Neuropeptide Y promotes adipogenic differentiation in primary cultured human adipose-derived stem cells

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Abstract. Neuropeptide Y (NPY) is an important neurotransmitter in the control of energy metabolism. Several studies have shown that obesity is associated with increased levels of NPY in the hypothalamus. We hypothesized that the release of NPY has coordinated and integrated effects on energy metabolism in different tissues, such as adipocyte tissue, resulting in increased energy storage and decreased energy expenditure. Whether NPY has role in the molecular mechanism of human adipocyte tissue remains unclear. We established the model of human adipose derived stem cells (hADSCs) from human adipose tissue and differentiated it into adipocytes in the presence of NPY at different concentrations (10^{-15}–10^{-6} mmol/L). We then assessed hADSCs proliferation and differentiation by quantifying lipid accumulation and examining the expression levels of related adipocyte markers after differentiation. Furthermore, the specific markers of white adipocyte tissue (WAT) in hADSCs were also analyzed. The results showed that low doses of NPY stimulated hADSCs proliferation (\( p < 0.05 \)), while high doses of NPY inhibited hADSCs proliferation (\( p < 0.05 \)). NPY significantly promoted lipid accumulation and increased the size of lipid droplets during human adipogenic differentiation; the levels of adipocyte markers PPAR-\( \gamma \) and C/EBP\( \alpha \) were also increased. At the same time, NPY also increased the levels of WAT markers Cidec and RIP140 after adipocyte differentiation. The results suggested high dose NPY inhibits the proliferation of hADSCs while promotes adipocyte differentiation and increases the expression of WAT markers. This may be the reason why increased levels of NPY can lead to a rise in body weight.

Key words: Neuropeptide Y, Human adipose-derived stem cells, Adipocyte differentiation, White adipose tissue

NEUROPEPTIDE Y (NPY) is a 36-amino acid neuropeptide, which is widely expressed in the central and peripheral nervous system. In the brain, NPY is found in many brain areas [1], including the hypothalamus, dentate gyrus, lateral thalamus and striatum, with the highest concentrations in the arcuate nucleus of the hypothalamus (Arc). NPY is implicated in the regulation of many physiological processes, including food intake and body energy balance. NPY also regulates cardiovascular, gastrointestinal, reproductive, endocrine and behavioural function [2-4]. Recently, studies have revealed that administration of NPY has profound metabolic effects throughout the body. One characteristic of obesity is the excessive intake, synthesis and storage of lipids as fat. This overproduction of fat is associated with increased lipogenesis in different organs, such as liver and white adipose tissue (WAT). Several studies found that obesity is associated with elevated levels of NPY in the hypothalamus [5-9]. Adipose tissue is important for regulation of energy metabolism. A disturbance of metabolic processes, such as thermogenesis, lipogenesis and fatty acid oxidation, may contribute to the pathology of obesity. In order to increase our understanding of how NPY could be involved in the adipocyte tissue, we investigated the effects of NPY on hADSCs proliferation and differentiation.

Previous work from our laboratory has demonstrated that 3T3-L1 preadipocyte proliferation and differentiation are promoted by NPY. Furthermore, 10^{-8} M NPY upregulates protein levels of peroxisome proliferator-activated receptor-\( \gamma \) (PPAR-\( \gamma \)) and CCAAT/enhancer-binding protein-\( \alpha \) (C/EBP-\( \alpha \)), which are the markers of WAT [10, 11].
In this study, we characterized the effects of NPY on hADSCs proliferation, differentiation and levels of WAT markers. We established the cell model of primary cultured ADSCs from human adipose tissue and then differentiated into adipocytes, and examined the effects of different doses of NPY on the adipogenesis through measuring the size of lipid droplets and protein levels of PPAR-γ and C/EBP-α as well as the proliferation of hADSCs.

Materials and Methods

Ethics statement

This research had been approved by the ethics committee of the Third Xiangya Hospital of Central South University and all subjects signed the informed consent form before taking part in the study.

Isolation and culture of human ADSCs

We selected three cases of pre-menopause women (Table 1), who were aged 30, 30 and 35, according to certain inclusion criteria and exclusion criteria and who were undergoing an elective abdominal gynecological surgery. The enrollment criteria included: BMI of 18.5–28 kg/m², smoking less than 20 cigarettes per day, drinking no more than 75 g of alcohol per day, no sepsis, no stress reaction, no cardiovascular system diseases, no endocrine and metabolic system disease, no autoimmune diseases, and no malignant tumors. About 30 g of fat tissue was obtained. The technology of isolation and culture of human adipose tissue-derived stem cells was mature [12-14]. Briefly, tissue was washed in PBS buffer and digested with 2 mg/mL Collagenase Type I for 1 hour at 37°C with constant shaking. The primary cells were passaged twice before being used for differentiation assays and the P3 cells were used for the following experiments (Fig. 1).

The proliferation of hADSCs in the presence of NPY

hADSCs were cultured in 96-well plates with DMEM medium, and the medium was changed every 3 days. After reaching 40–50% confluence, cells were washed and cultured in the medium containing 0.5% FBS for 24 h. Then cells were cultured in serum-free medium in the presence of NPY at 10⁻⁶–10⁻¹⁵ M for 24 h. Negative controls were cultured in serum-free medium in the absence of NPY. Positive controls were cultured in medium with 10% FBS in the absence of NPY. Each treatment was performed in parallel in six wells. After 24 h proliferation, 20 μL MTT (5 mg/mL) was added to each well and incubated for 4 h. Cells were then washed and dissolved in 150 μL DMSO by shaking for 15 min. Then the absorbance was measured with a plate reader for three times.

Induction of hADSCs differentiation in the presence of NPY

hADSCs were cultured in 6-well plates for 2 days until they reached confluence. Cells were then divided into 6 groups and cultured for additional 12 days (Table 2). Because adipogenic differentiation achieved the best condition on day 12, we could better observe and compare the effect between different differentiation cocktail through long term treatment. Group A was cultured in DMEM for 12 days. Group B was cultured in DMEM supplemented with hADSCs differentiation cocktail (MIX) (3-isobutyl-1-methylxanthine (IBMX, 0.5 mM), dexamethasone (0.25 μM), and insulin (1 g/mL)) for 3 days. Differentiation cocktail was then replaced with DMEM for the next 9 days. Group C was cultured in differentiation cocktail (MIX) for 3 days. Differentiation cocktail was then replaced with NPY for the next 9 days. Group C was divided into three subgroups C1, C2, and C3, which were treated with 10⁻¹¹, 10⁻⁹, and 10⁻⁷ M NPY, respectively. Group D was cultured with differentiation cocktail (MIX) until day 12. Medium was changed every 3 days. On the 12th day, differentiation was assessed by western blot analysis of the key adipogenic transcription factors PPARγ and C/EBP-α (described below). The white fat markers Cidec and RIP140 were also measured by western blot. On the 4th, 9th and 12th days, lipid droplets were stained with Oil Red O (described below).

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Oil Red O staining for lipid droplets

Cells were washed twice with PBS and fixed with paraformaldehyde (PFA, 4% in PBS) for 30 min. Cells were then washed twice with PBS and once with distilled water, and stained with Oil Red O dye (6:4, 0.6% Oil Red O dye in water; Sigma (Sintra, Portugal)) for 1 h at room temperature, and then washed three times with water. Finally, Oil Red O was dissolved in 200 µL iso-
propanol, and absorbance was measured at 500 nm using the Multiskan EX (Thermo Scientific; Waltham, MA, USA).

**Reverse transcription-PCR**

In order to detect NPY receptors expressed in hADSCs, we isolated RNA using Tri Reagent (Sigma) and synthesized cDNA using the iScript cDNA synthesis kit (Bio-Rad). In each sample, 18s rRNA served as a control. We performed Real time reverse transcription-PCR using the iCycler iQ Detection System (Bio-Rad). We amplified cDNA for 40 cycles using the TaqMan PCR Reagent Kit and pre-designed primers and fluorescein-labeled probes from Applied Biosystems for human NPY1R, NPY2R and NPY5R, as described [15], according to the manufacturer’s procedure.

**Western blotting**

Cells were lysed in RIPA buffer (50 mM Tris–HCl, pH = 8, 150 mM NaCl, 1% Triton, 0.5% SDC, 0.1% SDS) containing 100 M phenylmethylsulfonfyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 1 g/mL quimostatin, 1 g/mL leupeptin, 1 g/mL antipain, 5 g/mL peptstatin A (CLAP) and 1 mM orthovanadate, pH = 7.4. Protein concentration was determined by the bicinchoninic acid (BCA) assay (BCATM Protein Assay kit, Genstar). 6 × loading buffer (0.5 M Tris, 30% glycerol, 10% sodium-dodecyl sulfate (SDS), 0.6 M dithiothreitol, 0.012% bromophenolblue) was added and samples were heated (5 min, at 95°C). Equal amounts of protein were loaded on SDS-polyacrylamide gels and proteins were separated by electrophoresis. Proteins were then transferred electrophoretically to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 3% BSA in Tris-buffered saline (137 mM NaCl, 20 mM Tris–HCl, pH 7.6) containing 0.1% Tween-20 (TBS-T) for 1 h at room temperature. Membranes were incubated with primary antibodies (rabbit anti-PPAR (1:800); rabbit anti-C/EBPα (1:600); rabbit anti-Cidec (1:500); rabbit anti-RIP140 (1:200) in TBS-T containing 1% BSA) overnight at 4°C. The anti-GAPDH (1:10,000) was used as a loading control. Membranes were then incubated with alkaline phosphatase-linked secondary antibodies in TBS-T/1% BSA for 1 h at room temperature. After washing with TBS-T/0.5% BSA, membranes were incubated with ECF reagent for 5 min and the immunoreactive bands were visualized using the VersaDoc 3000 imaging system (BioRad, Hercules, CA, USA). Image-Pro Plus processing software was used to quantify band intensity, and values were normalized to loading control.

**Statistical analysis**

Data are expressed as mean ± SEM. Statistical significance was determined by one-way analysis variance (ANOVA) followed by Bonferroni’s or Dunnet’s post-hoc test as indicated. Differences were considered significant when \( p < 0.05 \).

**Results**

**Primary culture of hADSC**

We successfully established a cell model of primary cultured ASCs from human adipose tissue and then differentiated into adipocytes. Then we examined the effects of different doses of NPY on the proliferation and adipogenesis of hADSCs (described below).

**NPY receptors expressed in human ADSCs**

Before evaluating the direct effects of NPY on human ADSCs proliferation and adipogenic differentiation, we determined NPY receptor expression in human ADSCs by RT-PCR. The results showed that cells expressed Y2 and Y5 receptors (Fig. 2).

**Effects of NPY on hADSCs proliferation**

To determine the effect of NPY on human ADSC proliferation, increasing concentrations of NPY (10^{-15} - 10^{-6} M) were added to the cells. MTT results showed that low concentrations of NPY (10^{-15} - 10^{-11} M) promoted ADSC proliferation (\( p < 0.05 \)), while high concentrations of

![Fig. 2. Expression of NPY receptors detected by RT-PCR. 18s rRNA served as a control.](image-url)
NPY (10^{-10}–10^{-6} M) decreased the proliferation compared to the negative control (p < 0.05) (Fig. 3).

**NPY induces adipocyte differentiation**

To study the effects of NPY on adipogenic differentiation, cells were induced to differentiate using differentiation cocktail for 3 days, and then treated with NPY (10^{-7}, 10^{-9} or 10^{-11} M) in normal media without differentiation cocktail for 9 days. The differentiation was evaluated by Oil Red O staining for cytoplasmic lipid droplet accumulation (Fig. 3) and by the nuclear expression of the adipocyte differentiation markers PPAR-γ and C/EBP-α. Results showed that cytoplasmic lipid droplet accumulation was increased in NPY-treated cells (groups C1, C2 and C3) compared to control cells without NPY treatment (group B). However, 12 days of treatment with differentiation cocktail (group D) promoted adipocyte differentiation more strongly than NPY did. The PPAR-γ and C/EBP-α protein levels were also increased by NPY (groups C1, C2 and C3) compared to control (group B), as measured by western blotting (Figs. 4, 5). Of the NPY-treated cells, 10^{-7} M NPY treatment induced the largest increase of these markers. However, cells treated with differentiation cocktail for 12 d (group D) showed significantly higher levels of these markers than the NPY-treated groups (p < 0.01) (Fig. 5).

**NPY promotes expression of WAT marker proteins**

To study the effects of NPY on the differentiation of adipocytes into WAT, cells were induced to differentiate for 3 days, and then incubated with NPY (10^{-7}, 10^{-9} or 10^{-11} M) for 9 days. RIP140 and Cidec protein levels were then quantified by western blot analysis. NPY-treated cells (groups C1, C2 and C3) and cells treated with induction cocktail for 12 d (group D) showed an increase of RIP140 and Cidec protein levels compared to control (group B) (p < 0.01). However, protein levels were significantly lower in groups C1, C2, and C3 than in group D (Fig. 5). Of the NPY-treated groups, 10^{-7} M NPY induced the highest protein levels.

**Discussion**

We found that Low doses of NPY stimulated hADSCs proliferation (p < 0.05), while high doses of NPY inhibited hADSCs proliferation (p < 0.05). NPY could significantly increase the size of lipid droplets and increase the levels of PPAR-γ and C/EBPα in a dose-dependent manner during human adipogenic differentiation (p < 0.05) and treatment with 10^{-7} M NPY strongly inhibited hADSCs differentiation (p < 0.01). Actually, Adipose tissue is broadly divided into WAT and BAT. WAT and BAT have different gene expression profiles and lipid
Fig. 4. Oil red O staining at days 4, 9 and 12 of hADSC adipogenic differentiation.

(A) No induction of differentiation. (B) 3 days induction followed by 9 days normal medium. (C) 3 days induction followed by $10^{-11}$ (C1), $10^{-9}$ (C2), or $10^{-7}$ (C3) M NPY for 9 days. (D) 12 days differentiation induction. 200× magnification.

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droplet-associated proteins. In our experiment, we found the expression of RIP140 and Cidec, which are specific WAT markers, increased during hADSCs differentiation. Taken together, these results suggest that a high dose NPY inhibits hADSCs proliferation while promotes adipocyte differentiation and increases the expression of WAT markers.

Obesity and its related complications, including type 2 diabetic mellitus, are rapidly increasing problems in need of effective therapies. Multiple circulating hormones, including NPY, are known to regulate many physiological processes, including food intake and body energy balance. NPY is one of the most important adipocyte regulatory factors [13-16]. Several articles had provided evidence that NPY is produced in subcutaneous and visceral adipose tissue [17, 18]. Subcutaneous and visceral adipose tissue is the peripheral site of NPY biosynthesis. The current study gives insights for the first time to demonstrate that physiological relevant concentrations of NPY have a biphasic effect on hADSCs proliferation in vitro. Our study also shows that NPY can promote hADSC differentiation into mature adipocytes, and Oil red O staining shows that $10^{-11}$ M NPY increases the size of cytoplasmic lipid droplets. NPY can also promote the

**Fig. 5.** Western blot analysis of the differentiation marker PPAR-γ, C/EBPα and WAT marker RIP140, Cidec in differentiating hADSCs on day 12.

**p < 0.01 compared to group B. *n = 3.** A was cultured in DMEM for 12 days. B was cultured in DMEM supplemented with differentiation cocktail (MIX) (IBMX, dexamethasone, and insulin) for 3 days. MIX was then replaced with DMEM for the next 9 days. C was cultured in MIX for 3 days. MIX was then replaced with NPY for the next 9 days. C was divided into three subgroups C1, C2, and C3, which were treated with $10^{-11}$, $10^{-9}$, and $10^{-7}$ M NPY, respectively. D was cultured with MIX (IBMX, dexamethasone, and insulin) until day 12.

(a) Representative blots showing PPAR-γ, C/EBPα, RIP140, Cidec and GAPDH (as a loading control) bands.

(b) Quantification of PPAR-γ, C/EBPα, RIP140 and Cidec normalized to GAPDH. Data are presented as mean±SEM. **p < 0.01 compared to group B. *n = 3.
expression of WAT marker proteins in a dose-dependent manner. And these may be the potential mechanisms which lead to weight loses.

Tang [19] found that high dose NPY inhibits the proliferation of 3T3-L1 cells and promotes adipocyte differentiation. Zhang et al. [20] showed that NPY promotes adipogenesis in chickens by increasing production of new preadipocytes, lipid synthesis, and lipid storage. However, the effects of NPY on hADSCs differentiation have not been reported.

Our previous study [21] found that NPY promotes differentiation of 3T3-L1 cells, possibly via upregulation of PPAR-γ and C/EBP-α. Here, we used three concentrations of NPY (10\(^{-7}\), 10\(^{-9}\) and 10\(^{-11}\) M) to induce hADSCs differentiation. The physiological concentration of NPY in human body is 10-10-10-11 M [22, 23]. Before formal experiments, the lower doses of NPY at 10-13 M and 10-15 M which regulated differentiation had been checked and we found that the lower doses had no effects on differentiation. Finally we used the physiological and a higher concentration (10-11, 10-9 and 10-7 M) to intervene the differentiation in our formal experiments. Liu M et al. [21] have purified exogenous NPY through genetic engineering technology, PCR, the construction of a recombinant plasmid and transfection technologies. 10\(^{-7}\) M NPY comes from exogenous route. We found that physiological and a higher concentration could also promote the differentiation and a higher concentration (10-9 and 10-7 M) had a more potent effect on differentiation. Oil red O staining showed that NPY promotes lipid droplet formation, lipid accumulation and adipocyte differentiation. The action mechanism of NPY on hADSCs differentiation may be mainly through the PPAR-γ pathway. PPAR-γ and C/EBP-α are the two most important transcription factors for promoting adipocyte differentiation. C/EBP-α, which is active early in differentiation, induces and maintains PPAR-γ expression [24], which then up-regulates adipocyte gene expression [25]. Therefore, PPAR-γ and C/EBP-α are considered markers of adipocyte differentiation. Our results show that NPY (10\(^{-7}\), 10\(^{-9}\) and 10\(^{-11}\) M) upregulates PPAR-γ and C/EBP-α protein levels in a dose-dependent manner.

Rosmaninho-Salgado and colleagues [26] showed that NPY activates Y2 and Y5 receptors, thereby inducing PPAR-γ expression and lipid accumulation in 3T3-L1 cells. Rosmaninho-Salgado et al. [26] found that NPY could stimulate 3T3-L1 proliferation by Y2 and Y5 receptors, and the antagonists of Y2 and Y5 receptors could inhibit 3T3-L1 proliferation. Our current study gives insights for the first time to show that the Y2 and Y5 receptors are expressed in human ADSCs, and NPY may play its role in hADSCs proliferation and adipogenic differentiation through the two receptors.

Adipose tissue is broadly divided into WAT and BAT [27]. Human BAT is minimal in adults, and its main function is to generate heat to maintain body temperature [28-31]. In contrast, WAT is more abundant and is therefore the main source for engineering autologous tissue for transplantation [32]. WAT and BAT have different gene expression profiles and lipid droplet-associated proteins [33]. RIP140 and Cidec are specific WAT markers [34]. RIP140 is a nuclear receptor corepressor that regulates metabolic pathways and gene expression associated with cytoplasmic lipid droplets and glucose metabolism [35]. Cidec is a lipid droplet-associated protein mainly expressed in WAT [36]. Its overexpression causes aggregation of lipid droplets, and decreased expression decreases lipid droplet size while increasing mitochondria quantity [37]. RIP140 and Cidec are almost undetectable in BAT [38, 39]. Our results suggest that NPY induces hADSC differentiation into white adipocytes because it increases RIP140 and Cidec expression in a dose-dependent manner.

Our study has some limitations due to the limited time. We haven’t studied the underlying mechanisms for NPY-induced changes of PPAR-γ, C/EBP-α, Cidec and RIP140. The mechanism of dose-related reciprocal effect of NPY on hADSCs proliferation is unclear, and it is our next research direction. But notably, this is the first report to demonstrate the effects of NPY on human ADSCs proliferation and adipogenic differentiation. Our study demonstrated that NPY had a biphasic effect on hADSCs proliferation; NPY promoted human ADSCs differentiation by adjusting the expression of the adipocyte-specific markers PPAR-γ and C/EBP-α. And NPY could increase the expression of WAT marker proteins during adipogenic differentiation, which may decrease insulin sensitivity and consequently have a negative effect on weight control.

Acknowledgments

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Disclosure

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