

## Effect of blockade of neuropeptide Y receptor on aortic intima-media thickness and adipose tissue characteristics in normal and obese mice

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### ARTICLE INFO

**Article type:**  
Original article

**Article history:**  
Received: Sep 9, 2014  
Accepted: Mar 3, 2015

**Keywords:**  
Adipocyte  
Atherosclerosis  
Neuropeptide Y  
Obesity

### ABSTRACT

**Objective(s):** Atherosclerosis is an important risk factor for coronary heart disease. Neuropeptide Y (NPY) and its receptors, located in peripheral tissue such as white adipose tissue, have been linked to obesity and fat storage. The role of NPY in atherosclerosis has not yet been fully studied, so this study was conducted to further investigate the effect of BIIE 0246, an NPY receptor antagonist, on aortic intima-media thickness and size and number of adipocyte cells in normal and obese mice.

**Materials and Methods:** Tests were performed on 24 male C57BL/6 mice. The animals were divided into four groups as follows: control (normal), obese (high-fat diet), normal+NPY receptor antagonist (1  $\mu$ M, 100  $\mu$ l/Kg BIIE0246 intraperitoneally) and obese+NPY receptor antagonist (n=6 each). After 14 days, the animals were sacrificed and epididymal adipose tissue and thoracic aorta were removed. Evaluations were made for adipocyte cell number and size and for aortic intima-media thickness.

**Results:** The group on a high-fat diet showed a significantly decreased number of adipocyte cells and increased cell size ( $P<0.05$ ). BIIE0246 application changed the cell number of adipocyte in normal mice ( $P=0.05$ ); however, it did not change adipocyte cell size and aortic intima-media thickness in obese and normal mice ( $P>0.05$ ).

**Conclusion:** NPY receptor antagonist had no effect on adipocyte cell size and aortic intima-media thickness; however, it decreased cell number in the normal group indicating likely involvement in the progression of obesity.

### ► Please cite this paper as:

Alasvand M, Rashidi B, Khazaei M, Haghjooy Javanmard Sh. Effect of blockade of neuropeptide Y receptor on aortic intima-media thickness and adipose tissue characteristics in normal and obese mice. Iran J Basic Med Sci 2015; 18:443-448.

### Introduction

Atherosclerosis is a primary risk factor for coronary heart disease that affects peripheral arteries and cerebral circulation. Atherosclerosis begins with the transmigration of oxidized LDLs to the intima of the subendothelial space, and thereby causes injury to endothelial cells (1). Intima-media (IM) thickness is a noninvasive alternative marker and intermediate phenotype of atherosclerotic disease that has been used extensively since 1986 following the initial description by pignoli et al (2). IM thickness of the carotid artery is an established sonographic marker for early atherosclerosis, and thickening of the IM complex reflects generalized atherosclerosis. IM thickness is also a noninvasive method to detect and follow atherosclerosis. Determination of IM thickness is noninvasive, reproducible and has no side effects, so it is considered a superior method for assessment of coronary anatomy (3).

There are numerous risk factors for atherosclerosis including hypertension, hyperlipidemia, smoking and obesity. Obesity is a global health problem that affects much of the population. It has been reported that obesity is the fifth leading risk factor for death globally. Obesity is associated with metabolic dysfunction and multiple tissue system participation from conditions such as heart diseases, diabetes, dyslipidemia and many tumors (4).

Both, obesity and carotid IM thickness are interrelated; in addition, they are very important risk factors for ischemic stroke (5). Obesity is proliferation of white adipose tissue (WAT) mass that happens via a surge in cell size (hypertrophy) and/or an increase in cell number (hyperplasia). Hypothalamic neuropeptide Y (NPY) has been related to fat cell hypertrophy by exciting lipoprotein lipase activity in WAT (6, 7). NPY, a 36-amino-acid neuropeptide is known as the most

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potent physiological appetite transducer. NPY, peptide YY (PYY) and pancreatic polypeptide are part of the NPY family of peptides and affect food intake by interacting with G-protein-coupled Y receptors (8). Centrally infused NPY induce obesity in the long term, and research has determined that increasing concentrations of hypothalamic NPY seem to be a major factor contributing to the onset of obesity in several obese animal models (9).

NPY is extensively expressed in both the brain and the peripheral nervous system. Within the brain, NPY is highly active in the hypothalamus, particularly in the arcuate nucleus (10). Upon stimulation, NPY activates its Y receptors to motivate circuits that increase food intake and fat storage (11).

NPY and its receptors are located in peripheral tissue such as WAT, liver, and pancreas, but the role of NPY and its receptors in the regulation of energy homeostasis has not been fully identified. In the present study, the effect of BIIE 0246, an NPY receptor antagonist, was investigated on adipocyte size and cell number and IM thickness in normal and diet-induced obese mice.

## Materials and Methods

### Animals

A total of 24 male mice C57BL/6, weighing 20 to 30 g, and 5 weeks old were purchased from the Pasteur Institute of Iran. Animals were housed in cages, four in each cage in the animal facility under the following conditions: temperature  $25^{\circ}\text{C} \pm 2$  and lighting cycle of 12 hr (6:00 AM to 6:00 PM) with access to food and water *ad libitum*. After an adaptation period of 1 week, animals were assigned to four groups: obese, normal, obese+NPY Y2 receptor antagonist and normal+NPY receptor antagonist ( $n=6$ ). Body weight of mice was measured once a week. The Ethical Committee of the Isfahan University of Medical Sciences (Isfahan, Iran) approved the protocol for the study.

### Animal diet and treatment

For induction of diet-induced obesity, the obese groups were fed with a commercial high fat diet (HFD; BioServ Co., Cat #F3282, USA, protein 20.5%, fat 36%, carbohydrate 35.7%) for 16 weeks (12). The normal groups were fed with standard mouse chow (purchased from the Pasteur Institute). All animals had free access to food and water during the study. Body weight of animals was monitored weekly. After 16 weeks, half of the obese and normal animals received NPY antagonist. NPY antagonist (BIIE0246) was obtained from Tocris Co. (Bristol, UK), and to block Y2 receptors in melanoma tumor, animals were treated with BIIE0246 at concentration  $10^{-6}$  M and received 100  $\mu\text{l/kg}$  for 14 days intraperitoneal injection (18). The control group received normal saline of the same

volume. After 14 days, animals were sacrificed and epididymal WAT and thoracic aorta were removed.

### Histological examination

Epididymal WAT and thoracic aortae were removed and fixed in 10% formalin. Then, they were dehydrated and embedded in paraffin. After that they were removed from paraffin and cleaned with xylene and hydrated with decreasing concentrations of ethanol. Tissue blocks were sectioned into 5  $\mu\text{m}$  thickness and stained with hematoxylin and eosin (H&E). Adipocyte cell number was counted in 5 different fields through the camera of a light microscope equipped with computerized image analysis software (Advanced Motic Image 3.2). The size of adipocytes was determined by analyzing the cross-sectional area of white adipose tissue with the AxioVision 4.6 (Zeiss) software. Records were taken for diameter of adipocyte cells (10 cells from each specimen). Aortic IMT was measured from the endothelial surface to the adventitia in 13 different fields of samples from each animal (13). Images of five fields per section from each animal were captured with 40X magnification, and the adipocyte cell surface areas (H/E) were measured from at least 100 cells. A total of 1300 microscopic slides were evaluated.

### Statistical analysis

Data were expressed as means  $\pm$  SE and evaluated using analysis of variance (ANOVA) with a *post hoc* test, LSD. Significant difference was determined at the probability level of 0.05. All statistical analyses of data were performed using SPSS (version 16).

## Results

### Body weight and fat reposition

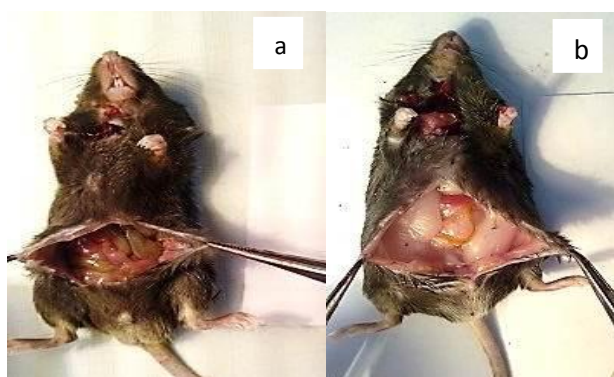
Figure 1 illustrates deposition of fat in the peritoneal cavity of the examined animals. Results showed that the body weight of mice fed with the high-fat diet was significantly higher than that of mice fed with normal chow ( $34 \pm 1.11$  g vs  $25.66 \pm 1.41$  g, respectively;  $P < 0.05$ ) (Figure 2).

### Effect of neuropeptide Y Y2 receptor antagonist on adipocyte cell number

Results showed significant difference in adipocyte cell number of the obese group and the normal groups ( $20 \pm 5.77$  vs.  $106 \pm 1$  number/field, respectively;  $P < 0.05$ ). Administration of NPY Y receptor antagonist reduced adipocyte cell number in the obese group ( $20 \pm 5.77$  vs.  $16.75 \pm 1.65$  number/field); however, results were not statistically significant ( $P > 0.05$ ). But cell number was reduced in normal groups ( $106 \pm 1$  vs.  $69.0 \pm 8.0$  number/field;  $P < 0.05$ ) (Figures 3A and B).

### Effect of neuropeptide Y Y2 receptor antagonist on adipocyte cell size

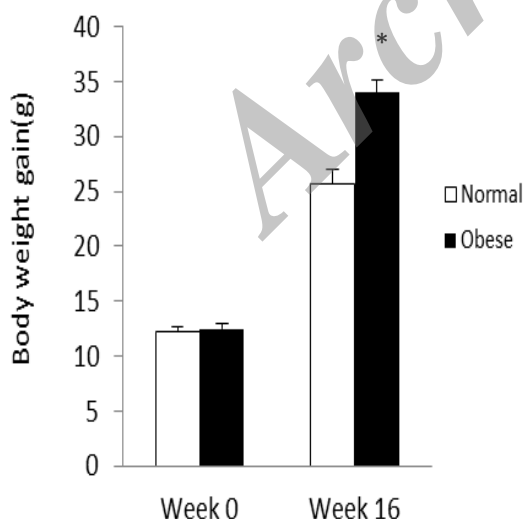
Adipocyte cell size in white adipose tissue in obese animals was significantly higher than in the normal



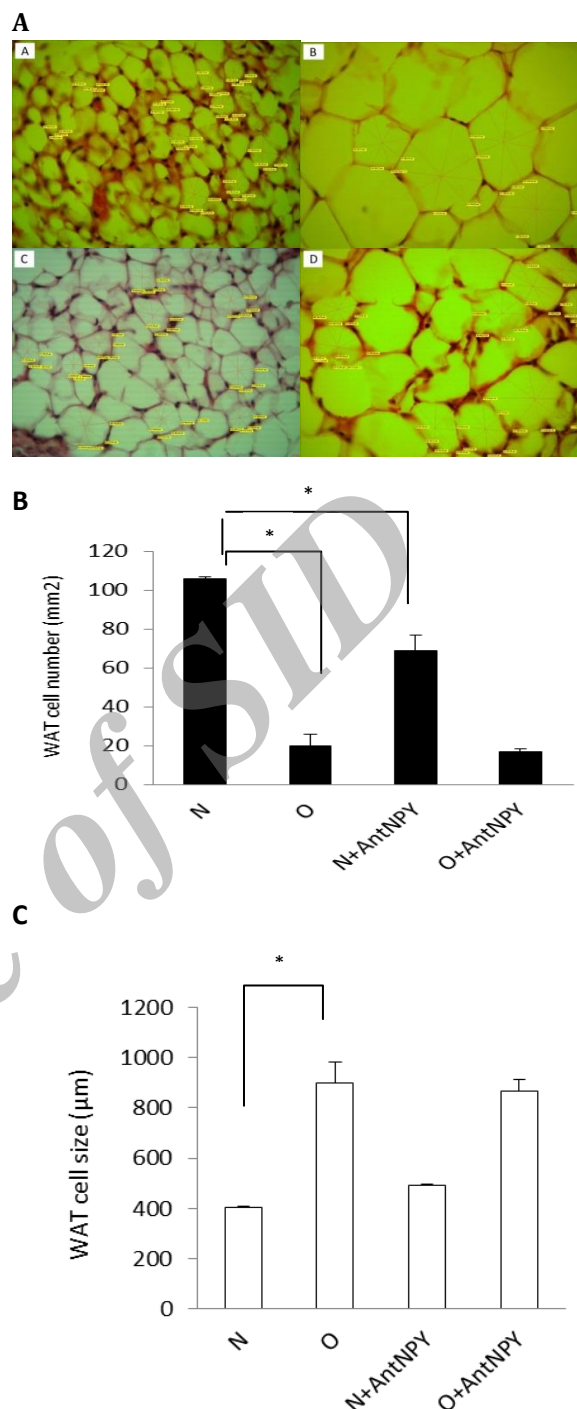
**Figure 1.** Images of normal (a) and obese (b) male mice showing increase in body size and repositioning of fat in the peritoneal cavity in obese animals

group ( $900.5 \pm 81.54$  vs.  $404.45 \pm 4.8 \mu\text{m}$ , respectively;  $P < 0.05$ ). Neuropeptide Y antagonist did not alter adipocyte cell size in obese groups ( $900.5 \pm 81.54$  vs.  $865.21 \pm 46.45 \mu\text{m}$ ;  $P > 0.05$ ) and normal groups ( $404.45 \pm 4.8$  vs.  $491.7 \pm 5.1 \mu\text{m}$ ;  $P > 0.05$ ) (Figure 3A & C).

Effect of neuropeptide Y2 receptor antagonist on aortic IM thickness Figure 4 (a-d) shows aortic rings from normal and obese mice stained by H&E. Aortic IM thickness was significantly greater in animals in the obese group compared to those in the normal group ( $603.55 \pm 19.44 \mu\text{m}$  vs.  $380.8 \pm 52.3 \mu\text{m}$ , respectively;  $P < 0.05$ ). Administration of neuropeptide Y antagonist did not alter aortic intima-media thickness in the obese group ( $528.6 \pm 75.36$  vs.  $450.56 \pm 34.21 \mu\text{m}$ ;  $P > 0.05$ ) and normal groups ( $380.8 \pm 52.3$  vs.  $459.43 \pm 12.74 \mu\text{m}$ ;  $P > 0.05$ ) (Figures 4A & B).

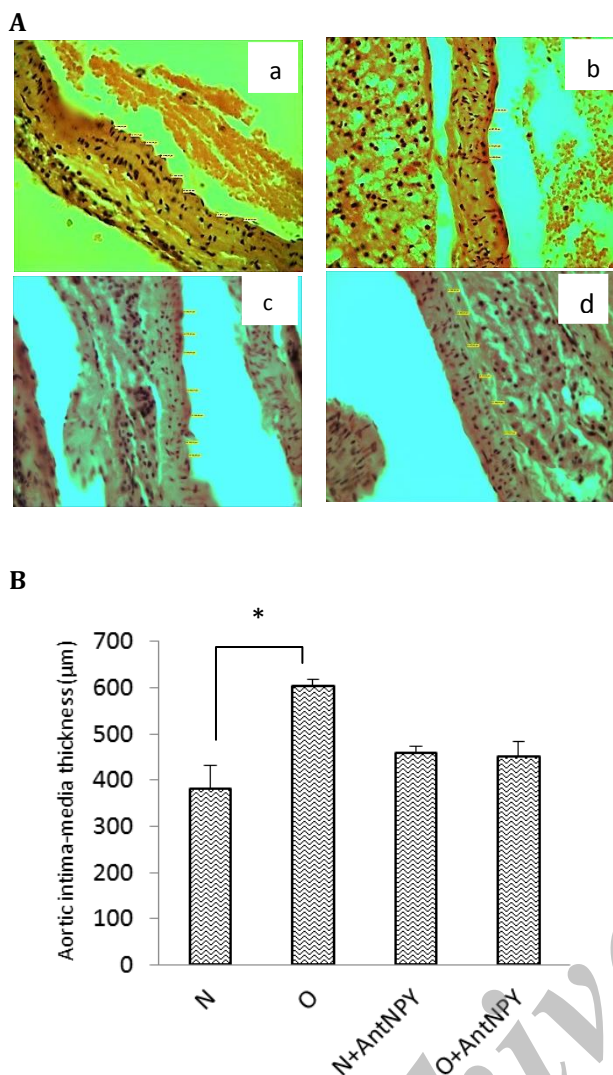


**Figure 2.** Body weight gain in normal and obese group



**Figure 3.** Effects of high-fat diet and NPY receptor antagonist administration on WAT. A: The histological sections were stained with hematoxylin & eosin. a: normal; b: obese; c: normal+ NPY receptor antagonist; d: obese+ NPY receptor antagonist. High-fat diet loading significantly decreased WAT cell number (B) and increased the size of epididymal adipocyte cells (C). NPY receptor antagonist administration changed the cell number of epididymal adipocyte in normal group but did not change adipocyte cell size in obese group (B&C). Data is shown as mean  $\pm$  SEM (n=6). \*  $P < 0.05$ . N: normal, O: obese





**Figure 4.** Effect of high-fat diet loading and NPY receptor antagonist administration on aortic IM thickness. A. The histological sections of aorta were stained with hematoxylin& eosin (a-d); A: normal; B: obese; C: normal+NPY receptor antagonist; D: obese+ NPY receptor antagonist. HFD loading increased aortic intima-media thickness in obese compared to normal animals. NPY receptor antagonist administration did not alter aortic IM thickness in obese and normal group (B). Data is shown as mean±SEM (n=6)

## Discussion

The aim of this study was to investigate the effect of NPY receptor antagonist on WAT characteristics including the number and size of adipocyte cells and aortic intima-media thickness in normal and diet-induced obese mice. Test results showed that BIIE0246, an NPY receptor antagonist, had no significant effect on aortic intima-media thickness and cell size in normal and obese animals. However, it did reduce adipocyte cell number in normal mice.

Research has shown that obesity is a condition that indirectly leads to the process of atherosclerosis, and its relation with other markers of atherosclerosis is to be evaluated (14). Atherosclerotic vascular disease may also be an important clinical result of adipose tissue

dysfunction. Dysfunctional adipocytes contribute to the development of vascular risk factors and vascular disease (15).

Results of the present study showed that administration of BIIE0246 did not alter body weight and IM thickness of aorta in normal and obese groups. NPY is a polypeptide containing 36 amino acids that has proven to be one of the most important regulators of energy homeostasis, thus it seems to be a therapeutic target for the management of disorders such as obesity. The known Y receptors – Y1, Y2, Y4, Y5 and Y6 – have distinctive tissue expression shape (16). NPY neurons exhibited increased adipose mass, and greater muscle protein expression of phosphorylated acetyl-CoA carboxylase, a key enzyme in fatty acid synthesis, demonstrating the obesogenic effect of selective blockade of Y2 receptor signaling in NPY neurons (17).

In this study, BIIE0246 was used as a selective NPY2 receptor antagonist. It was also determined that BIIE0246 reduced adipocyte cell number only in normal mice without affecting adipocyte cell size in obese and normal mice. Obesity is an enlargement of adipose tissue to store excess energy intake. Hyperplasia (cell number increase) and hypertrophy (cell size increase) are two possible growth mechanisms (18). Hypertrophy happens prior to hyperplasia to meet the need for additional fat storage capacity in the development of obesity (19). The importance of Y2 receptor agonists in the reduction of food intake and obesity is controversial in that some studies reported that these peptides may not produce a continued reduction of feeding in rodents (20) or primates (21), while other studies have supported the role of Y2 receptor activation in decreased body weight and confirmed that it has anti-obesity potential. Naveilhan *et al* showed that the germline Y2 receptor of the knockout mouse increased food intake, fat mass and body weight accompanied with leptin resistance that was indicated by an attenuated response to leptin in female mice (22). Another study on a Y2 deficient mouse model showed that female germline Y2 receptor of knockout mice also had increased food intake, but with reduced body weight, whereas male Y2 receptor of knockout mice had transiently reduced food intake and constantly decreased body weight associated with decreased adiposity at 16 weeks (23, 24). Subcutaneous injection of a Y2 receptor agonist, a polyethylene glycol-conjugated peptide agonist and 2-mercaptopyridine, reduced food intake in lean 18 hr fasted rodents, and this effect was abolished by pretreatment with the Y2 antagonist BIIE0246 (25). This supports the therapeutic potential of peripherally administered Y2 receptor agonists to reduce energy intake and treat obesity. However, there are some conflicting results. For example, Kuo, *et al* showed that Y2 receptors were involved in promoting proliferation and differentiation of adipocytes as well as stimulating angiogenesis of capillaries in adipose tissue (26).

Rosmaninho-S, *et al* showed that NPY induces adipocyte proliferation and differentiation, and lipid accumulation induced by NPY Y2 receptor activation occurs through PKA, MAPK and PI3K pathways (27).

In the present study, tests were performed on diet-induced obese mice, which was very close to clinical condition, and no effect of NPY receptor antagonist was observed on IM thickness and adipocyte cell size; however, adipocyte cell number was reduced in normal mice.

## Conclusion

Briefly, taking into consideration that central Y<sub>2</sub> receptor induces obesity, whereas activation of peripheral Y<sub>2</sub> receptor causes emaciation, presumably BIIE0246 acts mainly via inhibition of peripheral receptors, and decrease adipocyte cell number in normal group and peripheral receptor effects have been dominant. Although some investigations have suggested that for the interpretations of change in body weight and body composition, it is needed to consider the possibility of differential central versus peripheral effects, and/or hypothalamic or non-hypothalamic effects of the Y<sub>2</sub> receptor (18), future research needs to look more closely into the role of the Y<sub>2</sub> receptor on obesity and atherosclerosis.

## Acknowledgment

The authors would like to thank the Vice Chancellor of Research of Isfahan University of Medical Sciences for their financial support (Research project # 192039).

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