EFFECT OF DORSOMEDIAL HYPOTHALAMUS NEUROPEPTIDE Y KNOCKDOWN ON HEPATIC INSULIN SENSITIVITY

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Abstract

Objective. In this study we investigated the effect of dorsomedial hypothalamus (DMH) neuropeptide Y (NPY) knock-down on hepatic insulin sensitivity in high-fat (HF) diet-fed rats.

Methods. Forty-eight Sprague-Dawley rats were randomly assigned to receive bilateral DMH injections of adeno-associated virus AAVshNPY or AAVshCTL and then accessed to regular chow. Five weeks after viral injection, half rats in each group were given access to the HF diet. At 16 weeks, rat livers were collected. Insulin receptor substrate-1 (IRS-1) and phosphoinositide 3-kinase (PI3K) mRNA expression was measured by qRT-PCR. Blood glucose levels were measured by the oxidase method, serum insulin, triglyceride, and TC levels were measured by Elisa. Pathological changes in the liver were assessed by hematoxylin-eosin (HE) staining. AKT, p-AKT, and GSK-3 levels were measured by western blotting.

Results. Compared with AAVshCTL-injected rats, AAVshNPY-injected rats showed a significant decrease in blood glucose concentrations; serum insulin, triglyceride, and TC; HOMA-IR; and IRS-1 and PI3K mRNA levels ($P < 0.05$). ISI, GSK-3, and p-AKT levels were significantly increased ($P < 0.05$). HE staining showed that AAVshNPY-injected rats fed the HF diet had mild fatty degeneration.

Conclusion. These results suggest that DMH NPY knock-down improves hepatic insulin sensitivity in HF diet-fed rats by activating the hepatic PI3K/AKT insulin signalling pathway.

Key words: Dorsomedial hypothalamus, Insulin resistance, High fat, Liver.

INTRODUCTION

Obesity (when the standard body mass index exceeds 30 kg/m^2) has become a worldwide epidemic and causes serious public health problems that accompany changes in modern lifestyle. Obesity causes various metabolic diseases such as fatty liver disease, type 2

diabetes, cardiovascular disease, certain types of cancers, retinopathy, and arthritis; moreover, severe obesity can lead to myocardial infarction and stroke (1). The number of deaths from coronary heart disease and stroke is expected to reach 800,000 and 3,000,00, respectively, in China by 2030. The human body maintains energy homeostasis by regulating food intake and energy expenditure to balance energy intake and output. Long-term energy intake that exceeds energy expenditure leads to excessive energy accumulation in the form of fat in the adipose tissue, leading to obesity (2). Theoretically, controlling the diet and increasing energy expenditure can help control weight gain; however, body weight reduction through energy restriction is ineffective for impacting the obesity epidemic (3). Therefore, there is an urgent need to develop effective strategies for limiting food intake and increasing energy expenditure to combat obesity and its associated comorbidities.

Neuropeptide Y (NPY), a 36-amino-acid peptide, is one of the most potent orexigenic agents in mammals (4). NPY is abundantly distributed in the central and peripheral nervous systems. In the central nervous system, NPY is the most abundant neuropeptide and is expressed in the hypothalamus, cerebral cortex, and the brainstem (5-6). Within the hypothalamus, NPY is primarily expressed in the arcuate nucleus (ARC) and dorsomedial hypothalamus (DMH) (7). In contrast to ARC NPY, which acts as a downstream mediator of leptin to maintain energy homeostasis, DMH NPY is not regulated by leptin (7). NPY knock-down in the DMH promotes the inguinal white adipose tissue brown, increases interscapular brown adipose tissue (BAT) activity, and promotes body energy expenditure in addition to feeding (8). Knock-down of DMH NPY also prevents high-fat (HF) diet-induced obesity and ameliorates HF diet-induced hyperinsulinemia and glucose intolerance (8). The liver is a critical organ for regulating whole-body energy homeostasis because of

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its central role in lipid and glucose metabolism and its close association with nutrient uptake in the intestine via the portal vein (9). Non-alcoholic fatty liver disease (NAFLD) is increasing in parallel with obesity epidemic and insulin resistance (IR) (10). DMH NPY maintains energy homeostasis. However, it is unclear whether knock-down of DMH NPY affects hepatic IR and relevant signalling pathways. The present study mainly examined the effect of DMH NPY on hepatic insulin sensitivity in HF diet-fed rats.

MATERIALS AND METHODS

Animals

Eighty male Sprague-Dawley rats (4-5 weeks) weighing 130-150 g were purchased from the Animal Laboratory Center of Zhengzhou University. The rats were housed in a room with temperature-controlled (22-24°C), a 12-h/12-h light/dark cycle and relative humidity of 50-60% and were given access to running water and regular chow optionally. The HF diet (60% calories, 20% carbohydrates, 20% protein; total of the calories, 5.2 kcal/g) and normal diet (15.8% calories, 65.6% carbohydrate, 18.6% protein; total of the calories, 3.37 kcal/g) were purchased from the Animal Laboratory Center of Zhengzhou University. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC).

Groups

The experiment began with 60 animals. The rats were randomly assigned ($n = 30/\text{group}$) to receive bilateral DMH injections of AAVshNPY (which is a vector knocking down NPY) or AAVshCTL (which is the control vector) ($0.5 \mu\text{L}/\text{site}$ [1×10^9 particles/site] at a rate of $0.1 \mu\text{L}/\text{min}$ for 5 min; the injector was kept in the injection site for an additional 5 min before removal). DMH coordinates were as follows: ventral to skull surface, 7.6 mm; caudal to bregma, 3.1 mm; lateral to midline, 0.4 mm. When divided into AAVshNPY and AAVshCTL, five rats died of anaesthetic, seven rats died of large haemorrhages in brain, then each group (AAVshNPY and AAVshCTL) was left with 24 animals; several days after the operation, eight rats died of infection, the four groups comprised at the end respectively 9, 10, 10, and 11 animals. AAVshNPY group (24 animals) and AAVshCTL group (24 animals) were

given access to regular chow (RC) optionally. Five weeks after viral injection, half of the rats from each group were given access to HF diet optionally, the left is RC group which were given access to normal diet, (four groups are AAVshNPY+RC group (10 animals), AAVshNPY+HF group (11 animals), AAVshCTL+RC group (9 animals), AAVshCTL+HF group (10 animals)), and their body weights were measured weekly. At the end of 16 weeks after overnight fasting, all specimen samples were evaluated.

Enzyme-linked immunosorbent assay

At 16 weeks after viral injection, blood was collected from the orbital vein and centrifuged at 3000 r/min for 10 min. Serum insulin level was measured using an enzyme-linked immunosorbent assay kit (Jiancheng, Nanjing). The homeostasis model of assessment for IR index (HOMA-IR) was used to evaluate hepatic IR ($\text{HOMA-IR} = \text{fasting blood glucose [FBG, mM]} \times \text{fasting serum insulin [FINS, mU/L]} / 22.5$). The insulin sensitivity index (ISI) for evaluating systemic insulin sensitivity was calculated as follows: $\text{ISI} = 1/(\text{FBG} \times \text{FINS})$.

Quantitative reverse transcription-polymerase chain reaction

Total RNA of liver cells extracted by using TRIzol Lysis Reagent (TAKARA, Shiga, Japan). Two-step quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed to evaluate gene expression. Briefly, $1 \mu\text{g}$ total RNA was reverse-transcribed into first-strand cDNA by using RevertAid First-Strand cDNA Synthesis Kits (Thermo Scientific, Waltham, MA, USA). The resulting cDNA was quantified with the iQ SYBR Green Supermix Kit (ROCHE, Basel, Switzerland) and iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The reaction mixture for PCR contained $4 \mu\text{L}$ upstream and downstream primers ($1 \mu\text{M}$), $10 \mu\text{L}$ SYBR Green, $10 \mu\text{L}$ RNase-free water, and $2 \mu\text{L}$ cDNA. The following primers were used: β -actin forward, 5'-CATCACTATCGGCAATGAGC-3'; β -actin reverse, 5'-GACAGCACTGTGTTGGCATA-3'; insulin receptor substrate-1 (IRS-1) forward, 5'-AGGCACCATCTCAACAATCC-3'; IRS-1 reverse, 5'-GTTTCCCACCCACCATACTG-3'; phosphoinositide 3-kinase (PI3K) forward, 5'-GTGGGACTGTGACCGAAAGT-3'; PI3K reverse, 5'-GCTTAGGGCTGGTTCTCCTT-3'; NPY forward, 5'-GCTAGGTAACAAGCGAATGGGG-3'; and NPY reverse, 5'-CACATGGAAGGGTCTTCAAGC-3'.

Western blotting

SDS-PAGE on a 4-12% gel (Kangwei, China) separated Proteins and transferred the Proteins to an Immun-Blot PVDF membrane. The PVDF membrane was incubated with a rabbit anti-glycogen synthase kinase-3 antibody (dilution, 1:500; Abcam, Cambridge, UK), rabbit anti-protein kinases B (AKT) antibody (dilution, 1:1000; Abcam), and rabbit anti-p-AKT antibody (dilution, 1:500; Abcam), followed by incubation in the dark with horseradish peroxidase-labeled goat anti-rabbit antibody (1:8000; ProteinTech, Wuhan, China).

HE staining

The slices were dewaxed with xylol (Kangwei, China) in different jars, dehydrated by different concentrations of ethyl alcohol (Kangwei), and stained by hematoxylin (Kangwei) for 15 min and then eosin (Kangwei, China) for 1 min.

Statistical analysis

All data are presented as the mean $\pm$ SEM and were analyzed using SPSS17.0 software for statistical analysis (SPSS, Inc., Chicago, IL, USA). Data were analyzed by two-way analysis of variance. Two groups were compared using the least significant difference t-test, with $P < 0.05$ considered statistically significant.

RESULTS

Weight and intake

After the viral injection, the body weights of AAVshNPY-injected rats fed the regular diet were significantly lower than those of AAVshCTL-injected rats fed the regular diet. Furthermore, the body weights of AAVshNPY-injected rats were significantly lower than those of AAVshCTL-injected rats, even at 12 weeks after viral injection (Table 1). On the fifth week, the four groups of rats daily food intake was significantly increased in the HF groups; in this group, the AAVshNPY group consumed significantly less food than the AAVshCTL group (Fig. 1).

Table 1. The weights of rats in different groups ($x \pm s$, g)

Group	Number	Weight (g)
AAVshCTL+RC	9	530.3 $\pm$ 12.5
AAVshNPY+RC	10	508.4 $\pm$ 10.7*
AAVshCTL+HF	10	592.1 $\pm$ 10.7*
AAVshNPY+HF	11	565.7 $\pm$ 9.6 ^{#&}
P		<0.05

* $P < 0.05$ compared with AAVshCTL+RC rats, [#] $P < 0.05$ compared with AAVshNPY+RC rats, & $P < 0.05$ compared with AAVshCTL+HF rats.

Blood glucose level; serum insulin, triglyceride, and total cholesterol levels; HOMA-IR; and ISI

Compared with regular diet-fed rats, HF diet-fed rats showed a significant increase in blood glucose level; serum insulin, triglyceride (TG), and total cholesterol levels; and HOMA-IR ($P < 0.05$). Compared with AAVshCTL-injected rats, AAVshNPY-injected rats showed a significant increase in blood glucose level; serum insulin, TG, and total cholesterol levels; and HOMA-IR ($P < 0.05$). HF diet-fed rats showed a significant decrease in ISI compared to regular diet-fed rats ($P < 0.05$). Moreover, AAVshNPY-injected rats showed a significant decrease in ISI compared with AAVshCTL-injected rats ($P < 0.05$; Fig. 2, Tables 2 and 3).

NPY expression

We used a recombinant viral vector containing an RNA with NPY-specific short hairpin (AAVshNPY; Changjia, Zhengzhou, China) and humanized Renilla red fluorescent protein for AAV-mediated RNAi. We compounded the target gene, constructed the viral carrier, and then extracted the plasmid. Several days after transfection, cells transfected with the two different plasmids cotransfected into AAV-293 cells were harvested, and the recombinant viral vector was purified and concentrated. Virus titers were determined by qPCR, and 1×10^9 particles/site were used for each virus injection. We confirmed that the viral vectors infected at 1 week (72% left), 2 weeks (53% left), 4 weeks (51% left), and 16 weeks (64% left) significantly knock-down NPY expression in the DMH group compared to rats that received control vector injections (Fig. 3, 1:1000 μ m).

Figure 1. Daily food intake in the four groups of rats.

Values are means $\pm$ SEM, * $P < 0.05$ compared with AAVshCTL+RC rats, [#] $P < 0.05$ compared with AAVshNPY+RC rats, & $P < 0.05$ compared with AAVshCTL+HF rats.

Table 2. The concentrations of GLU, INS, HOMA-IR and ISI in different groups(x±s)

Group	Glucose (mmol/L)	Insulin (mU/L)	HOMA-IR (mmol·mU/L ²)	-Ln (ISI) (L ² /mmol·mU)
AAVshCTL+RC	4.59±0.162	5.58±0.296	1.14±0.081	3.24±0.067
AAVshNPY+RC	4.43±0.254*	4.91±0.248*	0.97±0.067*	3.08±0.068*
AAVshCTL+HF	5.83±0.258*	7.98±0.247*	2.07±0.058*	3.83±0.023*
AAVshNPY+HF	5.30±0.190 ^{#&}	6.91±0.324 ^{#&}	1.63±0.107 ^{#&}	3.60±0.065 ^{#&}
P	<0.05	<0.05	<0.05	<0.05

*P<0.05 compared with AAVshCTL+RC rats, [#]P<0.05 compared with AAVshNPY+RC rats, [&]P<0.05 compared with AAVshCTL+HF rats.

Table 3. The concentrations of TC and TG of rats serum in different groups (x±s)

Group	Number	TC (mmol/L)	TG (mmol/L)
AAVshCTL+RC	9	1.56±0.085	0.93±0.099
AAVshNPY+RC	10	1.41±0.101*	0.82±0.093*
AAVshCTL+HF	10	3.34±0.128*	1.81±0.123*
AAVshNPY+HF	11	2.42±0.136 ^{#&}	1.46±0.097 ^{#&}
P		<0.05	<0.05

*P<0.05 compared with AAVshCTL+RC rats, [#]P<0.05 compared with AAVshNPY+RC rats, [&]P<0.05 compared with AAVshCTL+HF rats.

Figure 2. Effects of DMH NPY Knockdown on glucose, serum insulin, triglyceride, total cholesterol, HOMA-IR, ISI.

A: Glucose concentrations in different groups at 16 weeks; B: Serum insulin concentrations in different groups at 16 weeks; C: HOMA-IR in different groups at 16 weeks; D: The ISI levels in different groups at 16 weeks; E: The concentrations of TC and TG of rats serum in different groups at 16 weeks.

Values are means±SEM, *P < 0.05 compared with AAVshCTL+RC rats, [#]P < 0.05 compared with AAVshNPY+RC rats, [&]P < 0.05 compared with AAVshCTL+HF rats.

Figure 3. A: The position of virus injection in rats brains; B: The expression of NPY gene in different time groups.

Values are means±SEM, *P < 0.05 compared with AAVshCTL rats.

Figure 4. The levels of IRS-1 and PI3K gene mRNA of liver in different groups.

Values are means±SEM, *P < 0.05 compared with AAVshCTL+RC rats, [#]P < 0.05 compared with AAVshNPY+RC rats, [&]P < 0.05 compared with AAVshCTL+HF rats.

Table 4. The levels of IRS-1 and PI3K gene mRNA of liver in different groups (x±s)

Group	IRS-1	PI3K
AAVCTL+RC	1.00±0.135	1.00±0.130
AAVNPY+RC	1.15±0.085	1.23±0.101*
AAVCTL+HF	0.57±0.092*	0.42±0.095*
AAVNPY+HF	0.78±0.095 ^{#&}	0.74±0.105 ^{#&}
P	0.001	<0.05

*P<0.05 compared with AAVshCTL+RC rats, [#]P<0.05 compared with AAVshNPY+RC rats, [&]P<0.05 compared with AAVshCTL+HF rats.

Figure 5. A: The expression of liver-related proteins in different groups; 1 is AAVshCTL+RC group; 2 is AAVshNPY+RC group; 3 is AAVshCTL+HF group; 4 is AAVshNPY+HF group; B: The comparison of gray level of liver tissue GSK3, p-AKT protein expression in different groups. Values are means $\pm$ SEM, * P < 0.05 compared with AAVshCTL+RC rats, # P < 0.05 compared with AAVshNPY+RC rats, & P < 0.05 compared with AAVshCTL+HF rats.

Insulin signalling pathway-related genes

HF diet-fed rats showed lower IRS-1 and PI3K mRNA expression than regular diet-fed rats (P < 0.05). In contrast, AAVshNPY-injected rats showed higher IRS-1 and PI3K mRNA expression than AAVshCTL-injected rats (P < 0.05; Figure 4, Table 4).

Expression of insulin signalling pathway-related proteins

GSK-3 and p-AKT protein expression was lower in the liver of HF-fed rats than in the livers of regular diet-fed rats (P < 0.05), but higher in the liver of AAVshNPY-injected rats than in the liver of AAVshCTL-injected rats (P < 0.05). However, no difference was observed in AKT expression among rats in different groups (Fig. 5).

HE staining of rat liver sections

Analysis of HE-stained liver sections of regular diet-fed rats under a light microscope showed that hepatocytes were arranged radially in regularly arranged rows around the central vein and that the cytoplasm of hepatocytes was stained red and the nucleus was located in the center. In HF groups, the AAVshCTL group rat liver tissue cells showed larger cytoplasmic lipid droplets than those in the AAVshNPY group (Fig. 6; 1:50 μ m).

DISCUSSION

DMH NPY plays a crucial role in maintaining energy homeostasis (8). Overexpression of DMH NPY

Figure 6. The HE stains of liver tissue in different groups. A is AAVshCTL+RC group; B is AAVshNPY+RC group; C is AAVshCTL+HF group; D is AAVshNPY+HF group.

induces hyperphagia and obesity, whereas knock-down of DMH NPY ameliorates hyperphagia and obesity in OLETF rats and prevents obesity development in HF diet-fed rats (11). In the present study, we observed that knock-down of DMH NPY delayed obesity development in HF diet-fed rats. We found that HF diet-fed rats developed IR at the end of the experiment and that knock-down of DMH NPY prevented the development of IR in these rats. These results are consistent with those of a study showing that knock-down of DMH NPY improved insulin sensitivity and glucose tolerance, prevented diet-induced hyperglycemia and hyperinsulinemia (8). Excess energy intake and obesity cause imbalances in human metabolism and increase free fatty acid, and TG in blood circulation, leading to abnormal insulin metabolism; this deposition will be affected by the insulin signalling pathway, causing IR in the liver, islet cells, skeletal muscle, and adipose tissue, as well as islet cell dysfunction (12). The occurrence of non-alcoholic fatty liver disease is closely associated with obesity and IR. HF diet consumption disturbs liver-specific gene expression, lipid and cholesterol levels, inflammatory responses, and oxidative pathways (13). NAFLD is thought to be a hepatic manifestation of more widespread and underlying metabolic dysfunction and is strongly associated with numerous metabolic risk factors, including insulin resistance, dyslipidemia, cardiovascular disease, and, most significantly, obesity (14). NAFLD induces the accumulation of lipids and lipid derivatives, which further aggravates inflammation, fibrosis, and oxidative stress (15-16). Fat accumulation in the liver may occur because of increased dietary fat intake, increased lipid synthesis, and/or reduced lipid oxidation. In addition, concurrent obesity and type 2

diabetes may increase non-fatty tissue via liver uptake of fats (16-17).

Under normal physiological conditions, insulin binds to and activates insulin receptors on the cell surface, subsequently inducing IRS-1 phosphorylation. Phosphorylated IRS-1 binds to and activates PI3K by activating downstream Akt and promotes glucose transporter-4 synthesis, thus increasing cellular glucose uptake. Glucose metabolism and IR development are closely associated with insulin signal transduction disorder. In obesity, increased activation of inflammation-related pathways, free fatty acids, and ERS affect insulin signalling pathways, leading to IR (18). This limits the cellular uptake of glucose. HOMA-IR and ISI were calculated from blood glucose and serum insulin levels, which showed that the HF diet induced IR in obese rats. Moreover, IRS-1 and PI3K mRNA expression and GSK-3 protein expression were significantly decreased in the livers of HF diet-fed rats. This is an important insulin signalling pathway in the liver and is involved in glucose uptake and transport. HF diet affects the insulin signalling pathway in the liver to decrease glucose uptake. Thus, the body appears to be in a hyperglycemic state, thereby affecting serum insulin, IR, dyslipidemia, obesity and further affecting the insulin signalling activation pathway.

NPY overexpression in the DMH induces obesity and overeating, whereas NPY downregulation in the DMH reduces food intake, increases activity, and delays HF diet-induced obesity and glucose intolerance (19). Rats lacking NPY in the DMH showed low body weights. To some extent, knock-down of DMH NPY reduces intake by rats, thus reducing energy intake to achieve an energy balance. This is consistent with the above findings. Moreover, knock-down of DMH NPY promotes the white adipose tissue brown, thus increasing the proportion of brown adipocytes and accelerating energy expenditure to achieve an energy balance (4). The DMH contains both glucoreceptive and glucose-sensitive neurons. The DMH lesions alter the feeding response to extrinsic glucose and insulin, indicating that DMH is involved in regulating glucose homeostasis, that knock-down of DMH NPY can adjust the insulin sensitivity and glucose tolerance. This result is consistent with those of Lin Li, who reported a distinct position for DMH NPY in regulating glucose homeostasis through the hepatic vagal efferents and of insulin in hepatic glucose production (20). However, this study focuses on the effect of DMH NPY on the insulin signalling pathway in the rat liver, which maintains the energy balance, IR, and insulin

susceptibility. Studies have shown that DMH NPY knock-out can improve insulin sensitivity in the liver of rats fed HF diets, significantly improving IR in rats. Moreover, knock-down of DMH NPY decreased fatty degeneration in the liver of HF diet-fed rats. However, further studies are needed to determine the mechanisms underlying the effects of DMH NPY on the hepatic insulin signalling pathway.

Knock-down of DMH NPY affected hepatic insulin sensitivity and glucose homeostasis in rats, enabling the body to reach a steady state. Future studies should examine how the NPY gene may improve obesity or IR state in patients to overcome the global problem of obesity.

Conflict of interest

All authors have no conflict of interest regarding this paper.

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