Neural control of hepatic lipid metabolism: A (patho)physiological perspective

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SYMPATHETIC AND PARASYMPATHETIC HEPATIC DENERVATION MODULATE DYSLIPIDEMIA IN ZUCKER RATS

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ABSTRACT

Human and animal studies increasingly point towards a neural pathogenesis of the metabolic syndrome, involving hypothalamic and autonomic nervous system dysfunction. We hypothesized that increased VLDL-Triglyceride (VLDL-TG) secretion by the liver in the obese Zucker (fa/fa) rat, is due to a relative hyperactivity of sympathetic or hypoactivity of parasympathetic hepatic innervation. To investigate this, we surgically denervated the sympathetic or parasympathetic hepatic nerve in obese Zucker rats. Our results show that cutting the sympathetic hepatic nerve lowers VLDL-TG secretion, finally resulting in lower plasma triglyceride concentrations. By contrast, a parasympathetic denervation results in increased plasma total cholesterol concentrations. The effect of a sympathetic or parasympathetic denervation of the liver was independent of changes in humoral factors or changes in body weight or food intake. In conclusion, a sympathetic denervation improves the lipid profile in obese Zucker rats with marked dyslipidemia, whereas a parasympathetic denervation increases total cholesterol levels.
INTRODUCTION

Increasing evidence in humans suggests an important role for the autonomic nervous system in the development of obesity, diabetes and dyslipidemia (1-3). These studies showed that increased sympathetic and decreased parasympathetic drive correlate with several factors of the metabolic syndrome, including hypertriglyceridemia. In the field of hypertension, the autonomic nervous system was regarded a treatment target already in the 1930s, when the first renal denervation was carried out (4). Due to major pharmacological discoveries the surgical strategy has been not used for many decades but is now again being investigated as a treatment of resistant hypertension in humans (5).

We therefore aimed to investigate in the present study if increased secretion of VLDL-TG, which is a major contributor to dyslipidemia, can be reduced with a surgical denervation of the liver in an animal model of dyslipidemia. The obese Zucker rat (fa/fa) has high triglyceride and cholesterol plasma levels, which are already apparent shortly after weaning, due to an overproduction of VLDL-TG by the liver (6-9). In contrast to reports of low sympathetic nerve activity to brown adipose tissue, obese Zucker rats show high levels of renal sympathetic nerve activity and elevated baseline greater splanchnic nerve activity, suggesting increased sympathetic nervous activity to the liver (10,11).

Previous studies in rats showed mounting evidence for the involvement of the autonomic nervous system in the secretion of triglycerides packed in VLDL particles (VLDL-TG) from the liver (12,13). These studies led us to believe that a sympathetic denervation of the liver may be beneficiary in reducing VLDL-TG secretion.

Based on the human studies showing autonomic dysfunction in dyslipidemia and rat studies showing effects of the autonomic nervous system on VLDL-TG secretion by the liver we hypothesized that a sympathetic denervation of the liver should have a beneficiary effect on plasma triglycerides and VLDL-TG secretion by the liver in obese Zucker rats. Conversely, a parasympathetic denervation might have an opposite effect. We tested our hypothesis in lean (Fa/fa) and obese (fa/fa) Zucker rats by following plasma TG levels and hepatic VLDL-TG secretion during a timeframe of six weeks after a surgical sympathetic or parasympathetic denervation of the liver.

MATERIAL AND METHODS

Zucker rats

In total 31 obese Zucker rats (fa/fa; Charles River Germany) were included in the experiment and received a hepatic sham denervation (fa/fa sham n = 10), hepatic sympathetic denervation (fa/fa Sx n = 12) or a hepatic parasympathetic denervation (fa/fa Px n = 9). For comparison, 7 age matched lean Zucker rats with a heterozygous mutation (Fa/fa) were included and received a sham denervation (Fa/fa sham n = 7). All rats were housed in individual cages with a 12/12 light dark schedule (lights on at 7.00 A.M.). Standard Rodent Chow and water were available ad libitum, unless stated otherwise. Body weight, food intake and water intake were measured on all weekdays. All procedures were approved by the animal care committee of the Royal Netherlands Academy of Arts and Sciences.
**Tail incision**

In the second week after arriving in the facility (week 0) a baseline blood sample was taken. All blood samples in week 0 to 4 were taken by tail incision (14). On the days of blood sampling, the rats were fasted from 09.00 A.M. and a blood sample was taken ~4 hours later between 1.00 and 2.00 P.M.

**Denervation and jugular vein surgery**

For both surgeries anaesthesia with 0.8 ml/kg Hypnorm i.m. (Janssen, High Wycombe, Buckinghamshire, UK) and 0.4 ml/kg Dormicum s.c. (Roche, Almere, The Netherlands) was used. One day after the baseline blood sampling in week 0, rats received a sham, selective parasympathetic or sympathetic denervation. Hepatic sympathetic or parasympathetic branches were denervated according to previous reports (15). The day after the blood sampling in week 4, the rats were fitted with an intra-atrial silicone cannula into the right jugular vein (16,17).

**Measurement of VLDL-TG secretion**

In week 5 rats were connected to an infusion swivel (Instech Laboratories, PA, USA) one day before the experiment for adaption. On the next day rats were fasted from 09.00 A.M. onwards and external lines for blood sampling were connected. At 1.00 P.M. a baseline blood sample from the jugular vein was taken and an intravenous dosage of 0.7 ml 15% tyloxapol (Sigma-Aldrich, Germany) was given. At 20-min intervals blood samples were drawn from the jugular vein catheter during 100 minutes.

**Tissue collection**

In week 6 rats were fasted from 09.00 A.M. onwards and at 1.00 P.M. a blood sample from the jugular vein was taken. Subsequently, rats were injected with an overdose of pentobarbital IV. Liver tissue, adipose tissue and the brain were removed and snap frozen for further analysis. When the cannula was blocked, decapitation blood was collected after intraperitoneal injection of pentobarbital.

**Plasma measurements**

Glucose concentrations were determined during the experiment in blood spots using a glucose meter (FreestyleTM, Abbott, The Netherlands). Triglycerides and cholesterol were assayed using a kit from Roche (Mannheim, Germany). The VLDL-TG secretion rate was determined by the slope of the rise in triglycerides over time by linear regression analysis. The WAKO NEFA HR kit (Wako. Chemicals, Neuss, Germany) was used to measure FFA in plasma. By using radioimmunoassay kits, plasma insulin (LINCO Research, St. Charles, MO, USA) and corticosterone (ICN Biomedicals, Costa Mesa, CA) were measured.

**Liver measurements**

Triglycerides in liver were measured after a single step lipid extraction with methanol and chloroform (18). The pellets were finally dissolved in 2 % Triton X-100 (Sigma-Aldrich, Germany) and triglycerides and cholesterol were measured (Roche, Mannheim, Germany). The effectiveness of the hepatic sympathetic denervation was checked by measurement of
liver noradrenaline concentration using an in-house HPLC method with fluorescent detection (17). RT-PCR was performed as previously described with the same primer sequences (13). Liver acylcarnitines levels were measured essentially as described (19).

Statistical analysis
Data are presented as mean ± SEM. For every experiment, two separate analyses were performed. First, Fa/fa sham and fa/fa sham rats were compared by a repeated measurements general linear model (GLM) for comparing outcome measures at multiple time points (with Genotype as between-animal factor and Time as within-animal factor) or a t-test for single outcome measures. Second, fa/fa sham, fa/fa Sx and fa/fa Px rats were compared by repeated measurements GLM for comparing outcome measures at multiple time points (with Denervation as between-animal factor and Time as within-animal factor) or one-way ANOVA for single outcome measures. A significant (P ≤ 0.05) global effect of repeated measurements GLM or ANOVA was followed by post hoc tests to detect individual group differences (Fisher’s protected least significant difference). Significance was defined at P < 0.05.

RESULTS AND DISCUSSION
Selective hepatic denervation of parasympathetic and sympathetic nerve
To investigate the effect of a selective sympathetic or parasympathetic hepatic denervation on the development of dyslipidemia in the obese Zucker rat (fa/fa), we surgically cut either the sympathetic (Sx) or parasympathetic (Px) branch to the liver and compared these groups with sham denervated rats. To evaluate the effect size, we included sham denervated heterozygous lean Zucker rats (Fa/fa) in the experiment. For the fa/fa Sx rats, a successful denervation was defined as achieving a liver noradrenaline concentration after the experiment of below 1 ng/g of liver tissue. All fa/fa Sx rats, except three, showed liver noradrenaline concentration below 1 ng/g (0.59 ± 0.07; mean ± SEM) compared to fa/fa sham rats (18.41 ± 4.03; mean ± SEM). We have previously validated our method for selective hepatic parasympathectomy by using retrograde viral tracing (15). During a timeframe of seven weeks, all rats were included in a protocol with weekly blood sampling by tail incision (week 0-4), a VLDL-TG secretion experiment in week 5 and a final blood sample and tissue collection (week 6) (Figure 1A). Additionally, daily measurements of body weight, food intake and water intake were undertaken.

A sympathetic denervation lowers VLDL-TG secretion and plasma triglycerides
Obese fa/fa sham rats show significantly higher plasma triglyceride concentrations compared to lean Fa/fa sham rats at all time points (week 0-4: Time p = 0.032, Time*Genotype p = 0.123, Genotype p = 0.001; week 5 and 6 p < 0.01) (Figure 1B). When comparing the Sham, Sx and Px fa/fa groups, a significant 39% decrease in plasma triglycerides was found in the fa/fa Sx rats compared to fa/fa sham (p = 0.040) and 56 % compared to fa/fa Px (p = 0.001) rats in week 6. It is well known that obese Zucker rats show increased lipoprotein lipase (LPL) activity in white adipose tissue, which partly compensates for the increase in
Figure 1. A sympathetic denervation lowers VLDL-TG secretion and plasma triglycerides and a parasympathetic denervation increases plasma total cholesterol in obese Zucker rats. (A) Graphical representation of study design (B) Plasma triglyceride measurements during the experiment (* p < 0.05 compared to fa/fa Sham) (C) VLDL-TG secretion rates calculated from the increase of plasma triglycerides after injection of tyloxapol (D) Plasma total cholesterol measured in the final blood sample (E) Body weight of all groups during week 0-6 (F) Average daily food intake of all groups during week 0-6. Values are mean ± SEM of 6-10 rats per group. * P < 0.05.
VLDL-TG secretion (20,21). In week 5 VLDL-TG secretion by the liver was therefore measured after injection of tyloxapol. Tyloxapol inhibits LPL-mediated triglyceride hydrolysis and thus prevents the removal of circulating triglycerides (8,22). We found that VLDL-TG secretion is more than twice as high in fa/fa sham rats compared to Fa/fa sham rats (p < 0.001; Figure 1C). Furthermore, this experiment showed lower VLDL-TG secretion rates in the fa/fa Sx rats compared to fa/fa sham (p = 0.026) and fa/fa Px (p = 0.012) rats before any changes in plasma TG concentrations occurred. Apparently, the clearance of triglycerides in peripheral tissues masked the effect of the reduced VLDL-TG secretion on plasma triglycerides in week 0-5. In fa/fa Sx rats, plasma total cholesterol concentrations showed no difference compared to fa/fa sham rats.

A parasympathetic denervation increases plasma total cholesterol concentrations

Obese fa/fa sham rats showed significantly higher plasma total cholesterol concentrations compared to lean Fa/fa sham rats at all time points (week 0-4: Time p = 0.081, Time*Genotype p = 0.487, Genotype p < 0.001; week 5 and 6 p < 0.01). In week 6 a significant increase of total cholesterol in fa/fa Px rats compared to fa/fa sham (p = 0.045) and fa/fa Sx rats (p = 0.002) was observed (Figure 1D). It is interesting to observe that the effects of a parasympathetic denervation are very different from a sympathetic denervation, with an increase of plasma triglycerides in week 5 (Figure 1B) and increased total cholesterol levels in the final sample. Human studies have also indicated that contrary to high sympathetic activity, low parasympathetic activity is correlated to dyslipidemia (2). To our knowledge, we are the first to investigate the effects of a sympathetic denervation to the liver in Zucker rats, although surgical parasympathetic denervations in Zucker rats have been performed previously (23,24). Ferrari et al., (24) investigated the effect of a subdiaphragmatic vagal deafferentation (SDA), cutting all vagal afferents from the gut, including the liver, and leaving half of the vagal efferents intact. Contrary to our results from a selective hepatic vagal denervation they found decreased body weight gain and food intake, decreased plasma insulin and triglyceride concentrations and reduced total liver fat content in obese Zucker rats treated with SDA. This indicates that the effects of the SDA are caused by a liver independent mechanism, as we did not observe any of these effects after a selective parasympathetic denervation of the liver.

Hepatic denervations do not influence body weight, food intake and water intake

As a sympathetic denervation lowered plasma triglycerides and a parasympathetic denervation elevated total cholesterol levels, we investigated whether these changes could be attributed to changes in body weight gain, food intake or water intake. All groups significantly increased their body weight during the experiment (Figure 1E). Obese fa/fa sham rats were heavier at baseline and showed increased body weight gain during the experiment compared to lean Fa/fa sham rats (Time p < 0.001, Time*Genotype p < 0.001, Genotype p < 0.001). On average obese fa/fa rats gained 117 ± 7 grams of body weight during the experiment, whereas lean Fa/fa rats gained 61 ± 3 grams. Comparison between fa/fa sham, fa/fa Sx and fa/fa Px rats revealed no significant differences in body weight.
during the experiment (Time \( p < 0.001 \), Time*Denervation \( p = 0.334 \), Denervation \( p = 0.857 \)). Average food intake during the study was significantly increased in fa/fa sham rats compared to Fa/fa sham rats \( (p < 0.001) \), but no differences were observed between the fa/fa groups (Figure 1F). Water intake was not significantly different for any of the groups. Therefore we conclude that the above described changes occurred independent of changes in body weight gain, food intake and water intake.

**Hepatic denervations do not influence insulin, glucose, FFA and corticosterone plasma levels**

VLDL-TG secretion is regulated by the availability of nutrients (e.g., FFA and glucose) and hormones (e.g., insulin and corticosterone). Obese Zucker rats are hyperinsulinemic in the face of unchanged plasma glucose concentrations and chronic hyperinsulinemia is known to increase VLDL-TG secretion. Studies have shown differences in plasma FFA and corticosterone levels in obese versus lean Zucker rats. To investigate if the changes in VLDL-TG secretion after a sympathetic denervation can be attributed to these factors blood glucose, plasma insulin, corticosterone and free fatty acids (FFA) were measured in the final sample. As expected, plasma insulin was significantly higher in fa/fa sham rats \( (7.71 \pm 0.54; \text{mean} \pm \text{SEM}) \) compared to Fa/fa sham rats \( (1.12 \pm 0.13; \text{mean} \pm \text{SEM}) \). Of note, no significant differences were observed between the fa/fa sham, fa/fa Sx and fa/fa Px rats. Furthermore, blood glucose, plasma corticosterone and plasma FFA were not significantly different between fa/fa sham and Fa/fa sham rats, nor between the fa/fa denervation groups. Therefore, these humoral factors do not explain the changes observed in fa/fa Sx and Px rats and support a direct control of autonomic nerves on VLDL-TG secretion.

**fa/fa Sx rats do not display changes in the expression of key enzymes involved in lipogenesis, VLDL-TG secretion and beta-oxidation**

To further dissect the hepatic mechanism responsible for the changes observed in plasma triglycerides, we measured liver triglyceride concentration and the mRNA expression profile of key enzymes involved in lipid metabolism. Obese fa/fa sham rats showed over 4-fold higher triglyceride levels in the liver compared to lean Fa/fa sham rats \( (p = 0.002) \). However, no differences were observed between the fa/fa sham, fa/fa Sx and fa/fa Px rats (Figure 2A). Therefore, we can conclude that the lower rate of triglyceride secretion by the sympathetically denervated liver does not result in an increased accumulation of triglycerides in the liver. Liu *et al.* (25) investigated the effect of a pharmacological blockade of the sympathetic nervous system for seven days with carvedilol in an animal model of alcohol fatty liver disease and found that this attenuates the progression of steatosis. Our data show that this is not the case with surgical sympathetic blockade in a model of non-alcoholic fatty liver disease. To further investigate if lipogenesis and TAG storage is changed in fa/fa Sx rats we measured mRNA levels of key genes in this pathway. It is known that the rate of lipogenesis is higher in obese fa/fa rats, secondary to the hyperphagia and hyperinsulinemia (26,27). Increased lipogenesis was also shown in our experiments by the increased mRNA expression of *Fas* (Figure 2B). However, no differences were observed between the denervation groups, showing that the lower VLDL-TG secretion rates in sympathetically denervated rats are not
Figure 2. Obese Zucker rats show higher liver triglycerides concentrations, higher mRNA expression of Fas and lower levels of long-chain acylcarnitines. (A) Liver triglyceride concentrations (B) Liver mRNA expression of key enzymes in hepatic lipid metabolism (C) Liver acylcarnitines concentrations. Values are mean ± SEM of 6-10 rats per group. * P < 0.05, ** P < 0.01.
due to a decreased expression of FAS resulting in lower lipogenesis rates. mRNA levels of other genes involved in lipogenesis (Acc1, Acc2, Scdn, Srebplc, Pparγ) were not affected by the genotype or the denervation. Apart from effects on lipogenesis, we measured mRNA expression of genes involved in the assembly and secretion of VLDL-TG, but no difference in the mRNA levels of Arf1, ApoB, Mttpl and Dgat1 was observed between the fa/fa sham and Fa/fa sham rats, nor was any effect of the denervation in the fa/fa groups observed. Finally, we investigated if fa/fa Sx rats increase their levels of fatty acid beta-oxidation, leaving less substrate for VLDL-TG secretion. It is known that obese Zucker rats favor triglyceride synthesis over fatty acid oxidation and are less capable to oxidize fatty acids in the liver (9). To this end, we measured mRNA levels of Cpt1a but found no significant differences between the Fa/fa and fa/fa groups or the different denervation groups. We also measured the levels of acylcarnitines in the liver to rule out any effect on beta-oxidation. We observed that most long-chain acylcarnitines were decreased, which is consistent with lower beta-oxidation in obese Zucker rats when compared to lean Zucker rats (Figure 2C). However, fa/fa Sx rats do not display elevated levels of these acylcarnitines and therefore beta-oxidation seems unaffected in the denervated rats. From these experiments we can conclude that decreased lipogenesis or TG accumulation and increased beta-oxidation within the liver cannot explain lower VLDL-TG secretion in fa/fa Sx rats. Alternatively, the sympathetic nervous system may regulate lipid metabolism by other mechanisms, e.g. by altering hepatic blood flow. Further studies are necessary to reveal these mechanisms.

CONCLUSIONS

In this study we confirm the hypothesis that cutting the sympathetic nerve to the liver lowers VLDL-TG secretion and results in subsequent lower plasma triglycerides in obese Zucker rats. On the contrary, a parasympathetic denervation results in increased plasma cholesterol and triglyceride concentrations. The effects of the sympathetic and parasympathetic hepatic denervations were independent of changes in body weight, food intake, water intake, plasma glucose, FFA, insulin and corticosterone. Thus in addition to hyperphagia and hyperinsulinemia, the autonomic nervous system is an independent determinant of VLDL-TG secretion and plasma cholesterol in obese Zucker rats. Therefore, in addition to caloric restriction and treatment of hyperinsulinemia in order to reduce hypertriglyceridemia selective hepatic denervations may be a useful intervention to modulate lipid metabolism.

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