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Effects of Neuropeptide Y on collateral development in a swine model of chronic myocardial ischemia

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Abstract

Objective—We investigated the role of neuropeptide Y (NPY), abundant in the myocardial sympathetic nervous system and endothelial cells, in angiogenesis during chronic myocardial ischemia.

Methods—Adult male Yorkshire swine underwent ameroid constrictor placement on the proximal left circumflex coronary artery. After three weeks, an osmotic pump was placed to deliver either placebo (control, n=8) or NPY₃₋₃₆ (NPY, n=8) to the collateral dependent region. Five weeks after pump placement, after cardiac catheterization and hemodynamic assessment, the heart was harvested for analysis.

Results—NPY treated animals demonstrated increased mean arterial pressures and improved left ventricular function (+dP/dt). Cardiac catheterization demonstrated a significant increase in the blush score in the NPY group (p<0.001). Blood flow to the ischemic myocardium was not different between groups at rest or during ventricular pacing. Immunohistochemical double staining for CD-31 and smooth muscle actin demonstrated an increase in capillary and arteriole formation in NPY treated animals (p=0.02 and p<0.001). Immunoblotting showed a significant upregulation of DPPIV (p=0.009) and NPY receptors 1 (p=0.008), 2 (p=0.02) and 5 (p=0.03) in the NPY treated group. Additionally, there was significant upregulation of VEGF (p=0.04), eNOS (p=0.014), phospho-

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eNOS(ser1177)($p=0.02$), and PDGF ($p<0.001$) in NPY treated group. The anti-angiogenic factors endostatin and angiostatin were significantly decreased in NPY treated animals (endostatin, $p=0.03$, angiostatin $p=0.04$).

Conclusion—Exogenous NPY₃₋₃₆ resulted in improved myocardial function and increased angiogenesis and arteriogenesis by stimulating growth factor, pro-angiogenic receptor upregulation, and decreasing anti-angiogenic expression, but did not increase blood flow to the ischemic myocardium. NPY may act as a good adjunct to primary agents of therapeutic angiogenesis.

Keywords

1. Chronic myocardial ischemia; 2. Neuropeptide Y; 3. Therapeutic angiogenesis; 4. Microvascular reactivity; 5. Vascular endothelial growth factor; 6. Endogenous inhibitors of angiogenesis

INTRODUCTION

Despite advances in percutaneous and surgical revascularization procedures, 20-37% of patients cannot receive any intervention because of the diffuse or severe nature of their coronary artery disease (CAD) [1]. A number of investigators have explored angiogenesis as a therapeutic modality by utilizing various factors to promote growth of new blood vessels into the ischemic myocardium [2-4]. The goal of therapeutic angiogenesis is to limit regions of myocardial impairment and ultimately preserve cardiac function by developing new vascular supplies to the ischemic myocardium. Although the concept of therapeutic angiogenesis in the ischemic heart has been studied in a number of basic laboratory experiments and human clinical trials, the ideal agent has not been fully characterized [5]. One major hurdle is the need to develop an agent that will induce a local angiogenic response in the setting of endothelial dysfunction, as seen in diabetes mellitus and dyslipidemia [6].

The angiogenic process requires intact endothelial and peripheral nervous systems [7]. During embryogenesis, vascular development occurs in parallel with the growth of peripheral nerves. One of the most important neurotransmitters released by the sympathetic nerves is neuropeptide Y (NPY), a 36 kDa mediator of neurogenic angiogenesis in tissues [8]. The arteries, in turn, not only supply oxygen and nutrients, but also express neurotrophic factors for nerve growth [7]. The levels of NPY, NPY converting enzyme dipeptidyl peptidase IV (DPP IV), and NPY receptors (NPYR) are highest during organogenesis and decrease later in life [9].

DPP IV cleaves full length NPY (NPY₁₋₃₆) to NPY₃₋₃₆, which has an affinity for NPYR₂ and NPYR₅, receptors that activate nitric oxide (NO)-dependent angiogenesis by stimulating endothelial adhesion, migration and proliferation [10]. NPY₃₋₃₆ also activates other growth factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), placental growth factor (PGF), and hepatocyte growth factor (HGF) [7]. Additionally, NPY₁₋₃₆ stimulates vascular smooth muscle proliferation via NPYR₁ and NPYR₅ receptors leading to the development of mature collateral vessels and cardiac hypertrophy [11].

Levels of NPY increase during hypoxia, cold, and exercise [12]. This is advantageous as it allows the body to respond locally to ischemic signals via the sympathetic nervous system [13]. The potency and efficacy of NPY in stimulating angiogenesis has been shown to be similar to FGF and VEGF [10]. It has been suggested that NPY may act upstream of VEGF and FGF, and may be a key factor or master “on switch” to initiate the cascade of events leading to angiogenesis [14,15].

At this time, there are no large animal studies examining local delivery of NPY₃₋₃₆ to ischemic myocardium. We hypothesized that intramyocardial infiltration of NPY₃₋₃₆ will initiate angiogenic pathways in a swine model of chronic myocardial ischemia by upregulating NPY

receptors, thus increasing NO directly and activating other growth factors. This should result in increased collateral development (capillaries and arterioles) and blood flow to the ischemic myocardium in the area at risk.

METHODS

Animal Model

Intact adult male Yorkshire miniswine (Parsons Research, Amherst, MA) underwent ameroid constrictor placement on the proximal left circumflex coronary artery (LCx). All animals were fed a regular diet and had free access to water. For all surgical procedures, anesthesia was induced with ketamine (10 mg/kg IM) and thiopental 2.5%, and maintained with a gas mixture of oxygen at 2 L/min and 2.0% isoflurane. The animals were intubated and mechanically ventilated at 16 breaths/min. During the first procedure (ameroid placement through a left mini-thoracotomy) gold-labeled microspheres (Biophysics Assay Laboratory, Worcester, MA) were injected into the left atrium during temporary occlusion of the LCx to determine the exact myocardial territory at risk. Next, the titanium ameroid constrictor (1.75 mm internal diameter) was placed around the proximal LCx.

Three weeks after ameroid placement, swine were divided into control (n=10) and NPY (n=10) groups. Swine were anesthetized and x-ray coronary angiography was completed via a 6 French introducer sheath placed in the right common femoral artery. A mini-thoracotomy through the original incision was completed. An osmotic pump (Alzet Inc., Cupertino, CA) was placed to deliver the NPY₃₋₃₆ solution (Sigma, Saint Louis, MO; NPY₃₋₃₆ 60µg with 50 units of heparin in a 2 ml solution of 0.1% bovine serum albumin in PBS delivered over 4 weeks at a rate of 3µl/hour) or placebo (0.1% bovine serum albumin in PBS) through a microcatheter placed in the myocardium at the level of the first marginal branch of the LCx in the ischemic territory.

Five weeks after pump placement, the heart was then exposed via median sternotomy, cardiac function measurements were taken, and microspheres were again injected at rest and pace followed by euthanasia. The heart was harvested and two 1 cm thick transverse slices were cut at the midventricular level and sectioned into 8 segments each. Samples were divided and rapidly frozen in liquid nitrogen (molecular studies), placed in 4°C Krebs solution (microvessel reactivity studies), 10% formalin (immunohistochemistry studies), or weighed and dried (microsphere perfusion analyses).

All experiments were approved by the hospital Institutional Animal Care and Use Committee. Animals were cared for in compliance with the Harvard Medical Area Institutional Animal Care and Use Committee and in accordance with the 'Principles of Laboratory Animal Care' formulated by the National Society for Medical Research and the 'Guide for the Care and Use of Laboratory Animals' (NIH publication no. 5377-3 1996).

Measurement of Global and Regional Myocardial Function

Indices of global and regional left ventricular (LV) function were obtained prior to animal sacrifice: mean arterial pressure (MAP); developed LV pressure (DLVP); positive (+dP/dt) and negative (−dP/dt) first derivatives of LV pressure, for 10 sequential beats using a Sonometrics system (Sonometrics Corp. London, ON, Canada).

Myocardial Perfusion analysis

Myocardial perfusion was determined during each procedure with isotope-labeled microspheres (ILMs), 15 µm diameter (Biophysics Assay Laboratory) using previously reported methods [16]. Briefly, 1.5×10^7 gold-labeled microspheres were injected during temporary LCx occlusion at the time of ameroid placement to identify the ischemic territory.

The segments with the lowest concentration of gold ILMs were considered the area at risk. Labeled ILMs were also injected at the pump placement and final procedures during rest and ventricular pace (150 beats/min) conditions. Following euthanasia, 10 transmural left ventricular sections were collected for ILM assays. The samples were exposed to neutron beams and microsphere densities were measured using a gamma counter.

X-ray coronary angiography

X-ray coronary angiography was carried out in order to ensure occlusion of the LCx and assess collateral formation. Recorded images were interpreted by an interventional cardiologist blinded to the treatment. Angiographic collateral formation was assessed according to the Rentrop grading system of 0–3, depending on the presence and extension of the collateral filling of coronary epicardial vessels. Myocardial blush grading system of 0–3 was used to assess the presence of developing collaterals as previously described [17].

Microvascular Responses

After cardiac harvest, coronary arterioles (100–180 μ m in diameter) from the LCx territory were dissected from the surrounding tissue and placed in isolated microvessel chambers [18]. Microvessels were cannulated with dual glass micropipettes and pressurized to 40 mmHg using 2 burettes containing Krebs solution. Vessels were bathed in Krebs (aerated with 95% oxygen–5% carbon dioxide) and precontracted by 25–50% of the baseline diameter with the thromboxane A₂ analog, U46619 (10^{-6} M). The microvascular responses to sodium nitroprusside (SNP, 10^{-9} to 10^{-4} mol/L, an endothelium-independent cGMP-mediated vasodilator) and endothelium-dependent vasodilators VEGF (10^{-15} to 10^{-10} mol/L), Substance P (SubP 10^{-12} to 10^{-7} mol/L), and adenosine diphosphate (ADP, 10^{-12} to 10^{-7} mol/L) were evaluated. All drugs were applied extraluminally. Relaxation responses were defined as the percent relaxation of the precontracted diameter. All reagents were obtained from Sigma-Aldrich (St Louis, MO).

Immunohistochemistry

Formalin fixed tissue samples were processed for immunostaining. Antibodies against platelet endothelial cell adhesion molecule (PECAM-1, CD-31, Santa Cruz Biotechnology, Santa Cruz, CA) and smooth muscle actin (SMA, Cell Signaling, Beverly, MA) were applied to the sections for 2 hours at room temperature. Detection was obtained using a biotinylated goat anti-mouse secondary antibody and the avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA). Color was developed using diaminobenzidine substrate (1 mg/ml in PBS and 0.03% H₂O₂). Sections were then counterstained with DAPI, dehydrated and mounted. Photomicrographs were taken with a Zeiss Axiolab microscope using a Plan-Apochromat objective lens (Carl Zeiss Inc, Thornwood, NY) equipped with a digital camera (Photodoc, Upland, CA) and vessels were counted in a blinded fashion. Vascular structure size and morphology were considered in the quantification process. Capillaries were defined as a single layer of CD31+ cells configured in a tube. Arterioles were defined as tubular structures staining with both CD31 and SMA, approximately 20 μ m, and with a VSMC layer between five and six layers thick.

Immunoblotting studies

Whole cell lysates were made from the ischemic territory of the control animals and ischemic (LCx) and non-ischemic (LAD) segments of the NPY animals using radioimmunoprecipitation assay buffer. Sixty micrograms of total protein was fractionated by 4–20% gradient, sodium dodecyl sulfate polyacrylamide gel electrophoresis (Invitrogen, San Diego, CA) and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA). Each membrane was incubated with specific primary antibodies (Cell Signaling, Beverly, MA and Abcam,

Cambridge, MA). Immune complexes were visualized with an enhanced chemiluminescence detection system (Amersham, Piscataway, NJ). Bands were quantified by densitometry of autoradiograph films. Ponceau staining was used to ensure equal protein loading.

Data Analysis

All results are expressed as mean $\pm$ standard error of the mean (SEM). Microvessel responses are expressed as percent relaxation of the precontracted diameter and were analyzed using two-way, repeated measures analysis of variance with a post-hoc Bonferroni test comparing treatment and dose. Western blots were analyzed after digitalization (ScanJet 4c; Hewlett-Packard, Palo Alto, CA) with NIH ImageJ 1.33 software (National Institute of Health, Bethesda, MD). Comparisons between two groups were analyzed by an unpaired, two tailed t-test, and three groups were analyzed by one-way, repeated measures analysis of variance with Newman-Keuls Multiple Comparison post-hoc test, using GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA). Probability values less than 0.05 were considered significant.

RESULTS

Animal Model

There were two animal deaths in the control group and two in the NPY group prior to completion of the study. There were a total of eight animals analyzed in each group. There was no difference in body mass indices (BMI) between the groups at the time of sacrifice, control $32.3 \pm 0.9 \text{ kg/m}^2$ and NPY $30.9 \pm 1.6 \text{ kg/m}^2$, $p = 0.45$.

Coronary Angiography

The ameroids were found to cause $>99\%$ occlusion of the LCx in all animals. There was no difference in Rentrop scores between groups (control 1.0 ± 0.2 and NPY 0.78 ± 0.17 , $p = 0.4$). There was a significant increase in blush scores in the NPY group as compared to the control group (Figure 1A-D).

Myocardial Functional Measurements

The animals in the NPY group demonstrated significantly increased MAP and DLVP as compared to the control group. The NPY group also demonstrated significantly increased global LV function, with higher maximum $+dP/dt$ and lower minimum $-dP/dt$ than the control group (Figure 2A-D).

Myocardial Perfusion

There was no difference in flow to the ischemic territory at rest (Figure 3A). There was no difference between the groups in blood flow to the collateral dependent area during ventricular pacing (Figure 3B). There was no difference in LAD flow (control 0.60 ± 0.08 and NPY 0.54 ± 0.05 , $p = 0.59$). There was also no difference in the ratio of LCx:LAD flow (control 0.92 ± 0.12 and NPY 0.95 ± 0.07 , $p = 0.85$).

Microvascular Reactivity

There was no difference between the groups in baseline diameter of the microvessels (control $157 \pm 12 \text{ }\mu\text{m}$ and NPY $149 \pm 15 \text{ }\mu\text{m}$, $p = 0.72$). After precontraction, there was no difference in microvessel diameters between the groups (control $99 \pm 7 \text{ }\mu\text{m}$ and NPY $109 \pm 13 \text{ }\mu\text{m}$, $p = 0.6$). The percentage of precontraction also was not different between the groups (control $-37 \pm 2 \%$ and NPY $-34 \pm 2 \%$, $p = 0.37$).

Microvascular relaxation to SNP, an endothelial-independent vasorelaxation agent, demonstrated no difference between the groups. The response to VEGF, an endothelial-

dependent agent, showed decreased relaxation in the NPY group. Microvascular relaxation responses to substance P and ADP, both endothelial-dependent relaxation agents, demonstrated no differences between the groups (Figure 4A-D).

Immunoblotting

In the NPY group protein expression was assessed in both the non-ischemic myocardium (LAD) and the ischemic myocardium (LCx). This allowed for the assessment of any paracrine effects exogenous NPY may exert on the adjacent non-ischemic myocardium. In the control group, only the ischemic territory (LCx) was examined. The expression of NPY was significantly increased in the LAD and LCx territories of the NPY group as compared to the control. The NPY receptor, NPYR_2 , was increased in the NPY-LAD vs. the control, and significantly increased in the NPY-LCx vs. NPY-LAD. NPYR_1 and NPYR_5 were upregulated in the NPY-LCx vs. control and NPY-LAD. DPPIV was upregulated in the NPY-LAD and NPY-LCx vs. control. Expression of the angiogenic proteins VEGF and FGF were significantly increased in the NPY-LCx vs. the control. Phospho-eNOS (ser1177) expression was increased in NPY-LCx vs. control and NPY-LAD. Expression of platelet derived growth factor (PDGF), important in vascular smooth muscle proliferation was increased in the NPY-LAD and NPY-LCx vs. control. Angiostatin and endostatin, endogenous inhibitors of angiogenesis, were downregulated in the NPY-LCx. Angiostatin was significantly lower in the NPY-LCx and NPY-LAD vs. control (Figure 5A-H). Endostatin was significantly decreased in the NPY-LCx vs. control ($p = 0.02$).

Immunohistochemistry

The density of CD-31 positive capillary endothelial cells was significantly increased in the ischemic territory of the NPY-treated group as compared to the control ($p = 0.02$). There was a significant increase in vascular structures double staining with CD-31 and alpha smooth muscle actin antibodies suggesting increased arteriole formation ($p < 0.001$) (Figure 6A-D).

DISCUSSION

Intramyocardial NPY_{3-36} administration in a swine model of chronic myocardial ischemia appears to have three advantages that could make it a valuable therapeutic agent. First, local delivery of NPY resulted in increased levels of VEGF and FGF in the ischemic myocardium, suggesting it may act upstream of these powerful growth factors (Figure 7). Second, it appears NPY may not only act to increase angiogenesis (the formation of new blood vessels from an existing vascular network), but also increases arteriogenesis (maturation of newly formed vessels by formation of a vascular smooth muscle layer). Finally, NPY treatment resulted in decreased expression of anti-angiogenic proteins, angiostatin and endostatin in the myocardium. While NPY treatment did not increase blood flow to the ischemic myocardium, it did improve the angiogenic potential of the local milieu. This could set the stage for further vessel growth leading to improved perfusion to the collateral dependent region in combination with other growth factors or cell-based therapy (i.e. stem cells).

NPY is thought to not only be pro-angiogenic, but it also upregulates other known pro-angiogenic factors such as VEGF and FGF [15]. This offers an advantage as numerous pro-angiogenic pathways are activated in the ischemic myocardium, and may result in greater collateral vessel formation than by an agent that activates a single pathway. Additionally, through synergistic actions, some pro-angiogenic factors may reach higher expression levels. In this case, activated eNOS was highly expressed in the ischemic territory of the NPY treated group. Both VEGF and NPY are known to activate eNOS [19], and this may account for the marked increase in phospho-eNOS (ser1177) levels.

NPY treatment resulted in increased NPY, DPPIV, and NPYR₂, and slightly increased VEGF and FGF, in the non-ischemic myocardium adjacent to the ischemic territory as well. NPY has been shown to induce mesenchymal stem cell mobilization and differentiation. In a study by Wang *et al.* [20] it was shown in a rat myocardial infarction model that NPY upregulates expression of stem cell homing and proliferation signals such as aurora B kinase, FGF-2, cyclin A2, eukaryotic initiation factor 4 E, and stromal cell-derived factor-1. These factors can work in a paracrine fashion to induce pro-angiogenic and pro-survival pathways in nearby myocardium [21]. Indeed, upregulation of NPY and its associated proteins in the non-ischemic territory suggests either direct extension of the drug or a paracrine effect.

The pro-angiogenic function of NPY is receptor subtype specific. There are five known NPY receptors, and all are G-protein coupled. NPY₁₋₃₆ is cleaved by DPP IV to form NPY₃₋₃₆, and this form of NPY is NPYR₂ and ₅ selective [22]. NPYR₂ is known to induce angiogenesis through endothelial cell proliferation, survival, and migration [9,15]. On the other hand, NPYR₅ is thought to induce angiogenesis and vascular smooth muscle proliferation [23]. This action can lead to the maturation of newly formed capillaries into arterioles. In our current study we demonstrated increases not only in capillary density, but also arteriole density. Pro-collateral growth factors VEGF, FGF, eNOS, and PDGF were all upregulated in the NPY treated animals. PDGF has been also been shown to be important in VSMC proliferation [24]. The action of NPY₃₋₃₆ appears to be unique in that it induces both vessel formation and maturation.

NPY treatment did not result in increased blood flow to the ischemic myocardium. Increased capillary density may not lead to improved perfusion, but usually an increase in the number of arterioles will improve flow. One possible explanation for this lack of increased collateral flow may be a lack of fluid shear stress. Shear stress is the force that blood exerts on the endothelium, and has been shown to be essential for successful arteriogenesis [25]. Though the NPY animals demonstrated increased MAP, if too many arterioles develop at one time the amount of stress each vessel experiences may not be increased. This can lead to limited flow and potentially regression of the vessel. Another possible explanation for the lack of increased blood flow to the ischemic territory is that the newly formed arterioles may have some degree of endothelial and/or VSMC dysfunction. Over time the vascular dysfunction may diminish and this could lead to increased collateral blood flow [26]. This dysfunction may be inherent to the newly formed vessels or secondary to the influence of NPY on the microvasculature. NPY did not improve microvascular reactivity, and in the case of VEGF there was a decrease in the relaxation response. The major NPY₃₋₃₆ receptor, NPYR₂, is thought to have little effect on vascular tone, and this is consistent with our findings [27]. However, the interaction of NPY₁₋₃₆ with NPYR₁ is known to result in potent vasoconstriction [28], and we did observe upregulation of NPYR₁. Additionally, VEGF may have a slight interaction with NPYR₁ leading to further decreased vasodilation. In fact, NPYR₁ receptor expression was increased in the normal and ischemic myocardium of the NPY treated animals suggesting this receptor subtype may be activated and contributing to local vasoconstriction, inhibiting blood flow increases. Further, while collateral vessel density was increased we did not demonstrate that these vessels were in continuity with existing blood vessels.

Interestingly, we found endostatin and angiostatin to be downregulated in the myocardium. Among the many growth factors investigated for use in therapeutic angiogenesis, NPY is the first pro-angiogenic signaling protein to demonstrate a decrease in anti-angiogenic protein expression. These two anti-angiogenic signaling molecules were first described by Folkman [29]. These proteins have subsequently been shown to be upregulated in comorbid diseases commonly associated with CAD such as diabetes mellitus, dyslipidemia, and hypertension [6]. Angiostatin and endostatin are known to inhibit native and therapeutic angiogenesis in animals and humans [30]. Boodhwani *et al.* [31] demonstrated that diabetes results in decreased

myocardial function and an impaired angiogenic response in a swine model of myocardial ischemia. Additionally, angiostatin and endostatin expression were significantly increased.

In a recent study of swine with metabolic syndrome and chronic myocardial ischemia, our lab illustrated that resveratrol, a polyphenol found in high concentration in red wine, had many metabolic benefits, but increased angiostatin expression. We demonstrated a lowering of cholesterol levels, blood pressure, and body mass index in animals treated with resveratrol. However, angiostatin expression was increased and the formation of new vessels was decreased to the level of the animals with untreated endothelial dysfunction (Robich, et al. *Circulation*, in press).

It is quite likely that endogenous inhibitors of angiogenesis are in large part responsible for the failure of therapeutic angiogenesis to transition from the bench to the bedside. Thus, if NPY treatment decreases the expression of angiostatin and endostatin in the setting of comorbid diseases, it may offer a powerful option for patients with endothelial dysfunction and coronary disease in combination with other growth factors or cell-based angiogenic therapies. Interest in the use of combination therapy to improve angiogenesis in ischemic myocardium is growing. Use of a drug to improve the angiogenic potential of another growth factor or stem cells is advantageous. The local environment in ischemic tissue can be hostile, with cell death signaling, inflammatory cells, and fibrosis predominating. Changing the overall signaling milieu to pro-survival and pro-angiogenesis can improve stem cell engraftment and/or vessel growth [32]. Additionally, NPY₃₋₃₆, via NYR5, may encourage maturation of newly formed capillaries to arterioles leading to improved perfusion of ischemic myocardium.

Limitations

There were limitations to the current study. Swine cardiac physiology and pathology are often similar to that seen in humans, though this may not be the case in NPY induced angiogenesis. This study was performed in healthy animals, and many cellular and growth factor therapies have been shown to work in this setting, but not in animals or humans with comorbid diseases. It will be necessary to examine the angiogenic potential of NPY in an animal model of endothelial dysfunction. Additionally, the time course of the experiment was relatively short. It is possible that the developing network of vessels seen on angiography (increased myocardial blush grade) and immunohistochemistry in the NPY group would eventually form significant collaterals leading to improved perfusion of the ischemic myocardium. Further, while we measured the level of DPPIV expression, assessment of the enzymatic activity would add insight into the effect of NPY.

Conclusion

NPY₃₋₃₆ delivered locally in a swine model of chronic myocardial ischemia resulted in improved left ventricular function and increased capillary and arteriole density. NPY treatment also upregulated VEGF and FGF expression in the ischemic myocardium, and decreased the expression levels of anti-angiogenic proteins, angiostatin and endostatin. These factors make NPY₃₋₃₆ a potential synergistic adjunct for use in growth factor or cell-based therapeutic angiogenesis.

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Abbreviations

DPP IV	dipeptidyl peptidase IV
CAD	coronary artery disease
ILM	isotope labeled microsphere
FGF	fibroblast growth factor
LV	left ventricle
NO	nitric oxide
NPY	neuropeptide Y
NPYR	neuropeptide Y receptor
VEGF	vascular endothelial growth factor

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Figure 1. Relevant anatomy and angiographic data and images

A) Schematic demonstrating the placement of the ameroid, the osmotic pump containing NPY₃₋₃₆, and the myocardium at risk. B) Assessment of myocardial blush grade demonstrates increased collateral formation in the NPY group, * $p < 0.001$. C) A representative angiogram from the control group demonstrating no collateral formation. D) Representative angiogram from the NPY group demonstrating collateral vessels with distal filling of the left circumflex coronary artery (LCx) and myocardial blush (block arrow in image inset). Asterisk-ameroid constrictor, small black arrow-left anterior descending artery (LAD), arrowhead-flow moderator within osmotic pump.

Figure 2. Myocardial function results

A) Mean arterial pressure (MAP) was significantly increased in the NPY group as compared to the control, *p= 0.03. B) Developed left ventricular pressure (DLVP) was increased in the NPY group, †p= 0.003. C) and D) LV function as measured by +dP/dt and -dP/dt was increased in the NPY treated animals as compared to control, ‡p= 0.02 and §p= 0.01, respectively.

Figure 3. Myocardial blood flow in the collateral dependent region

A) Measurement of blood flow at rest demonstrated no difference between the groups, $p=0.73$.

B) Perfusion of the ischemic territory during ventricular pacing (150 beats/min) was not different between groups, $p=0.45$.

Figure 4. Microvascular responses

A) The microvascular response to the endothelial-independent agent sodium nitroprusside (SNP) was not different between groups, $p=0.7$. B) The microvascular response to VEGF demonstrated decreased relaxation in the NPY group, $*p<0.001$. C) Endothelial-dependent relaxation agent Substance P (SubP) resulted in no difference between groups, $p=0.97$. D) Microvascular relaxation responses to ADP were not different between groups, $p=0.99$.

Figure 5. Immunoblotting results

Protein expression was assessed in the non-ischemic (LAD) and ischemic myocardium (LCx) of the NPY group, and both were compared to the ischemic myocardium (LCx) of the control group. A) NPY expression was increased in the normal territory of the NPY group (NPY-LAD), * $p < 0.05$ and the ischemic territory (NPY-LCx), † $p = 0.02$. B) NPY receptor 1 (NPYR₁) was upregulated in the NPY-LAD and NPY-LCx as compared to the control, ‡ $p = 0.008$. C) NPY receptor 2 (NPYR₂) expression was increased in NPY-LAD, § $p < 0.05$ and the NPY-LCx was increased compared to NPY-LAD and control, || $p < 0.001$. D) NPYR₅ was increased in the NPY-LCx as compared to control, # $p = 0.04$. E) DPP IV expression was increased in the NPY-LAD and NPY-LCx as compared to the control, ** $p = 0.009$. F) VEGF expression was increased in the NPY-LCx, †† $p = 0.04$. G) The expression level of FGF was also increased in the NPY-LCx, ‡‡ $p = 0.04$. H) Phospho-eNOS (ser1177) expression was increased in the NPY-LCx, §§ $p < 0.001$. I) Expression of angiostatin was decreased in the NPY-LAD as compared to the control, ||| $p < 0.05$ and in the NPY-LCx, ## $p = 0.007$. There was no difference between the NPY-LAD and NPY-LCx. J) Endostatin expression was significantly lower in the NPY-LAD as compared to the control (** $p = 0.02$). K) Representative images of the immunoblots.

Figure 6. Immunofluorescence images

Representative images taken from the ischemic territories of the A) control and B) NPY groups. Magnification for both images is 200x; scale bar represents 30 μ m. C) Capillary density in the ischemic territory demonstrates increased vessel formation in the NPY group (CD31+ cells/mm², *p=0.02). D) There is increased arteriole formation in the ischemic territory of the NPY group by CD31/smooth muscle actin (SMA) co-staining (CD31+/SMA+ cells/mm², †p<0.001).

Figure 7. Summary figure

Schematic drawing describing the proposed role of NPY in angiogenesis. NPY₁₋₃₆ is produced by sympathetic nerves in the myocardium. It is then cleaved by DPP IV to NPY₃₋₃₆ which is a pro-angiogenic signaling protein that works through NPY receptors 2 and 5. In the current experiment exogenous NPY₃₋₃₆ was delivered to the ischemic myocardium. NPY₃₋₃₆ upregulates VEGF, FGF and activates eNOS, resulting in increased nitric oxide (NO). This cascade leads to capillary and arteriole formation in the ischemic myocardium. NPY₃₋₃₆ also downregulates anti-angiogenic proteins such as angiostatin and endostatin, which leads to increased vascular tubule formation. NPY₁₋₃₆ stimulates NPY receptors 1 and 5 and results in increased vascular smooth muscle cell proliferation leading to maturation of capillaries to arterioles.