Original Article

Myocardial capillary density after neuropeptide Y antagonist administration in normal and high-fat diet C57BL6 mice

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Abstract

Background: Neuropeptide Y (NPY), a 36 amino acid peptide, has several effects on cardiovascular system. It is demonstrated that the angiogenic activity of NPY is similar to fibroblast growth factor and vascular endothelial growth factor (VEGF). The aim of this study was to evaluate the effect of systemic administration of antagonist of NPY receptor (BIIE0742) on coronary angiogenesis in normal and diet-induced obese animals.

Materials and Methods: Twenty-four male mice were received high-fat diet (HFD) or normal diet (ND) for 14 weeks. Then, each group was randomized to the treatment of antagonist of NPY receptor (BIIE0246) or saline as following: ND+ BIIE0246 (100 μl/kg; i.p.), ND+ saline, HFD+ BIIE0246, HFD+ saline. After 14 days, blood samples were taken, and myocardial tissue (left ventricle) from all experimental groups was evaluated by immunohistochemistry.

Results: Serum VEGF concentration and VEGF: Soluble VEGF receptor (sVEGFR)-1 ratio in obese animals was higher than normal group. Administration of BIIE0246 significantly reduced serum VEGF and VEGF: sVEGFR-1 ratio and increased serum sVEGFR-1 concentrations in obese animals ($P < 0.05$). In normal animals, BIIE0246 increased serum sVEGFR-1 level and decreased VEGF: sVEGFR-1 ratio. Serum nitrite did not alter after administration of BIIE0246 in both groups ($P > 0.05$). Myocardial capillary density expressed as the number of CD31 positive cells/mm^2 was reduced after NPY antagonist treatment in obese and normal animals ($P > 0.05$).

Conclusion: Administration of NPY antagonist impairs myocardial capillary density, reduces angiogenic factors and elevates anti-angiogenic factors, and there are no differences between obese and normal animals.

Key Words: Angiogenesis, coronary, neuropeptide Y

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INTRODUCTION

Angiogenesis is defined as the formation of new vessels from preexisting one and is a highly regulated process.[1] It occurs during several physiological conditions including wound healing, menstrual cycle, and some pathological conditions such as tumor growth or retinopathy.[2] Stimulation of angiogenesis

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is considered as a therapeutic approach in ischemic conditions, especially in the heart.[3]

During embryogenesis, neuropeptide Y (NPY) is a mediator of neurogenic angiogenesis.[4] In adult life, several studies reported that NPY has cardioprotective effects during myocardial ischemia.[5-7] In addition, it is suggested that NPY has an angiogenic properties.[8,9] It is indicated that NPY not only is a vasoconstrictor but also, it has a mitogenic activity for vascular smooth muscle cells. Moreover, NPY has angiogenic activity in *in vitro* and *in vivo* models.[10] The main stimulus for angiogenesis is hypoxia, and it was shown that the levels of NPY increase during hypoxia and exercise.[7] Furthermore, it is demonstrated that the angiogenic activity of NPY is similar to fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF), and it is suggested that it act as upstream of FGF and VEGF to initiate signaling which leads to angiogenesis.[9]

NPY is a 36 amino acid peptide which has six receptors. All receptors belong to a family of heptahelical receptors.[11] NPY Y2 receptor is a primary angiogenic receptor.[8,12] Since, angiogenesis process is altered during obesity which may accompanied with different consequences such higher tumor growth in obese subjects, and based on our knowledge, all previous studies have done on normal animals, in this study, we evaluated the effect of systemic administration of antagonist of NPY Y2 receptor (BIIE0742) on coronary angiogenesis in both normal and diet-induced obese animals.

MATERIALS AND METHODS

Animals

All experiments were approved by the Ethics Committee of Isfahan University of Medical Sciences. Twenty-four male mice (C57BL6, weight: 15–16 g, age: 4–5 weeks) were purchased from Pasteur Institute of Iran. The animals were kept four per cage at room temperature between 20°C and 25°C under 12 h light/dark cycles and had access to food and water *ad libitum*. After an initial period of 5 days for acclimatization, the animals were divided into two groups who received normal diet (ND) or high-fat diet (HFD).

Experimental groups

Mice received HFD or ND for 14 weeks and then each group was randomized to the treatment of antagonist of NPY receptor (BIIE0246) or saline. Therefore, we had four groups as following: ND+ BIIE0246, ND+ saline, HFD+ BIIE0246, HFD+ saline. BIIE0246 was administered 100 μl/kg at a concentration of 10^{-6} M, intraperitoneally.[13-15] The control groups received normal saline. After 14 days, the animals were sacrificed.

Materials and chemicals

To obtain the diet-induced obesity model, the obese groups consumed HFD (Laboratories BioServ, Cat #F3282, USA) included (59% fat, 27% carbohydrate, 14% protein) for 14 weeks. ND was purchased from Pasteur Institute. Antagonist of NPY receptor (BIIE0246) was purchased from Tocris Co., (Bristol, UK), VEGF (121, 165, and 189) from R and D systems (Minneapolis, MN, USA), NO from Promega Co., (Madison, WI, USA) and anti-CD31 antibody from Abcam (Cambridge, UK).

Immunostaining and measurement of vascularization

Formalin-fixed tissue samples were processed for immunohistochemistry. For this purpose, myocardial tissue (left ventricle) from all experimental groups was harvested. Histological sections with 5 μm thickness were made from each tissue sample and stained with monoclonal antibody against mouse CD31. Endothelial cells stained by the anti-CD31 antibody (anti-mouse CD31; 1:50; Abcam Co.) were counted under light microscopy. Fifteen random microscopic fields (×400) from three different sections in each tissue were examined by two blinded observers and counted for the presence of capillary endothelial cells (CD31 positive cells) using an Olympus light microscope at ×40 magnification. Then, the capillary density was expressed as the number of capillaries per mm^2.[16]

Serum angiogenic biomarkers

Serum VEGF and VEGFR-1 concentrations were measured using Sandwich Enzyme Immunoassay kits and reagents according to the manufacturer's instructions. The minimum sensitivity of VEGF and VEGFR-1 assays is 3.9 pg/ml and 3.8 pg/ml, with intra- and inter-assay coefficient of variation of <10% and 5%, respectively. Serum nitrite concentration, the main metabolite of NO, was determined by Griess reagent method (limit of detection is 2.5 μM).

Data analysis

All results were expressed as mean ± standard error. $P < 0.05$ was considered significant. The significance of differences between groups was compared with one-way ANOVA with Tukey's test, as *post hoc* test. Statistical analysis was carried out using SPSS (version 20, SPSS Inc. Chicago, USA).

RESULTS

Body weight

Figure 1 illustrated the changes of body weight in experimental groups. In the animals who received

HFD, the body weight was significantly higher than ND group (34 ± 1.11 g vs. 25.66 ± 1.41 g, respectively; $P < 0.05$).

Serum biomarkers of angiogenesis: Effect of neuropeptide Y receptor antagonist

Serum VEGF concentration in obese animals was higher than normal group ($P < 0.05$), while serum soluble VEGF receptor (sVEGFR)-1 was not different between groups ($P > 0.05$). Furthermore, VEGF: sVEGFR-1 ratio in HFD group was higher than ND group, although it was not statistically significant ($P < 0.05$) [Figure 2].

Administration of BIIE0246 significantly reduced serum VEGF concentration and increased serum sVEGFR-1 concentrations in obese animals and thus, decreased VEGF: sVEGFR-1 ratio ($P < 0.05$). In normal animals, BIIE0246 increased serum sVEGFR-1 level and reduced VEGF: sVEGFR-1 ratio [Figure 2].

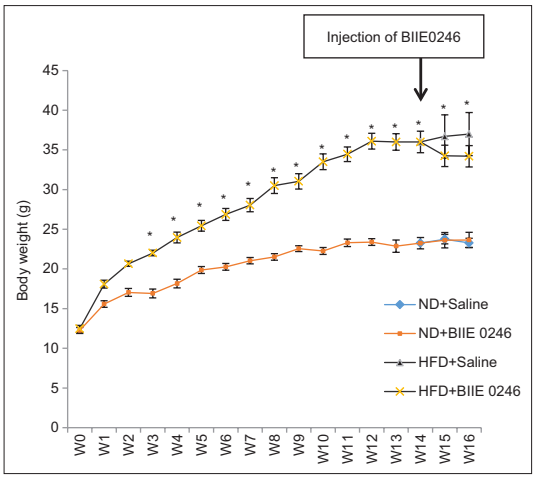

Figure 1: Changes of body weight during experimental periods. *$P < 0.05$ compare to ND. ND: Normal diet, HFD: High-fat diet, W: Week

Serum nitrite, the main metabolite of NO, was higher in HFD than ND group ($P < 0.05$) and administration of BIIE0246 did not alter it in both normal and obese group ($P > 0.05$).

Immunohistochemistry

Immunohistochemistry staining and analysis of capillary density in the hearts demonstrated that the number of CD31 positive cells was not significantly different between obese and normal ($P < 0.05$). Antagonist of NPY receptor administration reduced the number of CD31 positive cells in obese mice ($P > 0.05$), and the same results were also found in normal mice ($P > 0.05$). Samples of immunohistochemical staining were presented in Figure 3.

DISCUSSION

Our results showed that serum VEGF and VEGF: sVEGFR-1 ratio in obese and normal animals were reduced after administration of antagonist of NPY receptor. In addition, coronary angiogenesis expressed as capillary density/mm^2 was impaired after BIIE0742 treatment.

Recent studies indicated the role of NPY and its receptors in modulating tumor growth or treating obesity.[11] In this study, we found that the body weight of HFD group was higher than ND. Interestingly,

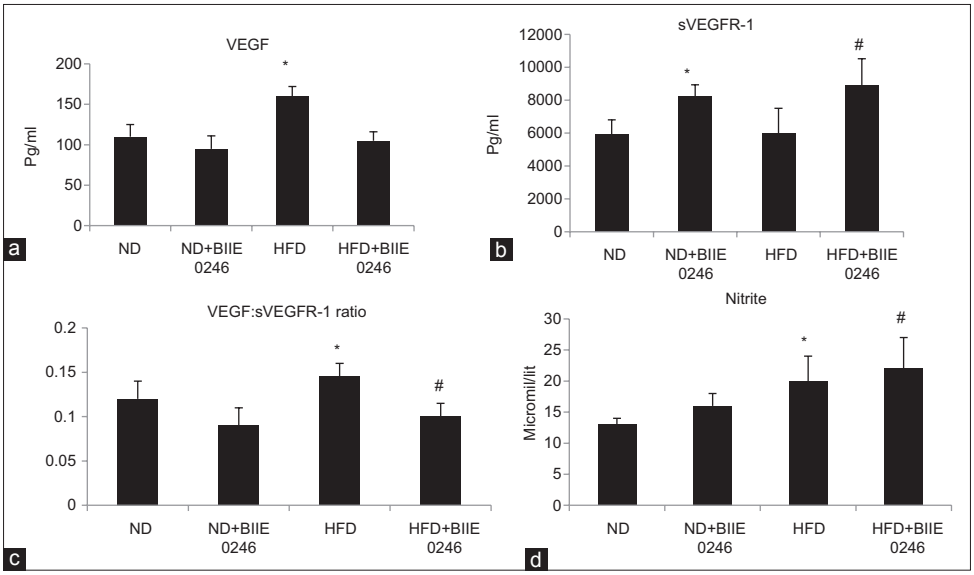

Figure 2: Serum vascular endothelial growth factor (a), soluble vascular endothelial growth factor receptor-1 (b) and nitrite (d) concentrations and vascular endothelial growth factor: Soluble vascular endothelial growth factor receptor-1 ratio (c). *$P < 0.05$ compare to normal diet. #: Compare to HFD group

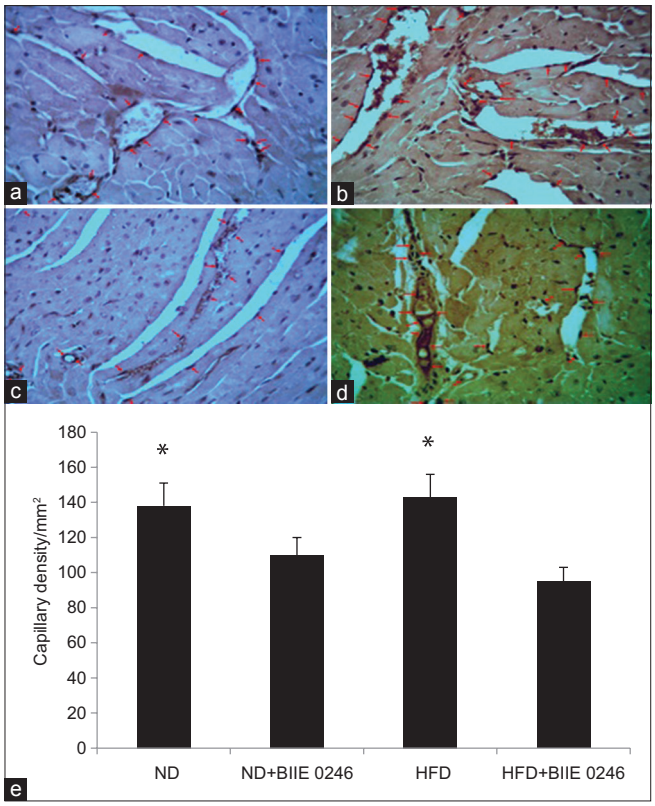

Figure 3: Immunohistochemical staining of left ventricle in all experimental groups (a-d). Arrows indicates CD31 positive cells. (a) ND, (b) HFD, (c) ND + BIIE0246, (d) HFD + BIIE0246. Changes of capillary density (expressed as number of CD31 positive cells/mm^2) (e). *$P < 0.05$ compare to treated-groups. ND: Normal diet, HFD: High-fat diet

as we showed in Figure 1, after administration of BIIE0742, the body weight in obese animals started to reduce. Although, in the present study, we did not measure food intake, and this is a limitation of the study, however, in agree to our observation, previous studies showed that the body weight of Y2 receptor knockout mice was lower than wild-type mice, and it was associated with adiposity.[17,18] Moreover, a study in ovariectomized female mice showed that increased body weight and adiposity after ovariectomy were normalized after global Y2 blocking and interestingly, not after ablation of hypothalamic Y2 receptor.[19] Thus, it seems that pharmacological agents which block Y2 receptor in peripheral tissue may be used for treating obesity.

NPY has five functional receptors, and it was shown that the pro-angiogenic properties of NPY are related to type of receptors. Y2 receptor is responsible for the angiogenic effect of NPY[8,12] and causes endothelial cell migration and proliferation. Thus, in this study, we used BIIE0742, as a specific Y2 receptor antagonist, and we found that the coronary angiogenesis was reduced after systemic administration of NPY Y2 antagonist in both normal and obese animals.

Our results also indicated reduced serum VEGF and elevated sVEGFR-1 after BIIE0742 administration in obese animals. VEGF is a known angiogenic factor *in vitro* and *in vivo* and VEGFR-1 has anti-angiogenic properties.[20] sVEGFR-1 binds with high affinity to VEGF, and decreases its activity and reduces angiogenesis.[20] NPY is not only act as angiogenic factors, but also it increases some angiogenic factors including FGF and VEGF.[9] Furthermore, it is suggested that VEGF to sVEGFR-1 ratio is a better marker for evaluation of angiogenesis status.[21,22] Thus, in the present study, we measured VEGF: sVEGFR1 ratio and we found that this ratio was decreased after BIIE0742 treatment in normal and obese animals. On the other hand, serum NO concentration was also decreased in treatment groups. NO is also a known angiogenic factor and it is indicated that NPY activated eNOS like VEGF.[7] Robich *et al*. reported that exogenous NPY increased myocardial capillary density and improved left the ventricular function in the normal animal.[7] They also showed that VEGF and FGF expression increased and anti-angiogenic proteins, endostatin, and angiostatin, increased after NPY treatment. Interestingly, they did not find increased blood flow to ischemic tissue. These results show that despite increasing in capillary density with elevated pro-angioenic factors, we may not have improving blood flow in myocardium. A recent study in patients with at least one coronary stenosis indicated that plasma NPY level in patients with well-developed collateral formation was higher than patients with poorly developed vessels while plasma FGF and VEGF were not different between groups.[5] Moreover, local injection of NPY improved capillary density and perfusion in chronic myocardial ischemia model.[6]

One of the limitations of the study was that the time course of treatment was relatively short. We also did not measure coronary blood flow in the heart. Some studies evaluated the role of NPY treatment in a model of cardiac or hindlimb ischemia,[7,9] but in the present study, we used normal and obese animals without induction of ischemia.

CONCLUSION

NPY Y2 antagonist treatment impairs myocardial capillary density, reduces pro-angiogenic factors and elevates anti-angiogenic factors. It seems that these effects are not different between obese and normal animals. Further studies need to evaluate more details of the effect of NPY receptors on myocardial tissue, flow, density, size, and strength to provide

stronger mechanisms to find pharmacologic agents with anti-obesity or anti-angiogenic properties.

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Conflicts of interest

There are no conflicts of interest.

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