HYPOTHALAMIC NEUROPEPTIDE Y (NPY) CONTROLS HEPATIC VLDL-TRIGLYCERIDE SECRETION IN RATS VIA THE SYMPATHETIC NERVOUS SYSTEM

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ABSTRACT

Abstract
Excessive secretion of triglyceride-rich very low-density lipoproteins (VLDL-TG) contributes to diabetic dyslipidemia. Earlier studies indicated a possible role for the hypothalamus and autonomic nervous system in the regulation of VLDL-TG. In the present study, we investigated whether the autonomic nervous system and hypothalamic neuropeptide Y (NPY) release during fasting regulates hepatic VLDL-TG secretion. We report that, in fasted rats, an intact hypothalamic arcuate nucleus and hepatic sympathetic innervation are necessary to maintain VLDL-TG secretion. Furthermore, the hepatic sympathetic innervation is necessary to mediate the stimulatory effect of intracerebroventricular (ICV) administered NPY on VLDL-TG secretion. Since the ICV administration of NPY increases VLDL-TG secretion by the liver without affecting lipolysis, its effect on lipid metabolism appears to be selective to the liver. Together, our findings indicate that the increased release of NPY during fasting stimulates the sympathetic nervous system to maintain VLDL-TG secretion to a postprandial level.
INTRODUCTION

The secretion of triglyceride-rich very low-density lipoproteins (VLDL-TG) is increased in type 2 diabetic patients (1). Licht et al. (2) showed that hypertriglyceridemia in patients with the metabolic syndrome strongly correlates with changes in activity of the autonomic nervous system (ANS). This indicates that besides the availability of free fatty acids (FFA) and hormones, such as insulin, the ANS might be involved in the regulation of VLDL-TG (3,4).

Recently, Stafford and colleagues reported that central infusion of Neuropeptide Y (NPY) increases VLDL-TG (5). The mechanism of this effect remains unclear. We hypothesized that the ANS mediates the effect of NPY on VLDL-TG secretion and that this mechanism is part of the physiological response during fasting, when lipids become the main energy source. First, NPY neurons in the arcuate nucleus (ARC) of the hypothalamus are activated in response to fasting, and the extracellular availability of NPY in the paraventricular nucleus (PVN) is increased (6-8). Second, Viñuela and colleagues (9) showed that intracerebroventricular (ICV) administration of NPY activates neurons in the PVN projecting to the sympathetic preganglionic neurons. Third, we and others showed that pre-autonomic neurons in the PVN are anatomically connected to the liver (10,11). These pharmacological and anatomical data support the concept that NPY neurons in the ARC communicate with peripheral metabolic organs via the ANS. Along this lines, the ICV administration of NPY induces insulin resistance and prevents the inhibitory effect of hyperinsulinemia on hepatic glucose production, via activation of the sympathetic nervous system (SNS) (12-14).

In this study, we tested the hypothesis that during fasting elevated hypothalamic NPY release regulates hepatic VLDL-TG secretion via autonomic inputs to the liver. We first investigated the importance of the ANS in VLDL-TG secretion during fasting by transecting either the sympathetic or parasympathetic nerves to the liver and measuring VLDL-TG secretion after fasting. Since NPY neurons in the ARC are activated during fasting, we then investigated if a central NPY infusion alters VLDL-TG metabolism via the SNS. Subsequently, we investigated VLDL-TG secretion after fasting in rats with a chemical lesion of the ARC, a component of the sympathetic outflow circuit to the liver (10). It was shown previously in Siberian hamsters that these ARC-lesioned animals do not shown increased NPY immunoreactivity after fasting (15). In the final experiment we investigated the effect of ICV NPY on the availability of substrate for VLDL-TG secretion through lipolysis.

RESEARCH DESIGN AND METHODS

Animals

Male Wistar rats weighing 280-310 grams (Harlan Nederland, Horst, The Netherlands) were ordered and 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. All procedures were approved by the animal care committee of the Royal Netherlands Academy of Arts and Sciences.
**Surgery**

After one week in the facility, rats underwent surgeries according to the different experimental designs. All rats were fitted with an intra-atrial silicone cannula into the right jugular vein and a second silicone cannula into the left carotid artery (16,17). For experiments involving denervation of the liver, hepatic sympathetic or parasympathetic branches were denervated according to previous reports (18). A total liver denervation was achieved by cutting the sympathetic and parasympathetic branches to the liver. The effectiveness of the hepatic sympathetic denervation was checked by measurement of noradrenaline content (17). We have previously validated our method for selective hepatic parasympathectomy by using retrograde viral tracing (18). For experiments involving acute ICV NPY treatment, a stainless steel guide cannula (Plastics One, Roanoke, VA) was implanted into the third ventricle (19). After recovery to pre-surgery body weight for at least 10 days, rats were connected to an infusion swivel (Instech Laboratories, PA, USA) one day before the experiment for adaption.

**Fasting experiments**

To investigate if the ANS regulates VLDL-TG secretion in the fasted state, rats received a sham, selective sympathetic, selective parasympathetic or total liver denervation. The fasted condition in our experiments was administered after recovery to pre-surgery body weight. Rats were placed in a clean cage without food from 5:00 P.M. onwards. The next day at 12:00 P.M. an arterial baseline blood sample was taken and VLDL-TG clearance was subsequently blocked by an intravenous dosage of 300 mg/kg tyloxapol (Sigma-Aldrich, Germany) (5,20). At 20-min intervals blood samples were drawn from the carotid catheter.

**NPY and sympathetic denervation experiments**

To determine that NPY stimulates VLDL-TG secretion via the SNS, rats were implanted with a third ventricle cannula and received a sham or sympathetic denervation of the liver. After recovery to pre-surgery body weight, rats were placed in clean solid-bottom cages with a measured amount of chow. The next morning at 8:00 A.M., food was removed from the cage and weighed. This represents the postprandial condition in our experiments. A baseline arterial blood sample was drawn at 12:00 P.M. and VLDL-TG clearance was subsequently blocked by an intravenous dosage of 300 mg/kg tyloxapol and at 20-min intervals arterial blood samples were taken. Forty minutes after the tyloxapol injection, we started an ICV infusion of NPY (1 µg/µl) or vehicle (purified water; Milli-Q) for 2 hours (bolus 5 µl/5 min, followed by 5 µl/h). In vitro control experiments showed that addition of 15 µl NPY or vehicle to 300 µl aCSF (pH = 7.5), the reported total volume of rat CSF (21), did not affect the pH of the aCSF. At the end of the experiment, 5 µl colored dye was injected via the ICV guiding probe to confirm the probe placement.

**MSG experiments**

To show that the ARC is necessary for regulating VLDL-TG secretion after fasting, we used monosodium glutamate (MSG) to chemically ablate the ARC. The offspring of pregnant Wistar dams (Harlan Nederland, Horst, The Netherlands) was treated with MSG (4 mg/g, sc; Sigma, St. Louis, MO) or saline on days 1, 3, 5, 7 and 9 postnatally (22). After reaching
a minimum body weight of 300 grams MSG treated rats were subjected to a sham, hepatic sympathetic or parasympathetic denervation as described above. After recovery, these rats underwent the same overnight fasting experiment as described above. One group of sham MSG rats was tested postprandially. MSG lesions were checked by NPY immunohistochemistry (Supplemental data 1).

**Stable isotope experiments**

To establish if central NPY affects lipolysis, rats received a cannula into the third ventricle. After recovery to their pre-surgery body weight, rats were fasted from 8:00 A.M. onward and at 11:00 A.M. a blood sample from the carotid catheter was drawn for background isotope enrichment. To study lipolysis (glycerol appearance) and endogenous glucose production, a solution of 1.69 mg/ml [1,1,2,3,3-d5]glycerol and 4.66 mg/ml [6,6-2H2]glucose was infused (as a primed 243 µl in 5 min – continuous 500 µl/h infusion). After 90 minutes a steady state was established and three baseline blood samples were taken at 5 minute intervals. Subsequently, the ICV NPY or vehicle infusion was started (i.e. under the same conditions as the previous experiment). Blood samples were taken every 20 minutes for 2 hours during NPY infusion.

**Analytical methods**

Glucose concentrations were determined during the experiment in blood spots using a glucose meter (Freestyle™, Abbott, The Netherlands). Triglycerides were assayed using a kit from Roche (Mannheim, Germany). The VLDL-TG secretion 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. Isotope enrichments were measured using gas chromatography-mass spectrometry (GCMS). Plasma glucose and [6,6-2H2]glucose enrichment were measured as described previously (23). EGP was calculated by the methods of Steele (24). [1,1,2,3,3-d5]glycerol was measured as described by Patterson et al. (25). Glycerol concentration and [1,1,2,3,3-d5]glycerol enrichment were used to calculate glycerol kinetics with Steele’s equation for steady-state conditions. Triglycerides in liver were measured after a single step lipid extraction with methanol and chloroform (26). The pellets were finally dissolved in 2% Triton X-100 (Sigma-Aldrich, Germany) and triglycerides were measured using the “Trig/GB” kit (Roche, Mannheim, Germany). Expression and phosphorylation of ACC protein from a liver homogenate was determined by SDS-PAGE Western blotting (Supplemental data 2).

**RNA isolation and real time PCR**

After the experiments, liver tissue was removed after an overdose of pentobarbital IV. Liver mRNA was isolated on the Magna Pure (Roche Molecular Biochemicals, Mannheim, Germany) using the Magna Pure LC HS mRNA kit. Approximately 10 mg of tissue was homogenized in the lysis buffer supplied with the kit using the Magna Lyser according to the kit protocol (Roche Molecular Biochemicals). The cDNA synthesis was performed using
the Transcriptor First Strand cDNA Synthesis kit for RT-PCR with oligo d(T) primers (Roche Molecular Biochemicals). All primers (Supplemental Table 1) were either intron spanning or checked for DNA contamination using a −RT reaction. Real-time PCR was performed using the Lightcycler 480 (Roche Molecular Biochemicals) and the Lightcycler 480 Sybr Green I Master kit (Roche Molecular Biochemicals). Samples were corrected for their mRNA content using HPRT as a reference gene. The reference gene was not altered by the different interventions used in the NPY and denervation experiment (NPY P = 0.51, Denervation P = 0.37) and fasting and denervation experiment (Fasting P = 0.16, Denervation P = 0.71). Samples were baseline corrected and individually checked for their PCR efficiency (27) using LC480 Conversion and LC9beta software, provided by Dr. J. M. Ruijter (Amsterdam, The Netherlands). The median of the efficiency was calculated for each assay and samples that had a greater difference than 0.05 of the efficiency median value were not taken into account (0-5%). The original amounts of target cDNA were calculated by LC480 Conversion and LC9beta software; calculation was based on the mean efficiency of the amplicon (28).

**Statistical analysis**

Data are presented as mean ± SEM. Data were first analyzed by one-way ANOVA. For the experiment combining denervation and NPY infusion, and the comparison between the sham and sympathetic denervation in the fasted and postprandial state, a two-way ANOVA was used. A significant (P ≤ 0.05) global effect of ANOVA was followed by post hoc tests of individual group differences (Fisher’s protected least significant difference). For continuous measurements during this study a general linear model (GLM) analysis with repeated measurements was used, with Treatment as between-animal factor and Time as within-animal factor. Significance was defined at P < 0.05.

**RESULTS**

**The sympathetic nervous system is necessary to regulate VLDL-TG secretion during fasting**

We hypothesized that in the fasted state the ANS is necessary to regulate VLDL-TG secretion. To test this hypothesis, we combined overnight fasting with a selective hepatic denervation (i.e. sham denervation, sympathetic denervation, parasympathetic denervation or a total denervation). We measured hepatic VLDL-TG secretion after injection of tyloxapol. Tyloxapol inhibits lipoprotein lipase, thereby blocking the uptake of triglycerides by the peripheral tissues. In the absence of chylomicrons carrying triglycerides from the gut, the increase in plasma triglycerides reflects VLDL-TG secretion (20). In the fasted state, VLDL-TG secretion in the selective sympathetically denervated rats (Sx) was significantly lower than in sham and selective parasympathetically denervated (Px) rats (Figs. 1A and B). Importantly, VLDL-TG secretion in Px rats was not significantly different from the sham controls. Moreover, a total hepatic denervation (Tx) did not add to the effect of sympathetic denervation alone (Fig. 1B). The effectiveness of the sympathetic denervation was confirmed by markedly reduced levels of noradrenaline in liver tissue (Fig. 1C). The decrease in VLDL-TG secretion in Sx rats did not result in an increase of liver triglyceride content (P = 0.54).
None of the denervation protocols affected body weight (P = 0.21) or food intake before fasting (P = 0.90), nor baseline plasma corticosterone (P = 0.25), insulin (P = 0.27), FFA (P = 0.71) and glucose (P = 0.61) concentrations. These experiments show that an intact hepatic sympathetic innervation is necessary to maintain VLDL-TG secretion during fasting.

**Sympathetic liver denervation prevents the stimulatory effect of NPY on VLDL-TG secretion**

To test our hypothesis that during fasting the increased release of hypothalamic NPY is responsible for stimulating hepatic VLDL-TG secretion via the SNS we combined the ICV infusion of NPY with a selective sympathetic liver denervation. In contrast to the fasted condition in the previous experiment, the rats were instead subjected to the postprandial (PP) condition (4 hr fast) to have a low endogenous NPY tone and nearly undetectable chylomicrons in plasma (5). In the PP condition, the sympathetic denervation itself did not change VLDL-TG secretion compared to the sham control (3.30±0.22 vs. 3.52±0.28 mmol/l/h). Infusion of NPY in the third ventricle of the brain in sham operated rats strongly increases VLDL-TG secretion compared to the vehicle control (4.82±0.35 vs. 3.52±0.28 mmol/l/h) (Figs. 2A and B). However, ICV NPY administration in Sx rats no longer resulted in a significant increase of VLDL-TG secretion compared to the vehicle control (4.02±0.14 vs. 3.30±0.22 mmol/l/h). Finally, the NPY-induced VLDL-TG secretion was significantly lower in Sx as compared to sham-denervated NPY-infused rats (4.02±0.14 vs. 4.82±0.35 mmol/l/h) (Fig. 2B). The marked decrease in liver noradrenaline levels confirmed a successful selective sympathetic liver denervation (Fig. 2C). There were no significant differences in body weight (P = 0.28) or food intake (P = 0.45) in the night before the experiment. During the experiment, we observed no differences in plasma corticosterone (Time*Treatment P = 0.85) and insulin (Time*Treatment P = 0.24) between the four groups.

To further dissect the metabolic pathways in the liver by which central NPY controls VLDL-TG secretion, we analyzed gene expression of key hepatic enzymes. NPY infusion increased mRNA levels of ADP-ribosylation factor (ARF-1), only in sham operated rats (Fig. 2D). The
mRNA levels of other genes involved in VLDL secretion, including apolipoprotein B (ApoB) and microsomal triglyceride transfer protein (MTTP) were not modified by NPY treatment. NPY infusion decreased carnitine palmitoyltransferase 1 alpha (CPT1α) mRNA levels in the sham denervated rats, but not in the Sx groups (Fig. 2E). We found no differences in expression of genes promoting lipogenesis, including acetyl-coenzyme A carboxylase alpha (ACC1) (Fig. 2F), acetyl-coenzyme A carboxylase beta (ACC2), fatty acid synthase (FAS), stearoyl-coenzyme A desaturase 1 (SCD1), peroxisome proliferator-activated receptor gamma and sterol regulatory element binding transcription factor 1c (SREB1c). Western blot analysis revealed no changes in total ACC protein or phosphorylated ACC/ACC protein ratio after NPY infusion (data not shown).
**Nutritional status has clear effects on genes regulating hepatic lipid metabolism**

With regard to nutritional status the previous experiments show that: 1) during fasting conditions a sympathetic liver denervation lowers VLDL-TG secretion, compared to sham-denervated animals; 2) in the postprandial condition, sympathetically denervated rats do not show a lower VLDL-TG secretion compared to sham-denervated animals, and 3) comparing experiments 1 and 2 shows that in intact animals VLDL-TG secretion is not significantly different between postprandial and fasted sham animals. Together these results clearly indicate that only during fasting conditions the SNS is necessary to stimulate VLDL-TG secretion in order to maintain VLDL-TG secretion at a postprandial level. We therefore compared expression levels of key genes involved in lipid metabolism, between the sham and sympathetic denervated rats in the fasted and the postprandial experiments, to determine which pathways are regulated by the SNS after fasting (Table 1). Lower baseline concentrations of plasma glucose and insulin, and higher baseline plasma concentrations of FFA illustrate the clear metabolic differences between the fasted and postprandial rats (Table 1). Fasted rats show decreased mRNA expression of genes involved in lipogenesis including, ACC1, ACC2, FAS, SCD1 and PPARγ, and increased mRNA expression of a gene promoting oxidation, CPT1α (Table 1). ACC 1 (P = 0.057) and MTTP (P = 0.084) showed a trend towards an interaction effect in the multivariate model including fasting and denervation. Western blot analysis revealed no changes in total ACC protein and the phosphorylated ACC/ACC protein ratio between the

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**Table 1.** Comparison of baseline plasma parameters and gene expression in liver tissue between fasted and postprandial (PP) rats with a sham or sympathetic (Sx) denervation.

<table>
<thead>
<tr>
<th></th>
<th>Fasted Sham</th>
<th>Fasted Sx</th>
<th>PP Sham</th>
<th>PP Sx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mmol/l)</td>
<td>4.1 ± 0.1</td>
<td>3.9 ± 0.2</td>
<td>5.8 ± 0.2</td>
<td>5.5 ± 0.4</td>
</tr>
<tr>
<td>FFA (mmol/l)</td>
<td>0.78 ± 0.08</td>
<td>0.72 ± 0.03</td>
<td>0.47 ± 0.05</td>
<td>0.41 ± 0.05</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>0.22 ± 0.05</td>
<td>0.33 ± 0.09</td>
<td>1.57 ± 0.22</td>
<td>1.57 ± 0.09</td>
</tr>
<tr>
<td>ACC 1 mRNA</td>
<td>0.07 ± 0.01</td>
<td>0.03 ± 0.01</td>
<td>0.09 ± 0.02</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>ACC 2 mRNA</td>
<td>0.03 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>0.11 ± 0.02</td>
<td>0.15 ± 0.04</td>
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<tr>
<td>ApoB mRNA</td>
<td>0.40 ± 0.07</td>
<td>0.52 ± 0.06</td>
<td>0.44 ± 0.08</td>
<td>0.56 ± 0.06</td>
</tr>
<tr>
<td>Arf1 mRNA</td>
<td>2.12 ± 0.33</td>
<td>2.13 ± 0.32</td>
<td>2.14 ± 0.32</td>
<td>1.95 ± 0.23</td>
</tr>
<tr>
<td>CPT1α mRNA</td>
<td>1.25 ± 0.20</td>
<td>1.14 ± 0.11</td>
<td>0.72 ± 0.08</td>
<td>0.71 ± 0.13</td>
</tr>
<tr>
<td>FAS mRNA</td>
<td>1.59 ± 0.61</td>
<td>2.07 ± 0.44</td>
<td>10.83 ± 1.53</td>
<td>10.74 ± 1.10</td>
</tr>
<tr>
<td>MTTP mRNA</td>
<td>0.63 ± 0.07</td>
<td>0.79 ± 0.02</td>
<td>0.74 ± 0.06</td>
<td>0.69 ± 0.05</td>
</tr>
<tr>
<td>PPARγ mRNA</td>
<td>0.32 ± 0.04</td>
<td>0.36 ± 0.07</td>
<td>0.43 ± 0.04</td>
<td>0.49 ± 0.06</td>
</tr>
<tr>
<td>SCD1 mRNA</td>
<td>0.25 ± 0.07</td>
<td>0.41 ± 0.13</td>
<td>6.05 ± 1.35</td>
<td>5.72 ± 1.69</td>
</tr>
<tr>
<td>SREBP1c mRNA</td>
<td>0.13 ± 0.04</td>
<td>0.26 ± 0.07</td>
<td>0.24 ± 0.04</td>
<td>0.30 ± 0.13</td>
</tr>
</tbody>
</table>

In a multivariate analysis including nutritional status and sympathetic denervation significant differences were observed between the fasted and PP groups in plasma glucose (P < 0.001), insulin (P < 0.001), FFA (P < 0.001) and gene expression levels of ACC1 (P < 0.01), ACC2 (P < 0.01), FAS (P < 0.001), SCD1 (P < 0.001), PPARγ (P < 0.05), and CPT1α (P < 0.01) (Table 1). ACC 1 (P = 0.057) and MTTP (P = 0.084) showed a trend towards an interaction effect in the multivariate model including fasting and denervation. Values are mean ± SEM of 7-9 animals per group.
Sx and sham groups (data not shown). Thus, although several genes involved in hepatic lipid metabolism are clearly affected by the nutritional status, analysis of mRNA expression of the fasted and postprandial denervated rats did not reveal the molecular pathways involved in lower VLDL-TG secretion in the Sx rats during fasting.

**Rats with a lesioned arcuate nucleus cannot maintain VLDL-TG secretion during fasting**

We subsequently investigated whether rats with a lesion of the ARC, an important component of the sympathetic outflow circuit to the liver (10), can maintain VLDL-TG secretion during fasting at a postprandial level. Adequate MSG treatment was shown by a pronounced decrease of NPY immunoreactive fibers in the ARC and PVN (Fig. 3A-D) in adult rats. First, comparing MSG-treated sham liver-denervated rats in the postprandial and fasted state, revealed a significant decrease in baseline plasma triglyceride concentration (Table 2) and a 42% decrease in VLDL-TG secretion (Fig. 3E). But in the fasted MSG-treated rats a sympathetic liver-denervation did not result in an additional significant decrease in VLDL-TG secretion as shown in the first experiment in rats with an intact ARC. The MSG-treated rats in the different groups did not differ in body weight on the day before the
experiment, nor in baseline plasma corticosterone levels (Table 2). Noradrenaline values were significantly lower in the MSG Sx group (P < 0.05). The fasted condition was clearly reflected in the significantly decreased plasma glucose concentrations compared to the PP rats (Table 2). With our sample size, we observed no significant effect of fasting on plasma insulin concentrations as seen in non-treated animals. However, MSG-treated animals were clearly hyperinsulinemic and showed larger variance in plasma insulin levels as compared to non-treated animals (Tables 1 and 2). These data show that fasted MSG rats cannot maintain VLDL-TG secretion to a postprandial level. Furthermore, in fasted MSG-treated rats sympathetic denervation does not affect VLDL-TG secretion as compared to fasted sham denervated rats.

Table 2. Comparison of baseline parameters between the MSG groups postprandial (PP) sham and fasted sham, sympathetically denervated (Sx) and parasympathetically denervated (Px) rats.

<table>
<thead>
<tr>
<th></th>
<th>PP MSG Sham</th>
<th>Fasted MSG Sham</th>
<th>Fasted MSG Sx</th>
<th>Fasted MSG Px</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mmol/l)</td>
<td>6.1 ± 0.1</td>
<td>5.0 ± 0.2*</td>
<td>4.9 ± 0.2*</td>
<td>4.9 ± 0.2*</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>2.26 ± 0.42</td>
<td>1.18 ± 0.20*</td>
<td>1.38 ± 0.23†</td>
<td>1.05 ± 0.19*</td>
<td>0.016</td>
</tr>
<tr>
<td>Corticosterone (ng/ml)</td>
<td>61 ± 21</td>
<td>53 ± 24</td>
<td>32 ± 9</td>
<td>100 ± 51</td>
<td>0.493</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>2.67 ± 0.48</td>
<td>1.77 ± 0.38</td>
<td>1.58 ± 0.64</td>
<td>1.66 ± 0.43</td>
<td>0.381</td>
</tr>
<tr>
<td>Bodyweight (g)</td>
<td>331 ± 3</td>
<td>332 ± 2</td>
<td>331 ± 3</td>
<td>334 ± 4</td>
<td>0.930</td>
</tr>
</tbody>
</table>

Values are mean ± SEM of 6-8 animals per group. * P < 0.01, † P < 0.05 compared to Sham PP.

Figure 4. ICV NPY does not affect lipolysis. A: Experimental protocol of the double stable isotope study to measure lipolysis and endogenous glucose production (EGP) during NPY or vehicle ICV treatment. B-G: Acute effect of ICV NPY (closed circles) or vehicle (open squares) on rate of appearance (Ra) of glycerol (Time*Treatment P = 0.90), endogenous glucose production (EGP) (Time*Treatment P < 0.01), plasma FFA (Time*Treatment P = 0.10), plasma glucose (Time*Treatment P = 0.06), plasma triglycerides (Time*Treatment P = 0.10) and plasma insulin (Time*Treatment P = 0.12) concentrations. Values are mean ± SEM of 6-7 rats per group.
Central NPY does not affect lipolysis

Finally, we investigated whether the increased VLDL-TG secretion after central NPY infusion could be due to increased substrate availability of FFA through lipolysis. We investigated the effect of ICV administered NPY on lipolysis with a double stable isotope technique measuring endogenous glucose production and lipolysis simultaneously. The stable isotopes [1,1,2,3,3-d5]glycerol and [6,6-2H2]glucose were used (Fig. 4A). No significant effects of central NPY on lipolysis, assessed either by glycerol appearance or plasma FFA levels, could be observed (Figs. 4B and D). On the other hand, a clear effect of central NPY on endogenous glucose production was observed (Fig. 4C). Plasma triglyceride and glucose concentrations showed a trend towards an increase (Figs. 4E and F). We observed no significant differences in plasma corticosterone (Time*Treatment P = 0.13), glucagon (Time*Treatment P = 0.81) or insulin (Fig. 4G) concentrations between the groups.

DISCUSSION

In recent years the important role of the central nervous system in controlling liver metabolism has become more and more evident. A number of insightful experiments considerably increased our understanding of the mechanisms employed by the hypothalamus to control glucose metabolism through the ANS (12-14,17,18). Our current experiments show that an intact hepatic sympathetic innervation and arcuate nucleus are also necessary to maintain VLDL-TG secretion during fasting. In agreement, a central infusion of NPY cannot increase VLDL-TG secretion in sympathetically liver denervated rats. Together these data indicate that the increased release of hypothalamic NPY during fasting maintains hepatic VLDL-TG secretion via the sympathetic input to the liver (Figure 5). This mechanism could be of physiological importance during fasting, when lipids are the main energy source. However, this mechanism could play a pathophysiological role in conditions characterized by a constant high activity of NPY as found in animal models of obesity and hypertriglyceridemia (6,8,29). Recent data in humans support this hypothesis, as high sympathetic activity and low parasympathetic activity significantly correlate with components of the metabolic syndrome, including hypertriglyceridemia (2). Additionally, genetic screening revealed a novel polymorphism in the NPY 1-5 gene to be associated with reduced serum triglyceride levels in a severely obese cohort (30).

The notion that the autonomic innervation of the liver is not only important for the control of carbohydrate metabolism, but also hepatic lipid metabolism is supported by a previous study applying phenol to the liver (31). In our study we used microsurgery to selectively denervate the sympathetic and parasympathetic inputs to the liver and dissect the separate roles of these antagonistic branches of the ANS. We conclude that during fasting specifically the SNS is important in the regulation of VLDL-TG metabolism. Our results also extend the study of Stafford et al. (5) showing that ICV NPY increases VLDL-TG secretion in the postprandial condition and ICV infusion of the Y1 antagonist decreases VLDL-TG secretion during fasting.

The MSG model induces a destruction of 80-90% of the neurons in the ARC, while sparing glial cells or axons passing through the nucleus (22,32). It was shown by others that
during fasting the increased NPY immunoreactivity in the ARC and PVN does not occur in the MSG model (15). We are, however, aware that the neurotoxic lesion is not restricted to NPY neurons, but affects other neurons in the ARC as well (22). In spite of the caveats of the MSG model, the data from our experiments in MSG animals support a role for the arcuate nucleus and possibly NPY in activating the SNS to control hepatic lipid metabolism during fasting as the experiments were performed in rats with similar body weights. Furthermore, MSG animals were still responsive to exogenous NPY (data not shown).

The strength of this study is that by a selective hepatic denervation we were able to show the neural pathway by which hypothalamic NPY increases VLDL-TG secretion. This is important as NPY is also known to exhibit endocrine effects (33). We propose that NPY neurons in the ARC activate second order neurons in the PVN, which in turn activate the preganglionic sympathetic neurons in the intermediolateral column (IML) of the spinal cord. This notion is supported by Viñuela et al. (9) who combined retrograde tracing from the IML with ICV administration of NPY and clearly demonstrated that sympathetic pre-autonomic
hypothalamic neurons are activated by NPY ICV. However, the lack of infected NPY neurons in the ARC after viral tracing from the liver (11) indicates that the NPY projection from the ARC to the pre-autonomic neurons might involve an interneuron. Alternatively, we cannot exclude from our experiments that NPY activates the SNS at the level of the brain stem. Although an inhibition of the melanocortin system could also be important during fasting, a previous study (5) did not find an acute effect of central melanocortin stimulation or blockade on VLDL-TG secretion. Moreover, contrary to the effect of chronic ICV NPY administration, chronic blockade of the central melanocortin system does not change plasma levels of triglycerides independently of increased food intake, although it does result in an obese phenotype (34-36). Therefore, in acute and chronic conditions hypothalamic NPY seems more important than the melanocortin system in regulating hepatic VLDL-TG secretion. Conversely, the effects of the melanocortin system on white adipose tissue are well established (36).

The molecular mechanism through which the activation of the SNS after ICV NPY regulates VLDL-TG secretion probably includes an increased VLDL-TG assembly and decreased β-oxidation. The second step in VLDL assembly is dependent on ARF1, which was increased in our NPY infused rats. This may reflect an increased production of mature VLDL particles (37). We also observed decreased mRNA levels of CPT1α indicating decreased oxidation of free fatty acids in the liver. When oxidation is inhibited, more substrate can be guided to the alternative route, i.e., resulting in a higher VLDL-TG secretion. Therefore, both of the above mentioned mechanisms may contribute to increased VLDL-TG secretion during the ICV administration of NPY. Surprisingly, the effects of exogenous NPY on oxidation were contrary to changes occurring during fasting (when endogenous NPY levels are high), when increased oxidation was observed. Our results point to the possibility that the central nervous system stimulates the liver to maintain VLDL-TG secretion in competition with the peripheral effects of fasting. This is in accordance with our observation that VLDL-TG secretion does not increase after fasting in spite of the fact that NPY levels are physiologically high.

We show that central NPY has no effect on lipolysis and this therefore does not attribute to increased VLDL-TG secretion. Others have shown that NPY inhibits lipolysis in vitro and after systemic administration, which possibly reflects a peripheral effect of NPY derived from the autonomic nerve endings on adipocytes (38-40).

In summary, we provide evidence that the activation of NPY neurons in the hypothalamus has a stimulatory role on hepatic VLDL-TG secretion through the SNS. We believe our data are of importance in understanding the physiological and pathophysiological role of the central nervous system in controlling lipid metabolism.

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REFERENCES


SUPPLEMENTAL DATA 1. IMMUNOHISTOCHEMISTRY

MSG lesions were checked by NPY immunohistochemistry and compared to saline infused rats. For immunohistochemical staining, animals were injected with pentobarbital IV and perfused intra-arterially with saline and 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). After post-fixation and equilibration for 48 hrs in 30% sucrose in 0.1M Tris-buffered saline (TBS), the brain tissue was cut on a cryostat into 35 µm sections into 4 groups. Free floating sections were incubated in the primary antiserum rabbit anti-NPY (1:1,000) overnight at room temperature. The NPY antibody (Niepke 26/11/1988, Netherlands Institute for Brain Research) was raised in rabbit against porcine NPY (N4509; Sigma) as previously described (Buijs et al. 1989; van der Beek et al. 1992; Goldstone et al. 2002). Sections were rinsed in TBS and incubated in biotinylated goat anti-rabbit antibody (1:800; Vector Laboratories, Burlingame, CA) for 1 h, rinsed three times in TBS, and incubated in avidin–biotin complex (Elite ABC, Vector Laboratories) for 1 h. After three rinses, sections were incubated in 1% diaminobenzidine (DAB) and 0.05% nickel ammonium sulfate and reacted with 0.01% hydrogen peroxide. Sections were washed, mounted on gelatin-coated glass slides, dehydrated in graded alcohols, cleared in xylene, and coverslipped using Entellan for observation by light microscopy.

Supplemental references

3. Goldstone AP, Unmehopa UA, Bloom SR, Swaab DF. Hypothalamic NPY and agouti-related protein are increased in human illness but not in Prader-Willi syndrome and other obese subjects. J Clin Endocrinol Metab 2002;87:927-937

SUPPLEMENTAL DATA 2. WESTERN BLOT ANALYSIS

A portion of liver tissue was homogenized in 1 ml buffer (0.05 M sodium phosphate, 2 mM EDTA, 25 mM DTT, 125 mM sucrose, 5% glycerol, 1 mM PMSF, peroxovanadate and protease inhibitors (Complete, Roche). Homogenates were subjected to centrifugation and total protein was determined in supernatant using a commercially available kit (Bio-Rad, Hercules, USA). Equal amounts of protein were diluted in Laemmli sample buffer. The samples were boiled for 5 minutes. 30 µg of protein was loaded on a 10% SDS-PAGE gel. Gels were blotted on Immobilon-P Transfer Membrane (Millipore, Bedford, USA). Phosphorylation and levels of several proteins were determined using the following antibodies: ACC (#3662, Cell Signalling Technology), phosphorylated ACC (Ser 79, #3661, Cell Signalling Technology) and rabbit-anti-goat-HRP (P0449, DAKO). All membranes were normalized with Actin (I-19, sc-1616, Santa Cruz). Bound antibodies were visualized by enhanced chemiluminescence and quantified by densitometry analysis of scanned images (Lumimages, Boehringer, Mannheim).
**Supplemental Table 1.** Primer sequences used for real-time PCR.

<table>
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<tr>
<th>Gene</th>
<th>Accession no.</th>
<th>Forward sequence</th>
<th>Reverse sequence</th>
<th>Product</th>
<th>Annealing T</th>
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<td>ACC1</td>
<td>NM_022193</td>
<td>GATGATCAAGGAGGCAGCTGT</td>
<td>CAGGCTACCATGCCAATCTC</td>
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<td>ACC2</td>
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<td>FAS</td>
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<td>GCCCTCCCGTACACTCACTC</td>
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<td>CPT1α</td>
<td>NM_031559</td>
<td>ACAATGGGACATTCCAGGAG</td>
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<tr>
<td>PPARγ</td>
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<td>SREBP1c</td>
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<td>SCD1</td>
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PCR product length (bp), Annealing temperature (°C).