG compared to insulin treatment.

In contrast, adipose lipogenesis was not differentially stimulated by S597 and insulin. Storage of newly synthesized palmitate and TG in adipose, as well as TG content, were all markedly and similarly stimulated by the two agents.

Can these apparent differential actions be explained by different glucose lowering effects of the two treatment regimens? Our studies were designed to achieve equal glucose lowering by S597 and insulin, in order to avoid any confounding of potential insulin effects on different lipid pathways. The treatments both significantly reduced HbA1C and glucose concentrations from pre-treatment levels, without any significant differences between the two agents.

Plasma glucose concentrations did fall more rapidly with insulin treatment, however, and HbA1C values were non-significantly reduced more by insulin while body weights were non-significantly higher compared to S597. The possibility that subtle differences in net insulin effect could explain the divergent hepatic lipid anabolic responses by S597 as compared to
insulin therefore seems unlikely, although it cannot be completely excluded. Of note, plasma glucose levels were identical in the two treatment arms at the start time of the two-hour labeling study (Study A), so divergent acute effects cannot be explained even by subtle differences in glycemic control.

What might then explain the divergent metabolic response exerted by S597 compared to insulin? Previous work with insulin receptor ligands and their interaction with the receptor suggests that there are several binding characteristics that have the potential to alter downstream signaling e.g. residence time of the ligand on the receptor, the ability of the ligand to induce more or less phosphorylation at distinct phosphorylation sites, or how internalization of the receptor/ligand complex takes place (24;25). Regarding S597 in particular, it seems to cause phosphorylation of the same number of insulin receptors but to a lower degree, and also it has been found that S597 remained associated with the receptor for longer periods of time than native insulin (25). It has to be kept in mind, however, that these changes were observed in tissue culture systems and it is unknown whether such changes take place and are of significance in whole animals. It is worth noting that ZDF rats exhibited remarkably active rates of de novo lipogenesis with either treatment. Insulin and S597 treatments each significantly stimulated adipose de novo lipogenesis in ZDF rats so that after five days of labeling, ~60% of newly deposited TG-palmitate in adipose tissue came from the de novo lipogenic pathway in both treatment groups. Moreover, after two hours of labeling, essentially 100% of newly secreted plasma TG-palmitate and 80% of newly stored hepatic TG-palmitate came from the de novo lipogenic pathway in each group. Endogenous synthesis of fatty acids is clearly a very active pathway in ZDF rats exposed to insulin receptor agonists. This marked induction of de novo lipid synthesis might partly explain why our studies showed increased TG levels in the plasma upon insulin or S597 therapy (this
effect was highly significant in study 2, see table 2), which is otherwise reported to improve hypertriglyceridemia in diabetic subjects in the clinic due to better glycemic control (26). We did not measure the effects of insulin or S597 treatment on plasma apolipoprotein B levels or production rates, which could have implications for atherogenesis. The effects on triglyceride-rich particle numbers and production will be an interesting area for future inquiry.

Insulin is also well known to be a potent stimulator of adipogenesis in vitro (27,28). To the best of our knowledge, we here show for the first time that insulin treatment of diabetic animals can increase adipogenesis in situ. Interestingly, it was the epididymal depot only that displayed increased adipogenesis in response to S597 treatment, both when compared to the ZDF vehicle group and to insulin treatment. It has previously been shown that in human diabetic subjects the subcutaneous depot displays less adipogenic potential when compared to visceral depots (29), which may partly explain this observation. Taken together with the substantial expansion of the subcutaneous depot in particular with insulin and S597 treatment, these adipogenesis results suggest that the subcutaneous depot responded to S597 treatment by expanding the already existing adipocytes, whereas the epididymal depot was also able to produce more adipocytes to incorporate the extra TG. Additional experiments with estimation of adipocyte size would be needed to address this hypothesis.

Finally, it is of interest to note than kinetic measurements were able to detect effects of insulin receptor-activating peptides or insulin on adipose lipids where traditional non-kinetics metrics could not detect effects. In ZDF rats, the content of palmitate in each fat pad was ~300-500 mg (suppl. figure 3). Treatment for 7 days did not generally cause measurable increases. In contrast, the kinetic measurement (fractional synthesis from the de novo lipogenic pathway) showed consistent and striking increases, and the total amount of newly synthesized
palmitate was even more strikingly elevated by treatment. The numbers involved may help explain the different sensitivity of kinetic vs static techniques. The fractional contribution over 7 days from *de novo* palmitate synthesis was 5-10%, representing ~20-40 mg new palmitate out of 300-500 mg total palmitate. It is easy to see how pool size measurements might not be able to detect these changes, whereas mass spectrometric measurements can sensitively distinguish between 5 and 10% values.

In summary, pathways of hepatic TG assembly (glyceroneogenesis, *de novo* fatty acid synthesis) and TG secretion (release into plasma) were significantly different between the peptide receptor agonist S597 and insulin itself, despite similar effects on adipose TG metabolism. These results reveal differential effects of insulin and the insulin receptor agonist S597 on lipid anabolic pathways in liver but not in adipose tissue. Subtle differences in glycemia are unlikely to explain these tissue-specific differences in lipogenesis. The capacity to modulate hepatic lipogenic pathways differentially with insulin receptor-activating peptides could have important therapeutic applications.

**Author contributions**

H.F.S., T.Å.P., C.F., G.S.O., S.D.B. and M.H. designed the study; H.F.S. performed the experiments; H.F.S. and M.F. analyzed collected samples; H.F.S., T.Å.P., C.F., G.S.O., S.D.B., and M.H. interpreted results of experiments; H.F.S. prepared figures; H.F.S. drafted manuscript; H.F.S, T.Å.P., C.F., G.S.O., S.D.B., and M.H. edited, revised, and approved final version of manuscript.

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M.H. 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.

Furthermore we declare that the study was funded by Novo Nordisk through which the authors T.Å.P., C.F., G.S.O., and S.D.B. all are employees and stakeholders. The insulin and the peptide used for the experiments have been provided by Novo Nordisk as well. This does not alter our adherence to all the Diabetes policies on sharing data and materials. All Intellectual Property Rights of the current study are owned by University of California Berkeley and there has been no compromise of the objectivity or validity of the data in the manuscript. T.Å.P., C.F., G.S.O., and S.D.B. all are employees and stakeholders of Novo Nordisk which owns the peptide receptor agonist S597. There are no other conflicts of interest to report.

Reference List


Table 1: Whole body composition by qMR scan.

Rats from study B were scanned on the day prior to treatment and again on the day prior to euthanisation.

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<th>Post-treatment fat mass (g)</th>
<th>Δfat mass (g)</th>
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<td>82.7±1.9</td>
<td>94.4±2.3*</td>
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Values are expressed as mean ± SEM.

*=p<0.05; ***=p<0.001 as compared to ZDF Vehicle.

(one-way ANOVA with Bonferroni post-tests).
Table 2: Lipid content of liver, plasma, and the subcutaneous adipose depot

TG, palmitate, and cholesterol were all quantified in the liver. In plasma TG, palmitate, FFA, and cholesterol were quantified. In the subcutaneous adipose depot palmitate was quantified and TG content was estimated based on fat pad mass.

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<td>31.9±3.0</td>
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<td>40.1±5.2**</td>
<td>2.60±0.38</td>
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<td>39.7±4.5*</td>
<td>2.56±0.33</td>
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Values are expressed as mean ± SEM.

*p<0.05; **=p<0.01; ***=p<0.001 as compared to ZDF controls

†=p<0.05; ††=p<0.01; †††=p<0.001 as compared to Insulin

(one way ANOVA with Bonferroni post-tests)
Figure legends

Figure 1. Blood glucose
Blood glucose concentrations after subcutaneous implantation of osmotic minipumps containing buffer in the Lean Vehicle group (white circles) and the ZDF Vehicle group (black circles), 0.2mM of insulin in ZDF rats (white squares), or 0.4mM of S597 in ZDF rats (black squares). Figure 1A shows the results from study A where a lean control group (Lean Vehicle) was included. 1B illustrates the glucose levels from study B. The time for heavy water injection is depicted by an arrow on the x-axis. **=p<0.01; ***= p<0.001 as compared to Lean Vehicle. †p<0.05; ††= p<0.01; †††= p<0.001 as compared to ZDF Vehicle, ‡=p<0.05; ‡‡=p<0.01 as compared to insulin (Bonferroni post-tests upon 2-way ANOVA). Error bars are SEM (in some groups error bars are not visible due to their small value).

Figure 2. Weight of liver and subcutaneous adipose depot
In study B the liver and subcutaneous adipose depot were carefully dissected and weighed. *=p<0.05; *=p<0.01; ***=p<0.001 as compared to ZDF Vehicle. ††= p<0.01 as compared to insulin (One-way ANOVA with Bonferroni post-tests). Error bars are SEM.

Figure 3: Palmitate synthesis
Fractional palmitate synthesis in liver, plasma, and the subcutaneous adipose depot is given in figure 3A, 3B, and 3C. The corrected fractional palmitate synthesis (3D for liver, 3E for plasma, and 3F for the subcutaneous adipose depot) is a measure of how large a fraction of palmitate in only the newly formed TGs is de novo synthesized Figures 3G (liver), 3H (plasma), and 3I
(subcutaneous adipose depot) are the quantified amounts of new palmitate present within each tissue. *=p<0.05  ***=p<0.001 as compared to ZDF Vehicle; †=p<0.05 as compared to Insulin (one-way ANOVA with Bonferroni post-tests). Error bars are SEM.

Figure 4: TG-glycerol synthesis and biosynthetic origin in liver, plasma, and subcutaneous adipose depot.

4A (liver), 4B (plasma), and 4C (subcutaneous adipose depot) are measures of fractional TG-glycerol synthesis with 2 hours (liver and plasma) and 5 days (adipose tissue) of heavy water labelling. The absolute quantified levels of TGs containing a new glycerol moiety are depicted in 4D (liver), 4E (plasma), and 4F (subcutaneous adipose depot). The biosynthetic origin of all new TG-glycerol e.g. how large a percentage of glycerol that originates from glyceroneogenesis versus glycolysis is given in figure 4G (liver), 4H (plasma), and 4I (subcutaneous adipose depot), respectively. *=p<0.05;**=p<0.01;***=p<0.001 as compared to ZDF Vehicle; †=p<0.05 as compared to the Insulin group (one-way ANOVA with Bonferroni post-tests). Error bars are SEM.

Figure 5: Adipogenesis in situ

Adipocyte and preadipocyte proliferation (adipogenesis) in the subcutaneous and epididymal depots, respectively, measured as fraction of DNA that has been newly synthesised within the 5 days of heavy water labelling. **=p<0.01; ***=p<0.001 as compared to ZDF Vehicle (one-way ANOVA with Bonferroni post-tests). Error bars are SEM.
Figure 1. Blood glucose concentrations after subcutaneous implantation of osmotic minipumps containing buffer in the Lean Vehicle group (white circles) and the ZDF Vehicle group (black circles), 0.2mM of insulin in ZDF rats (white squares), or 0.4mM of S597 in ZDF rats (black squares). Figure 1A shows the results from study A where a lean control group (Lean Vehicle) was included. 1B illustrates the glucose levels from study B. The time for heavy water injection is depicted by an arrow on the x-axis. **=p<0.01; ***=p<0.001 as compared to Lean Vehicle. †p<0.05; ††=p<0.01; †††=p<0.001 as compared to ZDF Vehicle, ‡=p<0.05; ‡‡=p<0.01 as compared to insulin (Bonferroni post-tests upon 2-way ANOVA). Error bars are SEM (in some groups error bars are not visible due to their small value).

214x284mm (300 x 300 DPI)
Figure 2. Weight of liver and subcutaneous adipose depot

In study B the liver and subcutaneous adipose depot were carefully dissected and weighed. 

\* = p<0.05; \** = p<0.01; \*** = p<0.001 as compared to ZDF Vehicle. \†† = p<0.01 as compared to insulin (One-way ANOVA with Bonferroni post-tests). Error bars are SEM.

124x55mm (300 x 300 DPI)
Figure 3: Palmitate synthesis

Fractional palmitate synthesis in liver, plasma, and the subcutaneous adipose depot is given in figure 3A, 3B, and 3C. The corrected fractional palmitate synthesis (3D for liver, 3E for plasma, and 3F for the subcutaneous adipose depot) is a measure of how large a fraction of palmitate in only the newly formed TGs is de novo synthesized. Figures 3G (liver), 3H (plasma), and 3I (subcutaneous adipose depot) are the quantified amounts of new palmitate present within each tissue. * = p < 0.05 *** = p < 0.001 as compared to ZDF Vehicle; † = p < 0.05 as compared to Insulin (one-way ANOVA with Bonferroni post-tests). Error bars are SEM.

186x129mm (300 x 300 DPI)
Figure 4: TG-glycerol synthesis and biosynthetic origin in liver, plasma, and subcutaneous adipose depot.
4A (liver), 4B (plasma), and 4C (subcutaneous adipose depot) are measures of fractional TG-glycerol synthesis with 2 hours (liver and plasma) and 5 days (adipose tissue) of heavy water labelling. The absolute quantified levels of TGs containing a new glycerol moiety are depicted in 4D (liver), 4E (plasma), and 4F (subcutaneous adipose depot). The biosynthetic origin of all new TG-glycerol e.g. how large a percentage of glycerol that originates from glyceroneogenesis versus glycolysis is given in figure 4G (liver), 4H (plasma), and 4I (subcutaneous adipose depot), respectively. * = p<0.05; ** = p<0.01; *** = p<0.001 as compared to ZDF Vehicle; † = p<0.05 as compared to the Insulin group (one-way ANOVA with Bonferroni post-tests).
Error bars are SEM.
195x140mm (300 x 300 DPI)
Figure 5: Adipogenesis in situ

Adipocyte and preadipocyte proliferation (adipogenesis) in the subcutaneous and epididymal depots, respectively, measured as fraction of DNA that has been newly synthesised within the 5 days of heavy water labelling. **=p<0.01; ***=p<0.001 as compared to ZDF Vehicle (one-way ANOVA with Bonferoni post-tests). Error bars are SEM.

95x41mm (300 x 300 DPI)
Supplemental figure 1: Bodyweight

Body weight after subcutaneous implantation of osmotic minipumps containing buffer (ZDF Vehicle, white circles), 0.2mM of insulin (black circles) or 0.4mM S597 (white squares). Figure 1A shows the results from study A whereas 1B shows the bodyweights of study B. * = p<0.05; ** = p<0.01; *** = p<0.001 as compared to ZDF Vehicle (Two-way ANOVA with Bonferroni post-tests). Error bars are SEM (in some groups the error bars are not visible due to small values).

192x250mm (300 x 300 DPI)
Supplemental figure 2: Weights of adipose depots

In study B the epididymal (2A), perirenal (2B), and mesenteric (2C) adipose depots were all carefully dissected and weighed. *=p<0.05 as compared to ZDF Vehicle (one-way ANOVA with Bonferroni post-tests).

Error bars are SEM.

279x568mm (300 x 300 DPI)
Supplemental figure 3. Palmitate synthesis in the epididymal, perirenal, and mesenteric adipose depots. Fractional palmitate synthesis in epididymal, perirenal and mesenteric adipose depots is given in suppl. figure 3A, 3B, and 3C. The corrected fractional palmitate synthesis (3D for epididymal, 3E for perirenal, and 3F for the mesenteric adipose depot) is a measure of how large a fraction of palmitate in only the newly formed TGs is de novo synthesized. Figures 3G (epididymal), 3H (perirenal), and 3I (mesenteric adipose depot) are the quantified amounts of total palmitate present within each tissue whereas the quantified of new palmitate only is given in 3J (epididymal), 3K (perirenal), and 3L (mesenteric adipose depot). *=p<0.05; **= p<0.01; ***=p<0.001 as compared to ZDF Vehicle (one-way ANOVA with Bonferroni post-tests). Error bars are SEM.

197x180mm (300 x 300 DPI)
Supplemental figure 4. TG-glycerol synthesis and biosynthetic origin in the epididymal, perirenal, and mesenteric adipose depots.

4A (epididymal), 4B (perirenal), and 4C (mesenteric adipose depot) are measures of fractional TG-glycerol synthesis with 5 days of heavy water labelling. The absolute quantified levels of TGs containing a new glycerol moiety are depicted in 4D (epididymal), 4E (perirenal), and 4F (mesenteric adipose depot). The biosynthetic origin of all new TG-glycerol e.g. how large a percentage of glycerol that originates from glyceroneogenesis versus glycolysis is given in figure 4G (epididymal), 4H (perirenal), and 4I (mesenteric adipose depot), respectively. **=p<0.01; ***=p<0.001 as compared to ZDF Vehicle; †=p<0.05; ††=p<0.01 as compared to the Insulin group (one-way ANOVA with Bonferroni post-tests). Error bars are SEM.

182x127mm (300 x 300 DPI)
Supplemental figure 1: Bodyweight

Body weight after subcutaneous implantation of osmotic minipumps containing buffer (ZDF Vehicle, white circles), 0.2mM of insulin (black circles)) or 0.4mM S597 (white squares). Figure 1A shows the results from study A whereas 1B shows the bodyweights of study B. *=p<0.05; **=p<0.01; ***=p<0.001 as compared to ZDF Vehicle (Two-way ANOVA with Bonferroni post-tests). Error bars are SEM (in some groups the error bars are not visible due to small values).
Supplemental figure 2: Weights of adipose depots

In study B the epididymal (2A), perirenal (2B), and mesenteric (2C) adipose depots were all carefully dissected and weighed. *=p<0.05 as compared to ZDF Vehicle (one-way ANOVA with Bonferroni post-tests). Error bars are SEM.
Supplemental figure 3. Palmitate synthesis in the epididymal, perirenal, and mesenteric adipose depots.

Fractional palmitate synthesis in epididymal, perirenal and mesenteric adipose depots is given in suppl. figure 3A, 3B, and 3C. The corrected fractional palmitate synthesis (3D for epididymal, 3E for perirenal, and 3F for the mesenteric adipose depot) is a measure of how large a fraction of palmitate in only the newly formed TGs is *de novo* synthesized. Figures 3G (epididymal), 3H (perirenal), and 3I (mesenteric adipose depot) are the quantified amounts of total palmitate present within each tissue whereas the quantified of new palmitate only is given in 3J (epididymal), 3K (perirenal), and 3L (mesenteric adipose depot). *=p<0.05; **= p<0.01; ***=p<0.001 as compared to ZDF Vehicle (one-way ANOVA with Bonferroni post-tests). Error bars are SEM.
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Supplemental table 1: Palmitate and TG content of the adipose depots

In all the remaining adipose depots palmitate content was quantified and TG content was estimated based on fat pad mass.

<table>
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<th>ZDF Vehicle</th>
<th>Insulin</th>
<th>S597</th>
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<tbody>
<tr>
<td><strong>Epididymal adipose depot</strong></td>
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<tr>
<td>TG (g/depot)</td>
<td>5.19±0.45</td>
<td>5.86±0.47*</td>
<td>5.53±0.38</td>
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<td>Palmitate (g/depot)</td>
<td>0.322±0.052</td>
<td>0.343±0.039</td>
<td>0.315±0.037</td>
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<tr>
<td><strong>Perirenal adipose depot</strong></td>
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<tr>
<td>TG (g/depot)</td>
<td>6.91±0.61</td>
<td>7.73±0.61*</td>
<td>7.75±0.48*</td>
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<tr>
<td>Palmitate (g/depot)</td>
<td>0.415±0.039</td>
<td>0.485±0.052**</td>
<td>0.459±0.031</td>
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<tr>
<td><strong>Mesenteric adipose depot</strong></td>
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<tr>
<td>TG (g/depot)</td>
<td>3.87±0.46</td>
<td>4.19±0.74</td>
<td>4.59±0.52</td>
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<tr>
<td>Palmitate (g/depot)</td>
<td>0.269±0.052</td>
<td>0.300±0.020</td>
<td>0.295±0.038</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SE.

*=p<0.05; **=p<0.01 as compared to ZDF Vehicle (one way ANOVA with Bonferroni post-tests)