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Insulin sensitivity : modulation by the gut-brain axis

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Citation
Heijboer, A. C. (2006, April 25). *Insulin sensitivity : modulation by the gut-brain axis*. Retrieved from https://hdl.handle.net/1887/4370

Version:	Corrected Publisher's Version
License:	Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden
Downloaded from:	https://hdl.handle.net/1887/4370

Note: To cite this publication please use the final published version (if applicable).

Chapter 3

High fat diet induced hepatic insulin resistance is not related to changes in hypothalamic mRNA expression of NPY, AgRP, POMC and CART in mice

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Peptides: 26(12):2554-8, 2005

ABSTRACT

The hypothalamic circuitry, apart from its impact on food intake, modulates insulin sensitivity to adapt metabolic conditions in the face of environmental fluctuations in nutrient availability. The purpose of the present study was to investigate the effects of 2 weeks high fat feeding in wildtype mice on (1) insulin sensitivity and triglyceride accumulation in liver and muscle in relation to (2) mRNA expression levels of Neuropeptide Y (NPY), Agouti-related protein (AgRP), pro-opiomelanocortin (POMC), and cocaine- and amphetamine-regulated transcript (CART) in the hypothalamus. Two weeks of high fat feeding induced hepatic insulin resistance in the presence of increased hepatic triglyceride accumulation. In muscle, however, 2 weeks of high fat feeding did not result in changes in insulin sensitivity or in triglyceride content. mRNA expression levels of NPY, AgRP, POMC, and CART in the hypothalamus were not different between the groups. This study shows that 2 weeks of high fat feeding in mice does not affect mRNA expression levels of NPY, AgRP, POMC or CART, in the whole hypothalamus, despite induction of hepatic, but not peripheral, insulin resistance. Therefore, a major physiological role of these neuroendocrine factors in the induction of hepatic insulin resistance during a high fat diet seems less likely.

INTRODUCTION

High fat (HF) diet is associated with the development of hepatic and muscle insulin resistance and the induction of liver steatosis and muscle triglyceride accumulation in rodents and humans. The induction of hepatic insulin resistance is an early event, whereas the indication of muscle insulin resistance occurs later in rats and dogs fed a HF diet for 3-12 weeks (11,12). The change in time of hepatic and peripheral insulin sensitivity during high fat feeding in mice has not been reported so far.

The hypothalamic arcuate circuitry, apart from its impact on food intake, modulates insulin sensitivity to adapt metabolic conditions in the face of environmental fluctuations in nutrient availability. Two types of neurons in the hypothalamus are of major importance for the control of these processes: neurons co-expressing neuropeptide Y (NPY) and Agouti-related protein (AgRP), and neurons co-expressing pro-opiomelanocortin (POMC), the molecular precursor of alpha-melanocyte stimulating hormone (α-MSH) and cocaine- and amphetamine-regulated transcript (CART) (8). These neuropeptides exert opposing effects on food intake and fuel homeostasis. We, and others, recently showed that, independent of its stimulating effect on food intake, NPY is also involved in controlling insulin action in peripheral tissues. Intracerebroventricular (icv) infusion of NPY induces hepatic insulin resistance in mice and rats (15,23). Conversely, subchronic (7 days) icv infusion of α-MSH enhances peripheral and hepatic insulin sensitivity in rats through stimulation of the melanocortin-3/4 receptor (16). These observations suggest that the balance between NPY/AgRP and POMC/CART neuronal activity may be a determinant of peripheral and hepatic insulin sensitivity.

To explore a potential physiological relationship between the arcuate NPY/POMC circuitry and the development of tissue specific insulin resistance during a HF diet, we studied the effects of 2 weeks HF feeding in wildtype mice on (1) insulin sensitivity and TG accumulation in liver and muscle in relation to (2) mRNA expression levels of NPY, AgRP, POMC, and CART in the whole hypothalamus.

MATERIAL AND METHODS

Animals

Male, 12-16 weeks old, C57Bl/6 mice were used in all experiments. Mice were kept in a temperature- and humidity controlled environment and had free access to food and water. Light were on from 07:00 h a.m. till 07:00 h p.m. The control group had

free access to standard chow diet whereas the experimental group had a high fat diet (21.5 wt.% fat or 43 energy% fat; saturated bovine fat) (Hope Farms, Woerden, the Netherlands) ad libitum. Before the experiments mice were fasted 4 h (postprandial) with food withdrawn at 05:00 h a.m., in order to exclude interfering effects of glucose uptake from the gastrointestinal tract. All experiments were performed at 09:00 h a.m. All animal experiments were approved by the Animal Ethic Committee from the Leiden University Medical Center and TNO-Prevention and Health, Leiden, the Netherlands.

Hyperinsulinemic euglycemic clamp study

The hyperinsulinemic euglycemic clamp study was performed as published earlier (7,22,24). In short, a continuous infusion of D-^{14}C glucose, 0.3 µCi/kg.min, (Amersham, Little Chalfont, UK) alone was started and blood samples were taken to determine basal glucose parameters. The hyperinsulinemic study started with a bolus (100 mU/kg, Actrapid, Novo Nordisk, Bagsvaerd, Denmark) followed by continuous infusion of insulin (6.8 mU/h) and of D-^{14}C glucose. A variable infusion of 12.5% D-glucose (in PBS) solution was adjusted to maintain euglycemia, as measured via tailbleeding (Freestyle, TheraSense, Disetronic Medical Systems BV, Vianen, the Netherlands). Blood samples (75µl) were taken when glucose levels in the blood were stable for determination of plasma [^{14}C]glucose and insulin concentrations. To estimate insulin-stimulated glucose uptake in individual tissues, 2-deoxy-D-[^{3}H]glucose (2-[^{3}H]DG; Amersham, Little Chalfont, UK) was administered as a bolus (1µCi), 40 minutes before the end of the clamp. After the last blood sample was taken, mice were sacrificed and liver and muscle were taken out, immediately frozen using liquid N_2 and stored at -20°C until further analysis. For the determination of plasma D-^{14}C glucose, plasma was deproteinized with 20% trichloroacetic acid, dried to remove water, resuspended in demi-water and counted with scintillation fluid (Ultima Gold, Packard, Meridien, CT, USA) on dual channels for separation of ^{14}C and ^{3}H, as described earlier (7,22,24).

Plasma parameters

Plasma glucose, free fatty acid and insulin levels were determined using commercially available kits (Sigma, St.Louis, MO; Wako Pure Chemical Industries, Osaka, Japan; Mercodia, Uppsala, Sweden, respectively).

Tissue analysis

Liver and muscle samples were homogenized (~10% wet w/v) in H_2O. Lipids were extracted according to Bligh and Dyer's method (2). In short, a solution was made of each sample of 200 µg protein in 800 µl H_2O, then 3 ml methanol/chloroform (2:1) was added and mixed thoroughly, after which 500 µl of chloroform, 100 µl internal standard and 1 ml demi-water were added. After centrifugation the chloroform layer was collected and dried. The remaining pellet was dissolved in 50µl chloroform and put on a HPTLC plate. The amount of TG in the tissues was quantified by scanning the plates with a Hewlett Packard Scanjet 4c and by integration of the density using Tina® version 2.09 software (Raytest, Staubenhardt, Germany).

For determination of tissue 2-DG uptake, the homogenate of muscle was boiled and the supernatant was subjected to an ion-exchange column to separate 2-DG-6-P from 2-DG as described previously (7,18,22,24).

Calculations

Under steady-state conditions for plasma glucose concentrations, the rate of glucose disappearance equals the rate of glucose appearance (Ra; i.e. endogenous glucose production plus exogenous D-glucose infusion). The latter was calculated as the ratio of the rate of infusion of D-^{14}C glucose (dpm/min) and the steady-state plasma [^{14}C]glucose specific activity (dpm/µmol glucose). Hepatic glucose production (HPG) was calculated as the difference between Ra and the infusion rate of exogenous D-glucose. Muscle tissue specific glucose uptake was calculated from tissue 2-DG content, which was expressed as percent of 2-DG of the dosage per gram of tissue, as described previously (18).

Extraction of hypothalamus

Two other groups of mice fed on chow or 2 weeks HF diet were, after 4 hours fasting, sacrificed at 9 a.m. by cervical dislocation. After death, the brain was dissected from the skull. A region bordered dorsally by the thalamus, rostrally by the optic chiasm, laterally by the internal capsule, and caudally by the mammillary bodies was excised, as described previously (9). Hypothalami removed were immediately frozen in liquid nitrogen.

Real Time Polymerase Chain Reaction (RT-PCR)

A real time polymerase chain reaction (RT-PCR) was used to measure NPY/AgRP/POMC/CART mRNA expression levels in the hypothalamus of mice after HF feeding and in control mice. Hypothalami were homogenised in 1,2 ml RNA-Bee

(Tel-Test, Inc, Texas, US) and total RNA was extracted according to Chomzcinsky and Sacchi (5). The amount of RNA was determined by spectrophotometry (ND-1000 spectrophotometer, Nanodrop®) at a wavelength of 260 nm. The quality was checked by the ratio of the absorption at 260 nm and the absorption at 280 nm. Complementary DNA (cDNA) was obtained of total RNA.

For RT-PCR, forward and reverse primers were designed from mice specific sequence data (*Entrez*, National Institutes of Health; and *Ensembl*, Sanger Institute) using computer software (Primer Express, Applied Biosystems). For each of the genes a Blast Search was done to reveal that sequence homology was obtained only for the target gene. Forward and reverse primers of NPY (5'CGCTCTGCGACACTACATCAA3'; 5'GGGCTGGATCTCTTGCCAT3'), AgRP (5' GGTGCTAGATCCACAGAACCG 3'; 5' CCAAGCAGGACTCGTGCAG 3'), POMC (5' CGAGGCCTTTCCCCTAGAGT 3'; 5' CCAGGACTTGCTCCAAGCC 3'), and CART (5' TGGATGATGCGTCCCATGA 3'; 5' CGGAATGCGTTTACTCTTGAGC 3') were used. Neuron-specific enolase (NSE) (5' ACGTGGTTCCATTTCAAGATGAC 3'; 5' CGAGCTTCAGTTAGTGCACCAA 3') and hypoxanthine phosphoribosyltransferase (HPRT) (5' GCTCGAGATGTCATGAAGGAGAT 3'; 5' AAAGAACTTATAGCCCCCCTTGA 3') were used as housekeeping genes.

PCR amplification was performed in a total reaction volume of 20 µl. The reaction mixture consisted of SybrGreen mix (Applied Biosystems, United Kingdom), forward and reverse primers, nuclease free water and cDNA. An identical cycle profile was used for all genes: 50°C for 2 min + 95°C for 10 min + [95°C for 15 sec + 60°C for 1 min] * 50 cycles. Specificity of NPY, AgRP, POMC, CART, NSE and HPRT amplifications was confirmed by analyzing the dissociation curves of the target amplicon. Data were analysed using a comparative critical threshold method as described previously (7).

Statistical analysis

Data are presented as means ± standard deviation. Data were analysed using a non-parametric Mann-Whitney *U*-test for independent samples. Differences were considered statistically significant at $P \leq 0.05$.

RESULTS

Body weight and plasma parameters

Two weeks of high fat diet did not lead to differences in body weight, plasma glucose, insulin or FFA levels compared to chow-fed littermates. We also achieved equal plasma glucose, insulin and FFA levels during the hyperinsulinemic euglycemic clamp (table 1).

Table 1. Body weight and plasma glucose, insulin, and FFA concentrations of HF fed and control mice under basal and hyperinsulinemic conditions.

	Body weight (g)	Glucose (mmol/l)		Insulin (ng/ml)		FFA (mmol/l)	
		basal	hyper-insulinemia	basal	hyper-insulinemia	basal	hyper-insulinemia
control	27±2	5.9 ± 0.7	8.7±1.2	0.8±0.5	4.1±1.0	1.0±0.2	0.5±0.2
HF	27±2	6.7±1.2	8.8±0.7	1.9±1.4	3.7±1.7	1.0±0.2	0.6±0.2

Values represent the mean ± SD of at least 8 mice per group

Hepatic glucose production

Basal HGP was not different between the HF fed group and the control group (48±8 µmol/kg.min versus 43±9 µmol/kg.min, ns). Under hyperinsulinemic conditions, HGP was significantly higher in the HF fed group versus the control group (30±9 µmol/kg.min versus 16±10 µmol/kg.min, P<0.05). The relative inhibition of HGP was significantly less after HF feeding compared to the control group (35±20% versus 61±23%, P<0.05), indicating hepatic insulin resistance (figure 1).

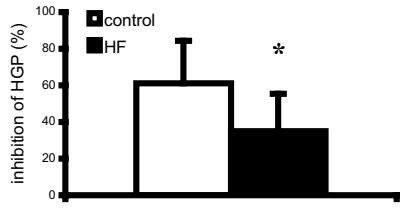

Fig. 1. Inhibition of hepatic glucose production of HF fed and control mice. Data are means ± SD for n=9 in the control group and n=8 in the HF group. *p < 0.05 vs control mice, as assessed by the Mann-Whitney *U* test.

Whole body glucose uptake and muscle specific 2-DG uptake

Basal whole body glucose uptake was not different between the HF fed group and the control group (48±8 µmol/kg.min versus 43±9 µmol/kg.min, ns). Under hyperinsulinemic conditions, whole body glucose uptake was not different between the HF fed group and the control group (66±10 µmol/kg.min versus 59±8 µmol/kg.min, ns).

Under hyperinsulinemic conditions, muscle-specific glucose uptake in the HF fed group was not significantly different from the control group (1.3±0.6 versus 1.3±0.3%/g tissue).

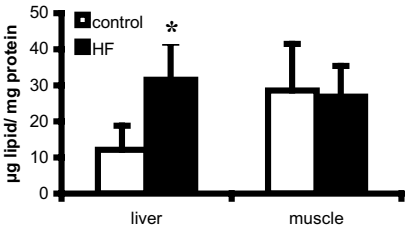

Fig. 2. TG-content determined in liver and skeletal muscle of HF fed and control mice. Data are means ± SD for n=7 in the control group and n=6 in the HF group. *p < 0.05 vs control mice, as assessed by the Mann-Whitney U test.

Tissue lipid levels

Hepatic TG content of HF fed mice was 2.5-fold higher compared to the control group (32±10 versus 12±6 µg/mg protein, P<0.05), whereas muscle TG content was not significantly different between the two groups (27±9 versus 23±7 µg/mg protein, ns) (figure 2).

mRNA expression levels

Hypothalamic mRNA expression levels of NPY, AgRP, POMC, and CART were not different between HF fed and control mice (100±11% versus 111±20%; 100±20% versus 113±20%;100±47% versus 140±54%;100±33% versus 81±12%, respectively, ns).

DISCUSSION

This study shows that 2 weeks of high fat feeding in wildtype mice causes hepatic insulin resistance, reflected by decreased inhibition of hepatic glucose production, without affecting muscle specific insulin sensitivity. However, this was not associated with differences in hypothalamic mRNA expression of NPY, AgRP, POMC, and CART. Therefore, the hypothalamus does not seem to play a major role in the primacy of hepatic insulin resistance during a HF diet in mice.

Although it was concluded from studies in the past in rats (17) and primates (4) that peripheral insulin resistance precedes the development of hepatic insulin resistance, our findings are in line with recent studies in rats (12) and dogs (11) documenting the primacy of hepatic insulin resistance during high fat feeding. Kraegen et al (12) used an extremely high fat diet (59% calorie fat) and found already after 3 weeks muscle specific insulin resistance as well. As this extreme diet is not relevant for the human situation we used a more human like high fat diet. However, even after 6 weeks, we did not observe muscle specific insulin resistance (unpublished data).

In our study, 2 weeks of high fat feeding resulted in hepatic insulin resistance in the presence of hepatic steatosis. These findings are in line with observations of

other groups [18;19] and could imply that hepatic steatosis is either a consequence of metabolic alterations elsewhere in the body and/or an active player in the pathophysiology of the metabolic syndrome.

The mechanisms underlying the impairment of insulin action during a high fat diet are only partly known. Studies performed in vitro and in vivo showed an increase in gluconeogenesis (6,13) and a decrease in the suppressive effect of insulin on glycogenolysis (3). Shulman *et al.* postulated a more direct effect of FFA on hepatic glucose production, by interference of fatty acid derivatives with insulin signalling (10,20).

The past years, data have become available on the potency of the hypothalamus to modulate insulin sensitivity during environmental fluctuations in nutrient availability. Independent of its stimulating effect on food intake and body weight, intracerebroventricular (icv) infusion of NPY causes hepatic insulin resistance in mice and rats (15,23). Conversely, subchronic (7 days) icv infusion of α-MSH enhances peripheral and hepatic insulin sensitivity in rats through stimulation of the melanocortin-3/4 receptor (16). These observations suggest that the balance between NPY/AgRP and POMC/CART neuronal activity is a determinant of peripheral and hepatic insulin sensitivity. In our study, however, we did not find significant changes in NPY, AgRP, POMC and CART mRNA expression levels in the whole hypothalamus during a 2 weeks HF diet, despite tissue-specific differences in insulin sensitivity. Since in our study, mRNA levels were measured in whole hypothalamus without regional dissection of the data, we cannot totally exclude involvement of certain specific hypothalamic areas in the regulation of hepatic insulin resistance during a high fat diet. To our knowledge, differential activation of NPY neurons in the medial and lateral part of the arcuate nucleus is possible (14,21), but no reports of opposite activation have been published. Therefore, a small effect could theoretically be masked by differential expression, but unlikely the absence of an effect, as we report in our study. The same holds true for POMC. In addition, the fasting condition might have a potential masking effect on neuropeptide expression, however, every feeding state could have a masking effect, depending on the neuropeptide of interest.

Our observations are partly in line with those of other groups, who did not find an effect of a 2 weeks HF diet on NPY and AgRP mRNA expression levels in the hypothalamus of C57Bl/6 mice (25) and on NPY mRNA expression level in the hypothalamus of two strains of rats (Osborne-Mendel and S5B/Pl) (19) either. However, in contrast to our findings, Ziotopoulou *et al*. found a significant increase in POMC mRNA expression levels after 2 weeks of HF feeding in mice (25). They

hypothesized that the observed overexpression of POMC, a precursor of melanocyte-stimulating hormone, a potent inhibitor of food intake, is a response to overfeeding and positive energy balance to counteract the effect of high fat diet to promote increased food intake in an effort to maintain energy homeostasis. This is in line with findings of Bergen et al., who found compensatory hypothalamic changes in mRNA expression levels of NPY and POMC in A/J mice (resistant to diet-induced obesity) fed a high fat diet for 14 weeks (1). In line with our study, these studies imply that changes in hypothalamic mRNA expression levels of NPY, AgRP, POMC and CART are rather secondary or compensatory to high fat feeding, in order to maintain energy homeostasis, than primary, i.e. causing increased food intake during high fat feeding and thereby obesity and insulin resistance.

From this study, we conclude that 2 weeks of high fat feeding in mice does not affect mRNA expression levels of NPY, AgRP, POMC or CART in the whole hypothalamus, despite induction of hepatic, but not peripheral, insulin resistance. Therefore, a major physiological role of these neuroendocrine factors in the induction of hepatic insulin resistance during a high fat diet seems less likely.

This study was supported by the Netherlands organisation for Scientific Research (NWO) (grant 907-00-002 to EPMC). This study is conducted in the framework of the 'Leiden Center for Cardiovascular Research LUMC-TNO). We thank Anita van den Hoek and Edwin Parlevliet for their technical assistance.

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