Journal of Molecular and Cellular Cardiology xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

Original article

Neuropeptide Y improves myocardial perfusion and function in a swine model of hypercholesterolemia and chronic myocardial ischemia

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ARTICLE INFO

Article history:
Received 31 July 2012
Received in revised form 24 August 2012
Accepted 30 August 2012
Available online xxxx

Keywords:
Angiogenesis
Metabolic syndrome
Chronic ischemia
Neuropeptide Y

ABSTRACT

Pharmacologically induced angiogenesis could be a promising option in clinical situations with diffuse inoperable coronary artery disease e.g. metabolic syndrome and diabetes mellitus. The failure of focused cytokine, stem cell and gene therapies to achieve both perfusion and functional improvement in clinical trials suggests a more centralized control mechanism. Neuropeptide-Y (NPY) is one such natural neurotransmitter that is known to exert a multifaceted role during neo-angiogenesis and can possibly act as the central control. To date, the ability to harness the 'master switch' nature of NPY in a specific experimental model of metabolic syndrome and chronic myocardial ischemia has not been conclusively demonstrated. We hypothesized that infiltration of NPY into an area of chronic ischemia in a metabolic syndrome swine model would induce angiogenesis and improve myocardial perfusion and function. An osmotic pump was inserted three weeks after surgical induction of focal myocardial ischemia. We delivered either NPY or placebo for five weeks, after which the myocardial tissue was harvested for analysis. Assessments of myocardial perfusion and function were performed at each stage of the experiment. Local infiltration of NPY significantly improved collateral vessel formation, blood flow and myocardial function. We believe activation of NPY receptors may be a potential target therapy for patients with diffuse coronary artery disease.

1. Introduction

Therapeutic neovascularization including angiogenesis (capillary formation) and arteriogenesis (arteries with smooth muscle cell) with cytokines and growth factor therapy has demonstrated variable success [1–6]. Neo-angiogenesis is a complex process, which involves multiple cytokine pathways, and requires an intact endothelium and smooth muscle migration/proliferation [7]. The equivocal nature of results with specific and targeted cytokine therapy, e.g. vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and colony stimulating growth factor (CSGF), also suggests a multifactorial nature of the process of angiogenesis [2,3,8,9]. Angiogenic signaling is especially altered in patients with coronary artery disease associated with co-morbidities, e.g. diabetes, hypercholesterolemia, and metabolic syndrome [10,11]. The associated diffuse coronary artery disease pattern has led to limited success after both percutaneous and surgical treatments [12]. Importantly, apart from the altered angiogenic signaling there is increased anti-angiogenic signaling in this patient population [13,14]. Thus, overcoming the local anti-angiogenic milieu in this patient population with inoperable diffuse disease associated with diabetes and metabolic syndrome would be a highly desirable option. Currently no such therapy exists for clinical use.

Neuropeptide Y, a 36-amino acid peptide, is one of the most important trophic neurotransmitters released by the sympathetic nerves. During embryogenesis, NPY is a mediator of neurogenic angiogenesis [15]. In adult life, capillary angiogenesis is limited to certain organs, e.g. uterus during gestation, skeletal muscle during training, or in ischemic tissue to restore impaired blood flow. NPY is present in abundance in the myocardium, and has both pre- and post-junctional effects. It is co-stored and co-released with noradrenalin and is involved in acute and chronic stress responses. NPY is positioned at the beginning of the cascade that activates endothelial

Abbreviations: HCT, high cholesterol treated; HCT LAD, high cholesterol treated-left anterior descending (non-ischemic area); HCT LCx, high cholesterol treated-left circumflex (ischemic area); HCP, high cholesterol placebo; NDP, normal diet placebo; NPY, neuropeptide Y; NPYR, neuropeptide Y receptor.

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0022-2828/$ – see front matter © 2012 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.yjmcc.2012.08.027

migration, vascular smooth muscle cell (VSMC) proliferation and cardiomyocyte hypertrophy [16,17]. The full length NPY_{1-36} is cleaved by NPY-converting enzyme dipeptidyl peptidase IV (DPPIV) to NPY_{3-36} in the tissues which stimulates endothelial adhesion, migration and proliferation [16,18,19]. Both animals and humans with diabetes mellitus express decreased tissue levels of NPY, possibly due to autonomic dysfunction and sympathetic neuropathy [18,20,21]. NPY_{3-36} plays an important role in ischemic revascularization and myocardial hypertrophy in animals [17,19,22,23]. We have previously demonstrated the trophic and angiogenic properties of local infiltration of NPY_{3-36} in a normal-cholesterol swine model of myocardial ischemia [24]. NPY may also improve myocardial perfusion in conditions associated with endothelial dysfunction, neuropathy, reduced vascular endothelial growth factors and increased production of angiogenesis inhibitors [1,25]. This study evaluated the role of locally delivered NPY_{3-36} in up-regulating angiogenic factors and improving myocardial perfusion and function in a swine model of metabolic syndrome and chronic myocardial ischemia. We hypothesize that NPY_{3-36}, would improve collateral vessel formation and perfusion, thus improving myocardial function.

2. Materials and methods

2.1. Animal model

All experiments were approved by 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).

Twenty-one six-week-old male Yorkshire miniswine (Parsons Research, Amherst, MA) were used. After an initial period of five days for acclimatization the animals were divided into two main groups. Seven animals were fed 500 g of a commercially available pig chow daily while the remaining fourteen were fed 500 g of a hypercholesterolemic diet composed of 4% cholesterol, 17.2% coconut oil, 2.3% corn oil, 1.5% sodium cholate, and 75% regular chow daily. This hypercholesterolemia diet is known to induce metabolic syndrome (obesity, hypercholesterolemia, hypertension and insulin resistance leading to glucose intolerance and type II diabetes) [26]. The separate diets were sustained for five weeks before the induction of myocardial ischemia and were continued for the duration of the experiment.

2.2. Surgical protocol

After five weeks of dietary modification, all animals underwent the first surgical procedure. The day before each surgery, all animals received aspirin (325 mg orally) and were fasted for 12 h. Prior to each surgical procedure, all animals received prophylactic antibiotics and buprenorphine (0.03 mg/kg, intramuscular) for pain control. All survival procedures were conducted in a sterile fashion. For all surgical procedures animals were given general anesthesia after sedating with Telazol (4 mg/kg, intramuscular), followed by endotracheal intubation and ventilation with a volume-cycled ventilator (North American Dragger). General anesthesia was maintained with a gas mixture of oxygen at 1.5–2 l/min and 3% isoflurane. The animal's vital signs were recorded during and throughout postoperative recovery. Femoral access via a percutaneous stick was achieved for arterial access, blood draws and blood pressure monitoring. An ameroid constrictor (internal diameter 1.75 mm) was placed on the proximal left circumflex coronary artery (LCx) via small left thoracotomy through the fourth intercostal space. During the first surgical procedure isotope-labeled microsphere (ILM) injection was performed to determine the myocardial territory at risk by temporary occlusion of the left circumflex artery and ILM injection in the left atrium.

Thirty minutes prior to the end of each procedure, a dose of buprenorphine (0.03 mg·kg, intramuscular) was administered. After each procedure, the dosage of aspirin was continued for five days, while a fentanyl patch (4 μg/kg) was applied for 72 h for pain control. Animals demonstrating severe pain by refusing to eat, prostration, tachypnea tachycardia and listlessness were treated with an additional administration of fentanyl patch.

Three weeks after ameroid placement, the animals were anesthetized for the second time and coronary angiography was performed through femoral artery sheath to ensure occlusion of the LCx. Microsphere injection in the left atrium at rest and during ventricular pacing at a rate of 150 beats/min was performed through a mini-left thoracotomy. An osmotic pump (Alzet Inc. model 2ml4, Cupertino, CA, USA) was placed to deliver the peptide NPY_{3-36} solution (Sigma, Saint Louis, MO; NPY 60 μg mixed with 50 U of heparin in a 2 ml solution of 0.1% bovine serum albumin in phosphate buffered saline (PBS) delivered over 4 weeks at a rate of 3 μl/h) to seven animals on high cholesterol diet (HCT). Osmotic pump with placebo (0.1% bovine serum albumin in PBS) was placed in two groups, seven high cholesterol diet animals (HCP) and seven animals on normal diet (NDP). The osmotic pump delivered directly into the ischemic territory via a catheter implanted into the myocardium at the first marginal branch of the LCx.

Five weeks after the osmotic pump placement, the animals were anesthetized for the third and final procedure. Coronary angiography was again performed through the femoral artery sheath, the heart was exposed through sternotomy and microspheres were injected at rest and with pacing in the left atrium. Lastly, sonomicrometer crystals (Sonometrics Corp. London, ON, Canada) were used to assess myocardial function. Following this, euthanasia was performed under deep anesthesia.

Myocardium was harvested and two 1 cm thick transversal slices cut at the mid-ventricular level and then sectioned into 8 segments identified clockwise starting from the anterior junction of the right and left ventricles. Separate samples were weighed and dried in a 60 °C oven for microsphere perfusion analysis. Samples from the anterior and lateral walls were taken for rapidly freezing in the liquid nitrogen for molecular studies or in 10% formaldehyde for paraffin section slides for immunohistochemistry.

The measurements for height and weight were made at the end of each surgery. Glucose tolerance test was performed at 0, 30 and 60 min after dextrose infusion. Blood samples were collected after the femoral sheath placement and spun for serum and plasma collection.

2.3. Animal data

The measurements for height and weight were made at the end of each surgery. Glucose tolerance test was performed at 0, 30 and 60 min after dextrose infusion. Blood samples were collected after the femoral sheath placement and spun for serum and plasma collection.

2.4. Measurement of global and regional myocardial function

Indices of global and regional left ventricular function prior to cardiac harvest using single-sensor pressure catheters (Millar Instruments, Houston, TX) and the Sonometrics system (Sonometrics Corp. London, ON, Canada) were obtained [27]. The mean arterial pressure (MAP), developed left ventricular pressure, positive (+dP/dt) and negative (−dP/dt) was obtained for ten sequential beats.

2.5. X-ray coronary angiography

Intraoperative X-ray coronary angiography was performed in order to ensure occlusion of the left circumflex artery and assess

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radiographic evidence of collateral formation. An interventional cardiologist blinded to the treatment groups interpreted the recorded images. Angiographic collateral formation and myocardial blush scores were assessed according to the Rentrop grading system of 0–3 to evaluate the presence and extension of the collateral filling of coronary epicardial vessels. A score of 0 would indicate no myocardial blush or contrast density. A score of 1 would indicate minimal myocardial blush or contrast density. A score of 2 would indicate moderate myocardial blush or contrast density but less than that obtained during angiography of a contralateral or ipsilateral non-infarct-related coronary artery. Lastly, a score of 3 would indicate normal myocardial blush or contrast density, comparable with that obtained during angiography of a contralateral or ipsilateral non-infarct-related coronary artery [28].

2.6. Myocardial perfusion analysis

Myocardial perfusion was determined with ILMs, 15 μm diameter (Biophysics Assay Laboratory, Worchester, MA) as previously described [24]. 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 microspheres were also injected during the pump placement and at the time of final procedures during rest and ventricular pacing (150 beats/min). Following euthanasia, 10 transmural left ventricular sections were collected for assays. The samples were exposed to neutron beams and microsphere densities were measured using a gamma counter.

2.7. Immunohistochemistry

Formalin fixed tissue samples were processed for immunohistochemistry. Myocardial tissue from the HCT group was harvested from both the ischemic LCx territory as well as the non-ischemic LAD territory. This further subdivided the tissue from the HCT group into an ischemic group with NPY_{3-36} infusion (HCT LCx region) and a non-ischemic group (LAD region), allowing for the assessment of the paracrine effects of exogenous NPY_{3-36} on the adjacent non-ischemic myocardium.

Antibodies against platelet endothelial cell adhesion molecule (PECAM-1, CD31, Santa Cruz Biotechnology, Santa Cruz, CA) and smooth muscle actin (SMA, Cell Signaling, Beverly, MA) were applied to the sections for 2 h at room temperature. Detection was obtained using a biotinylated goat anti-mouse secondary antibody and an avidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, CA). Color was developed using diaminobenzidine substrate (1 mg/ml in PBS and 0.03% H_2O_2). 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. All capillaries of approximately 85 μm and above size were counted. Arterioles were defined as tubular structures stained with both CD31 and smooth muscle actin, with a vascular smooth muscle cell layer between five and six layers thick. All arterioles of size 100 μm and above were counted.

2.8. Immunoblotting

Whole cell lysates were made from ischemic territory of the HCP group as well as the tissues from the HCT LCx and the HCT LAD regions of HCT group. Sixty micrograms of total protein was fractioned by a 4–12% gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis (Life Technologies, Grand Island, NY) and transferred to PVDF membranes (Millipore, Billerica, MA) with a semi-dry transfer cell (Bio-Rad Trans-Blot, Hercules, CA). Ponceau staining was used to ensure equal protein loading. Each membrane was incubated with specific primary antibodies overnight at 4 °C and secondary antibodies for 1 h at room temperature. We examined NPY Receptors ($NPYR_1$, $NPYR_2$, Alpha Diagnostics, San Antonio, TX) ($NPYR_5$, US Biological, San Antonio, TX), growth factors (PDGF-b, Santa Cruz Biotechnology, Santa Cruz, CA) (FGF2b, US Biological, San Antonio, TX) (p-eNOS, eNOS, Cell Signaling, Danvers, MA) and anti-angiogenic factors (angiostatin, endostatin, Millipore, Billerica, MA). Immune complexes were visualized with an enhanced chemiluminescence detection system (Amersham, Piscataway, NJ).

2.9. Data analysis

All results were expressed as mean ± standard error of the mean. Probability values of less than, or equal to, 0.05 were considered significant. Coronary angiography was used to document circumflex ameroid occlusion and evaluate changes in lateral myocardial collateralization. A cardiologist blinded to the study quantified collateral development. The regional myocardial functional assessment by crystals was measured by mean arterial pressure (MAP), left ventricular systolic function (+dP/dt), left ventricular diastolic function (−dP/dt) and fractional shortening. Myocardial blood flow (ml/min/g tissue) was derived from the myocardial microsphere concentration data, which was analyzed by Biophysics Assay Laboratories (Worcester, MA). The blood flow among the HCP, HCT and NDP groups, was compared using a one-way ANOVA, with repeated analysis of variance, with Newman–Keuls multiple comparison post-hoc test. GraphPad Prism 4 (GraphPad Software, CA) was used for analysis. Immunoblots were analyzed after digitalization (ScanJet 4c; Hewlett Packard) with NIH ImageJ 1.33 software (National Institute of Health, Bethesda, MD). Comparison between the HCT group and the HCP group was analyzed by an unpaired, two-tailed, *t*-test and between the LCx and the LAD regions of the HCT swine by a paired two-tailed *t*-test.

3. Results

3.1. Animal model

There was one death in the NDP group and two in each of the HCP and the HCT groups. Table 1 details the comparisons between groups. The animals fed with a high cholesterol diet exhibited higher body mass index, glucose intolerance, and abnormal lipid profiles compared to the control group. The average ratio of low-density lipoprotein to high-density lipoprotein (LDL/HDL) was increased, but not significantly, in both the HCP group and the HCT groups by the end of the experiment ($p=0.32$ $p=0.16$ respectively) (Table 1).

3.2. Myocardial functional measurements

The HCT and HCP groups demonstrated significantly increased mean arterial pressure as compared to the control ($p=0.02$). There was an improvement of systolic function as measured by +dp/dt in the HCT group as compared to the HCP and NDP groups, but this did not reach significance ($p=0.06$) (Table 1).

3.3. Coronary angiography

The ameroid constrictor caused 100% occlusion of the proximal left circumflex coronary artery (LCx) in all animals. There was a significant increase in collateral formation evaluated by Rentrop score in the HCT group when compared to the HCP and NDP groups ($p=0.01$). The blush score was increased in the HCT group but it was

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Table 1
Baseline characteristics and hemodynamic data. Comparison of percentage change in body mass index (BMI) between first surgeries and the final surgery showed a significant increase in both high cholesterol groups. Final blood sugar was calculated at the time of final surgery at fasting and 30 min after dextrose infusion. The low-density lipoprotein to high-density lipoprotein ratio (LDL/HDL) was calculated during the first surgery and again at the time of final surgery. The table also includes invasively driven hemodynamic data comparing mean arteriole pressure (MAP) and left ventricular systolic function (+dp/dt) between the three groups.

	High cholesterol placebo	High cholesterol treated	Normal placebo	p value
% change BMI	51.3±5.0	45.2±4.6	17.6±5.0	<0.001*
Blood sugar fasting (mg/dl)	50.7±5.5	64.4±2.0	41.1±6.8	0.007*
Blood sugar 30 min after dextrose infusion (mg/dl)	157.1±7.7	156.9±6.3	113.3±5.9	0.001*
Pre LDL/HDL	0.03±0.03	0.06±0.02	–	0.42
Post LDL/HDL	0.24±0.24	0.11±0.04	–	0.49
MAP (mm Hg)	90.3±6.5	87.4±6.9	62.2±7.6	0.02*
+dP/dT (mm Hg/s)	1130±160	1415±75	1021±95	0.06

* $p<0.05$.

not statistically different as compared to the HCP and NDP ($p=0.26$) (Fig. 1).

3.4. Myocardial perfusion

Blood flow to the ischemic territory at rest was significantly increased in the HCT group as compared to the HCP and NDP groups ($p=0.01$), and also during ventricular pacing ($p=0.03$) (Fig. 2).

3.5. Immunohistochemistry

There was a significant increase in the cluster of differentiation 31 (CD31) positive capillary in HCT group as compared to the HCP group ($p=0.001$) and the NDP group ($p=0.003$). There was also a significant increase in the number of CD31/SMA co-stained arterioles in the HCT LCx region as compared to the non-ischemic left anterior descending (LAD) region ($p=0.003$) and also compared to the HCP LCx region ($p=0.002$) (Fig. 3).

3.6. Immunoblotting

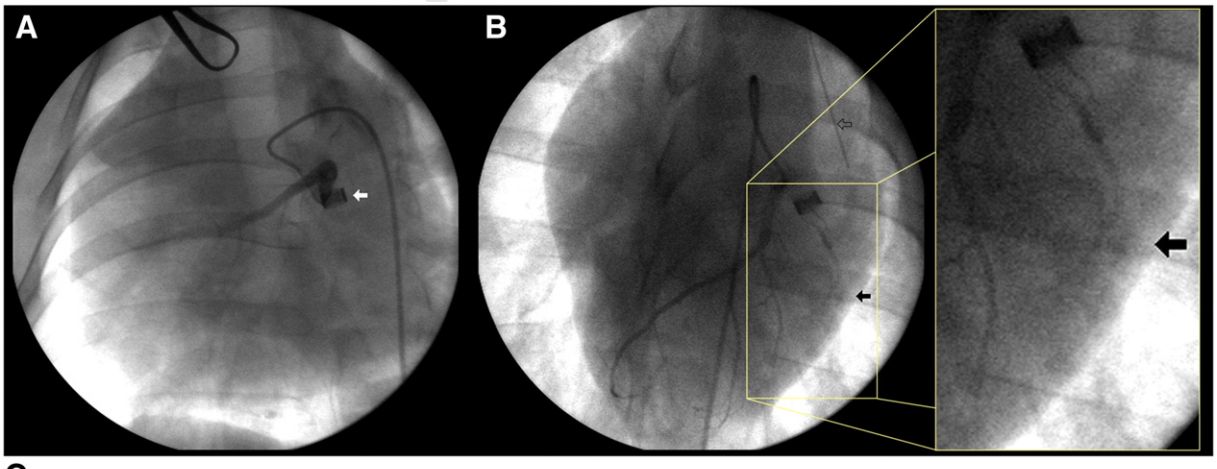
The expression of NPY receptors, $NPYR_1$ and $NPYR_5$, was significantly increased in the HCT group compared to the HCP group ($p=0.03$ and $p<0.001$, respectively). There was an upward trend of the expression of $NPYR_2$ in the HCT group as compared to HCP ($p=0.06$) but a significant decrease in the HCT LCx region as compared to LAD region ($p=0.05$) (Fig. 4).

There was a significant upregulation of platelet-derived growth factor (PDGF) in the HCT group as compared to the HCP group ($p=0.04$); however, the angiogenic protein fibroblast growth factor (FGF2b) was decreased ($p=0.04$). Also, phosphorylated endothelial nitric-oxide synthase (p-eNOS) was decreased in the HCT LCx region as compared to both the LAD region and the HCP control ($p=0.001$ and $p=0.05$ respectively).

The endogenous inhibitors of angiogenesis were decreased compared to controls. Angiostatin was significantly lower in the HCT LCx region when compared to both the LAD region and the HCP control ($p=0.02$ for both). Similarly, endostatin was significantly lower in the HCT group when compared to the HCP group ($p=0.004$) (Table 2).

4. Discussion

Our study has demonstrated the favorable effects of localized infiltration of NPY_{3-36} in a metabolic syndrome swine model of chronic myocardial ischemia. Histologically, NPY_{3-36} treatment was associated with an increase in both capillary and arteriolar collateral vessel formations. Physiologically, we found a significant improvement in myocardial perfusion both at rest and also during myocardial stress induced with ventricular pacing. We also demonstrated significant improvement in myocardial function in the region of chronic ischemia. Biochemically, the most angiogenic cytokines were increased and anti-angiogenic factors decreased in the NPY infiltrated ischemic myocardium. Interestingly, we also noticed an up-regulation of NPY receptors and increase of early pro-angiogenic factors in the NPY treated non-ischemic LAD territory possibly suggesting a paracrine effect. The demonstration of an improvement in myocardial perfusion and function at the microscopic, physiologic, biochemical



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	High Cholesterol Placebo	High Cholesterol Treated	Normal Placebo	p value
Collateral Score	0.33 ± 0.33	1.57 ± 0.20	0.33 ± 0.33	0.01*
Blush Score	0 ± 0	0.57 ± 0.30	0 ± 0	0.26

Fig 1. X-ray coronary angiography. (A) A representative cardiac catheterization image showing 100% occlusion of left circumflex artery by the ameriod constrictor (white arrow). (B) New collaterals are demonstrated in the treated ischemic area (black arrow). The osmotic pump catheter is visible (white arrow). (C) Cardiac catheter data with collateral and blush scores. *$p<0.05$.

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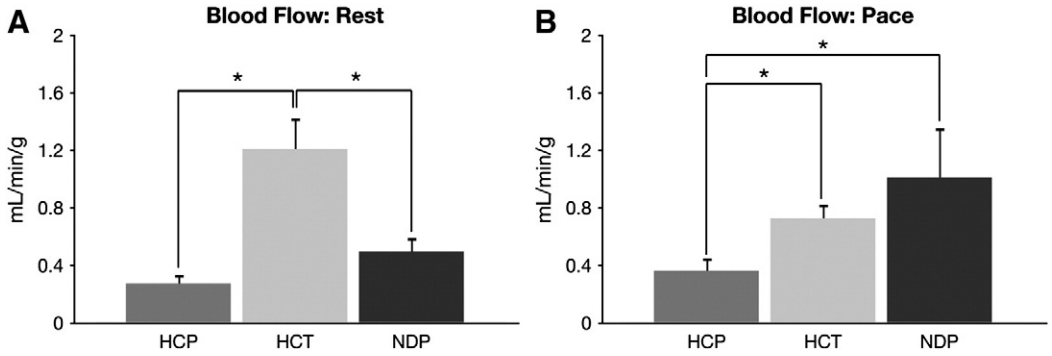

Fig. 2. Myocardial blood flow analysis. (A) There was significantly increased blood flow at rest in the HCT group as compared to the other two groups ($p = 0.01$). (B) Perfusion during ventricular pacing (150 beats/min) showed a significant increase in blood flow in the HCT group. *$p < 0.05$.

and functional levels in NPY treated myocardium suggests that the NPY receptor has the potential to be an important target for therapy in chronic myocardial ischemia.

Therapeutic angiogenesis requires the development of both micro-vascular and macro-vascular changes, and is clearly a complex process. Stimulation of neovascularization at the microscopic level of capillary formation and at macroscopic level for collateral vessel development is a unique and important finding of our study. Multiple studies have shown that NPY by activating $NPYR_2$ and $NPYR_1$ can stimulate endothelial cells for angiogenesis [18,22,29]. Also, activated $NPYR_5$ amplifies arteriogenesis and the development of collateral vessels [30]. The angiogenic potency of NPY and its ability to form normal arteries with layers of smooth muscle is unique and previously established, and is distinct from the abnormal vessels formed by either VEGF or FGFb [31]. VEGF-A and PDGF may result in a synergistic restoration of normal blood flow to the ischemic area via capillary formation (VEGF-A) and maturation (PDGF) [32]. The increase in PDGF in the NPY treated myocardium may help explain the proliferation of smooth muscle cells associated with neovascularization [22,33]. The simultaneous up-regulation of NPY receptors and cytokines in the ischemic myocardium further suggests the key role of NPY in initiating angio and arteriogenesis [34].

Another important finding in our study was the significant decrease in the plasminogen derived endostatin and angiostatin in the ischemic area treated with NPY_{3-36}. Angiostatin and endostatin inhibit collateral vessel formation in dog models of repeated myocardial ischemia [35]. The increased expression of these angiostatic factors observed in type I and type II diabetic animals leads to significantly lower collateral vessel density. A similar finding has been noted in

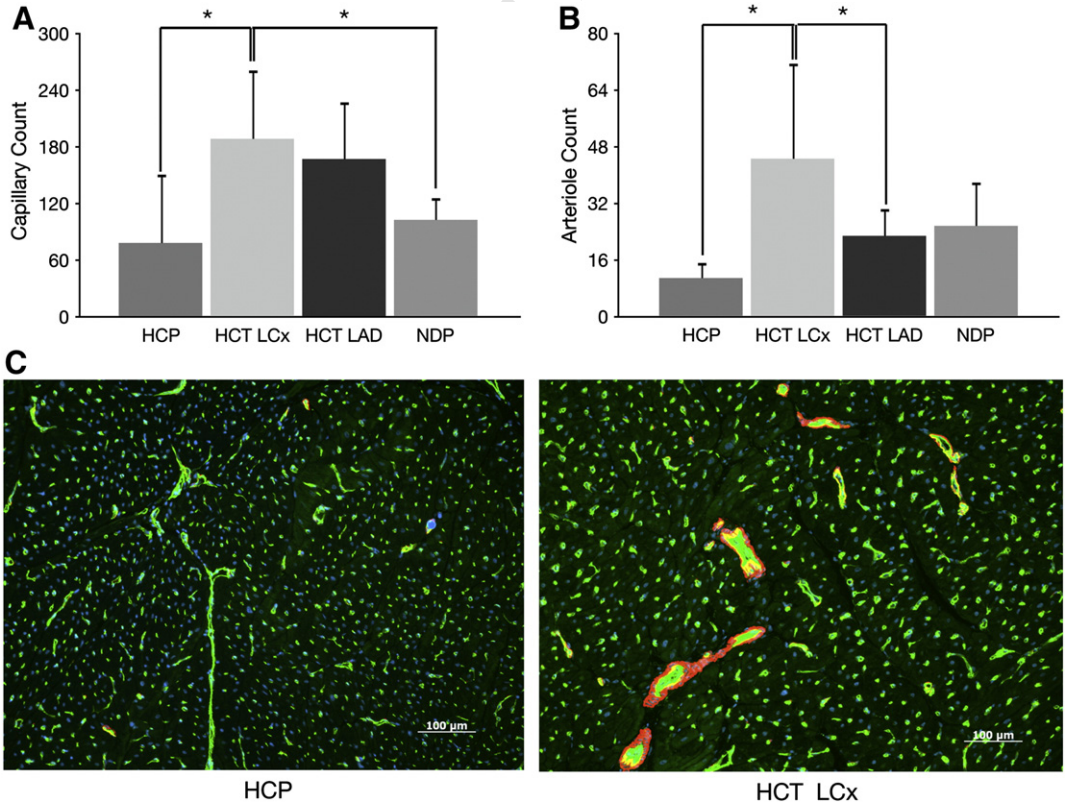

Fig. 3. Immunohistochemistry analysis. The HCT group was further divided into HCT LCx region (NPY infused ischemic area) and LAD region (non-ischemic treatment control area). All capillaries larger than 85 μm^2 and all arteries larger than 100 μm^2 have been counted. (A) There was a significant increase in capillary density in the HCT LCx region as compared to the HCP group ($p = 0.001$) and the NDP group ($p = 0.003$). (B) There was a significant increase in arteriole density in HCT LCx region as compared to LAD region ($p = 0.003$) and HCP group ($p = 0.002$). (C) Representative immunohistochemistry slide showing the ischemic area from the HCP and the HCT LCx groups. Capillaries (CD31, green), nuclei (DAPI, blue) and arterioles with smooth muscle (actin, red) are stained. *$p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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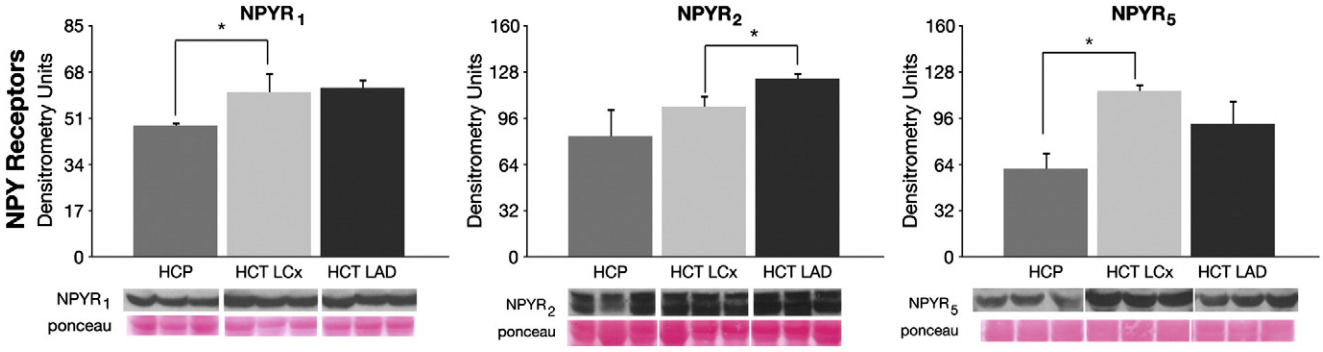

Fig. 4. Neuropeptide Y receptor expressions. Two neuropeptide Y receptors, $NPYR_1$ and $NPYR_5$, were significantly increased in the HCT LCx regions as compared to HCP ($p=0.03$ and $p<0.001$ respectively). $NPYR_2$ expression in the HCT LCx region was elevated as compared to HCP ($p=0.06$) and significantly decreased as compared to LAD territory ($p=0.05$). All blots were normalized to total protein stain with Ponceau. *$p<0.05$.

the right atrium of patients with type II diabetes mellitus [13,20,36,37]. Interestingly, in a normal-diet swine model of chronic myocardial ischemia, local infiltration of NPY_{3-36} significantly decreased the levels of these anti-angiogenic factors [24]. In the current study, we also demonstrated that these anti-angiogenic factors are decreased in the metabolic-syndrome swine model. This dual action of NPY (up-regulation of pro-angiogenic proteins while down-regulating anti-angiogenic factors) may be essential when attempting to overcome the detrimental anti-angiogenic milieu that exists in patients with metabolic syndrome.

In our current experiment, we noted an increase in the NPY receptors in the non-ischemic LAD territory in the NPY treated animals. We hypothesize that this may be a paracrine effect of NPY, with significant up-regulation of $NPYR_2$ resulting in increased FGF2b and p-eNOS. By up-regulating these angiogenic factors, NPY facilitated recruitment of surrounding endothelial progenitor cells for ischemic area. The aforementioned paracrine effects of NPY have been observed in animal models of ischemic skeletal muscle and myocardial infarction [22]. The proliferation signaling factors (cycline A2, stromal cell-derived factor-1 and aurora B kinase) released by local infiltration of NPY caused activation of pro-angiogenic factors in adjacent non-ischemic tissue [38].

Considering the multi-faceted effects of NPY, the results of our study are unique but not surprising. As a neurotransmitter, NPY is ideally positioned at the beginning of a cascade with multiple downstream angiogenic, arteriogenic and neurogenic effects (Fig. 5) [16,18,39]. The lack of unequivocal success in focused clinical trials of utilizing cytokines such as VEGF, FGF, proangiogenic genes, and delivery of autologous stem cells is also suggestive of the complexity of the process of angiogenesis [3,8,40]. Increasing the angiogenic and cell survival signaling milieu via NPY_{3-36} may be is especially important in the presence of endothelial dysfunction in diabetics and patients with metabolic syndrome and its component diseases [41,42]. Furthermore, the inhibitory effect of NPY_{3-36} on angiostatic factors can possibly provide another benefit in this patient population.

By activating early in the cascade, exogenously administered NPY_{3-36} may also improve structural and functional recovery of the myocardium in older patients presenting with chronic myocardial ischemia, as the levels of NPY and associated angiogenesis is also decreased in this cohort of patients. Recently, it has been shown that NPY can trigger neonatal and adult cardiomyocytes into cell growth and division, and proliferation of already differentiated mesenchymal cells into angiomyogenesis when transplanted into an acute ischemic area [43]. This method of tissue recovery may be more effective in patients with type II diabetes and metabolic syndrome. Thus NPY, acting both as messenger and activator of multiple pathways and cell types, leads to angiogenesis, collateral formation and improved perfusion, promoting functional recovery.

Table 2
Growth and anti-angiogenic factor expressions. There was a significant increase in platelet-derived growth factor (PDGF) ($p=0.004$) in HCT LCx area but a significant down-regulation of the angiogenic protein fibroblast growth factor (FGF2b) ($p=0.04$) and phosphorylated endothelial nitric-oxide synthase (p-eNOS) ($p=0.001$). Angiostatin expression in the HCT LCx area was significantly downregulated when compared to both the LAD region ($p=0.02$) and the HCP group ($p=0.02$). Endostatin was significantly downregulated in the HCT LCx area when compared to the HCP group ($p=0.004$). All blots were normalized to total protein stain with Ponceau.

	HCP	HCT LCx	HCT LAD	p value (HCP and HCT LCx)	p value (HCT LAD and HCT LCx)
PDGF-b	43.5±6.1	72.3±12.3	59.8±13.0	0.004*	0.16
FGF2b	129.0±3.5	104.5±7.1	135.5±2.7	0.04*	0.26
p-eNOS	40.6±1.8	21.8±11.9	46.7±14.3	0.05*	0.001*
Angiostatin	30.8±19.3	5.5±4.9	14.5±4.8	0.02*	0.02*
Endostatin	86.1±10.3	43.8±19.3	44.3±10.2	0.004*	0.84

* $p<0.05$.

5. Limitations

Although we were able to show changes in physiological and molecular mechanisms in metabolic syndrome, the abbreviated time period given for the development of metabolic syndrome in our swine model may not completely mimic the chronic nature of these diseases in humans. Another limitation of our study is that we had a small number of animals, which was complicated by the demise of five animals. A few of our results may not have achieved statistical significance due to this. We do believe that the remarkable results of our study warrant a larger study. Also, we recognize that we did not directly study cardiomyocyte proliferation and hypertrophy. However, we had a strong immunohistochemical and functional evidence of myocardial benefit, which would argue for the validity of our results. Future studies should examine myocardial cell density, size, and contractile strength, directly. Finally, we examined protein signaling at only a single time point, which may not reflect protein levels over the course of angiogenesis. It remains unknown if longer periods of treatment time would result in greater benefit, or if the beneficial effects of NPY infiltration would diminish over time.

6. Conclusions

NPY is an important neuropeptide in cardiac sympathetic nerve terminals that activates multiple angiogenic promoting cell growth such as angiogenesis and arteriogenesis, through smooth muscle cell proliferation. This study demonstrated a successful use of NPY as an angiogenic treatment to increase arteriolar density, improve collateral-dependent perfusion, and improve myocardial function in the setting of metabolic syndrome. We believe that our results support that this was accomplished via an increase in angiogenic factors

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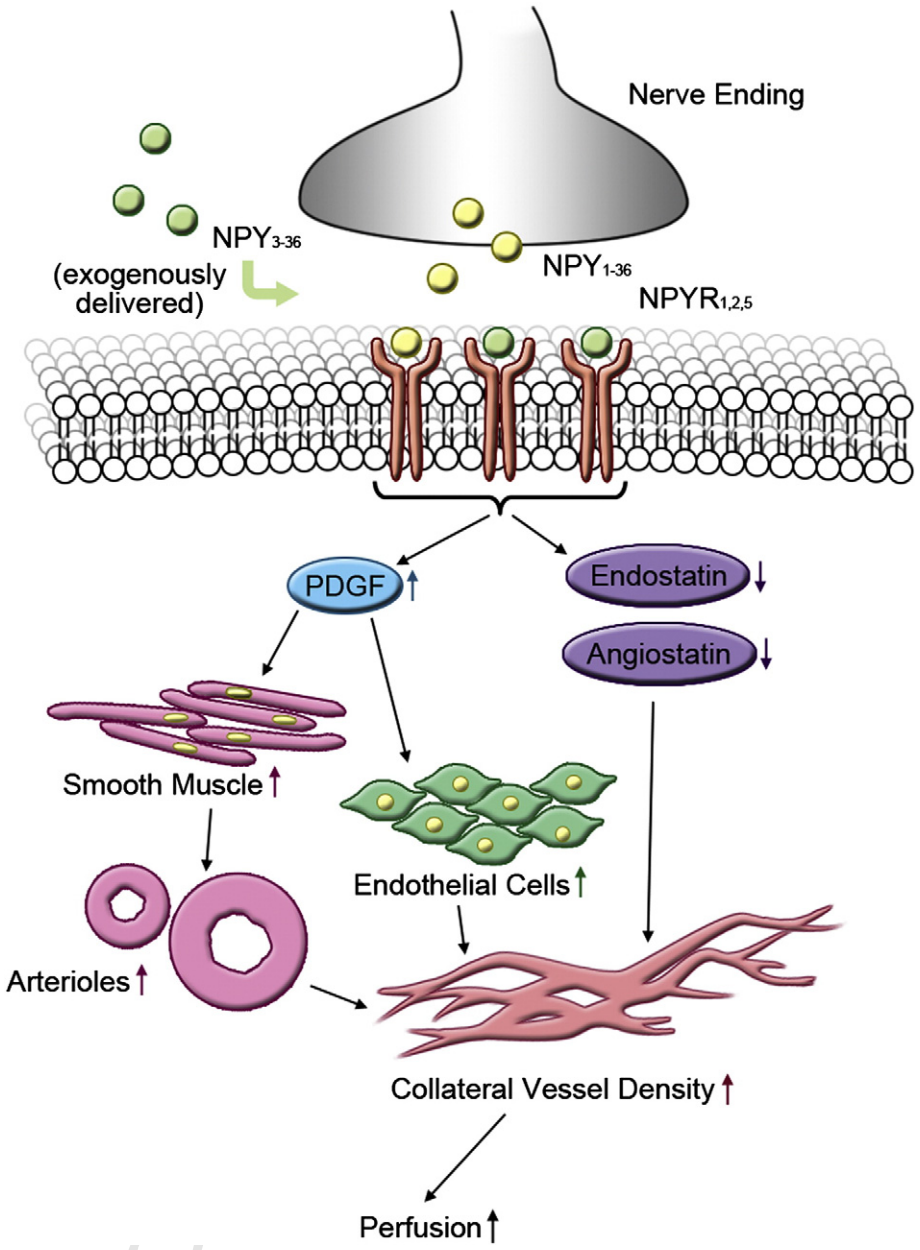

Fig. 5. A summary of the proposed mechanism for exogenous NPY_{3-36}-associated angiogenesis. NPY_{1-36} is released by sympathetic nerve endings and can act directly on NPY receptors. In the current experiment, the exogenous infiltration of NPY_{3-36} leads to activation of $NPYR_{1,2,5}$, causing the observed upregulation of PDGF and downregulation of angiostatin and endostatin. This results in the activation of endothelial cells, leading to capillary formation. Simultaneously, the proliferation and hypertrophy of smooth muscle cells lead to arteriole formation and the growth of collaterals.

and a decrease in anti-angiogenic factors. NPY receptors may provide an attractive target for the treatment of ischemic coronary disease in the setting of obesity, metabolic syndrome, and type II diabetes. This development, together with the recent advances in the drug delivery techniques such as nanotechnology, may pave the way for the generation of new classes of drugs to treat cardiovascular disease.

Disclosures

None.

Funding sources

Research was funded by the Department of Anesthesia and Critical Care at the Beth Israel Deaconess Medical Center and the National Institute of Health Training Grants 5T32-HL094300 (LMC) and T-32 HL007734 (MPR).

Acknowledgments

We acknowledge Varun Kumar, M.B.B.S., Lakshmi Gopalakrishnan, M.B.B.S., and Ujjwal Rastogi, M.B.B.S. for helping us to evaluate the images from the X-ray coronary angiography. We would also like to acknowledge Jayant Jainandunsing, MD for his help in manuscript preparation and Eitezaz Mahmood for his work in the laboratory.

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Please cite this article as: Matyal R., et al, Neuropeptide Y improves myocardial perfusion and function in a swine model of hypercholesterolemia and chronic myocardial ischemia, J Mol Cell Cardiol (2012), http://dx.doi.org/10.1016/j.yjmcc.2012.08.027