Neuropeptide Y–Induced Acceleration of Postangioplasty Occlusion of Rat Carotid Artery

Lijun Li, Edward W. Lee, Hong Ji, Zofia Zukowska

Objective—Attempts to restore blood flow through atherosclerotic vessels by angioplasty often result in restenosis. Because the role of nerves in this process is unclear, we investigated whether neuropeptide Y (NPY), a sympathetic cotransmitter with vascular mitogenic activities, contributes to postangioplasty restenosis.

Methods and Results—Carotid artery balloon angioplasty upregulated vascular expression of NPY and its processing enzyme (DPPIV/cd26) and receptors (Y1, Y2, Y5 mRNA and protein) within 6 to 24 hours and stimulated neointima formation and accumulation of NPY in platelets after 14 days. NPY pellets (1 to 10 μg/pellet for 14 days) inserted next to the injured artery elevated platelet and vascular NPY immunoreactivity to stress-like levels and dose-dependently augmented angioplasty-induced neointima. Strikingly, 10 μg NPY for 14 days led to vessel occlusion with an atherosclerotic-like lesion, with thrombus and neointima containing neovessels, macrophages, matrix, and lipids. Y1 or Y5 receptor antagonist completely prevented the effect of NPY and reduced angioplasty-induced neointima by 50%.

Conclusions—Angioplasty upregulates platelet and vascular NPY systems, which then contribute to neointima formation via Y1 and Y5 receptor activation. Increasing NPY to high stress levels triggers formation of a thrombotic atherosclerotic-like lesion and vessel occlusion. Thus, NPY may be a risk factor for accelerated atherosclerosis, and NPY receptor antagonists may be a possible new treatment for restenosis. (Arterioscler Thromb Vasc Biol. 2003;23:1204-1210.)

Key Words: neuropeptide Y ▪ NPY receptors ▪ atherosclerosis ▪ restenosis ▪ neointima

Atherosclerosis is a multifactorial, pathological process and a leading cause of ischemic vascular diseases. Despite identification of multiple genetic and environmental risk factors such as hyperlipidemia, obesity, and smoking, it remains poorly manageable by conventional therapy. The most commonly used procedures are surgical vascular interventions such as percutaneous transluminal coronary angioplasty. Although effective acutely, they often, however, result in restenosis of occluded arteries.3

Multiple growth factors have been implicated in restenosis,4 most of them thought to derive from mesenchymal cells. Surprisingly, the role of perivascular sympathetic nerves that richly innervate these vessels is less known. Sympathetic activity has been believed to promote atherosclerosis indirectly, either by its vasoconstrictive effects or by stimulating platelet aggregation or insulin resistance.6 However, these views have been rapidly changing in recent years because both norepinephrine7 and its cotransmitter, ATP,8 have been found to exert direct trophic actions on vascular smooth muscle cells (VSMCs) both in vivo and in vitro.

NPY is also a sympathetic and central cotransmitter, mediating pleiotropic activities such as stimulation of appetite and anxiolysis by activating multiple G\textsubscript{i/o}-coupled Y1-Y5 receptors.9-11 In the cardiovascular system, NPY cooperates with norepinephrine (NE) and causes, via Y1 receptors, a slow-onset, long-lasting vasoconstriction in many arteries, including coronary and cerebral.9-11 Plasma NPY levels are elevated by stress, such as cold exposure or treadmill exercise9,12,13 and, in patients with myocardial ischemia, congestive heart failure and hypertension.9,12,13 Recently, the vasoconstrictive Y1 receptor has also been identified as mediating proliferation of VSMCs in vitro.14,15

In addition to neurogenic, extraneuronal NPY synthesis has been identified in endothelial16 and immune cells17 and megakaryocytes/platelets.9,18-20 Similar to VSMCs, NPY potently promotes growth of endothelial and immune cells,14,16 but specific receptor subtypes mediating its effects appear to differ. For example, the angiogenic activity of NPY seems to be mediated not by Y1 but by Y2/Y5 receptors. The switch from the Y1-receptor–mediated vasoconstriction and VSMC mitogenesis to non–Y1-receptor–mediated angiogenic actions of NPY is provided by DPPIV,16 which converts NPY1-36 to NPY3-36, a Y2/Y5-selective agonist. The latter is also the second major form of circulating NPY.21

Recently, a common (6% to 14%) polymorphism of the signal peptide of the NPY gene with leucine(7)-to-proline(7)
substitution has been discovered in the European population. Interestingly, the mutation correlates highly with elevated total and LDL cholesterol levels and increased carotid artery intima-media thickening. Our experimental data, together with these human findings, led us to hypothesize that NPY exerts proatherosclerotic activity; its proliferative receptors are activated by vascular injury, which then results in VSMC proliferation and restenosis.

**Methods**

**Rat Carotid Artery Angioplasty**

Angioplasty was performed as described. In male anesthetized Wistar rats (350 to 375 g), a 2F Fogarty balloon catheter was inserted into the left common carotid artery through the left external carotid artery, and angioplasty was performed by retracting the balloon catheter 3 times. On catheter removal, the left external carotid artery was ligated, and blood flow was restored. As controls, intact unoperated and sham-operated (tying off the external carotid artery, without catheter insertion) rats were used. All procedures were approved by the Animal Care and Use Committees of Georgetown University and conducted in accordance with the National Institutes of Health guidelines.

**Drugs**

The Y1 (H409/22 acetate) and Y5 (CGP71683A) antagonists (60 mg/mL each, gifts from AstraZeneca, structures identical to BIBP32232 and the Novartis compound, respectively, with established specificity) or vehicle (60% PEG-400) were delivered at 0.02 μmol/kg per min for 14 days into the right jugular vein via osmotic minipumps. These concentrations (0.1 μmol/L) of Y1 and Y5 antagonists blocked proliferation of rat aortic VSMC in response to 0.01 μmol/L NPY in vitro. NPY-containing (1 to 10 μg/pellet for 14 days) or placebo-containing pellets were inserted during angioplasty at the site of the left carotid artery (or contralateral vessels in some rats) and secured in place with a neighboring muscle.

**Morphometry**

After 14 days, in reanesthetized and artificially respired rats, both common carotid arteries were harvested and fixed. Tissues were paraffin-embedded and stained with H&E or Masson’s trichrome for morphometry. Intima was calculated as the area luminal from the internal lamina, and media as area between external and internal lamina (averaged from 15 arterial sections and imaged) (Optimax-Nikon computer with NIH image) (additional information available online on http://atvb.ahajournals.org).

**Histocytochemistry and Immunocytochemistry**

Tissue sections were deparaffinized and stained with primary antibodies against the endothelial markers, cd31 and von Willebrand factor (vWF), the macrophage scavenger receptor (cd68), and hyaluronan, a marker for matrix deposition (HA). Additionally, sections were stained with Oil Red O to identify lipid deposition and Masson’s Trichrome to identify matrix and neovascularization (additional information online).

**Reverse Transcriptase–Polymerase Chain Reaction**

Both common carotid arteries were harvested, samples were immediately snap frozen, and total RNA was isolated using TRI-Reagent. cDNA was synthesized with random hexamer and MMLV reverse transcriptase, and 18s rRNA served as an internal control. NPY, DPPIV, and NPY-Y1-Y5 receptor primer sequences are provided online. Polymerase chain reaction (PCR) was performed using Taq DNA polymerase (Promega) as described. After electrophoresis, the products were visualized by ethidium bromide staining.

**Western Blotting**

Carotid arteries were homogenized in RIPA buffer and centrifuged, and supernatants were collected. Protein samples were resolved on a Tris-Glycine gel and transferred onto a nitrocellulose membrane, and blots were blocked with nonfat milk in TBST buffer and incubated with rabbit polyclonal anti-human Y1, Y2, or Y5 antibodies (Astra-Zeneca). The membranes were then washed with TBST buffer, incubated with horseradish peroxidase–conjugated anti-rabbit IgG antibody (Amersham Pharmacia Biotech), and visualized using ECL reagents (Amersham Pharmacia Biotech) and visualized by autoradiography (additional information online).

**NPY Immunoreactivity**

NPY immunoreactivity (NPY-ir) was measured using ELISA in platelet-poor plasma (PPP) and platelet-rich plasma (PRP), carotid arteries, and neighboring skeletal muscle from intact, angioplasty-subjected (with and without NPY pellet), and stressed rats (placebo treatment and cold stress) (additional information online). Plasma NPY-ir levels in PPP and PRP were measured under the influence of angioplasty (angioplasty) or placebo (placebo) (angioplasty) or placebo (placebo).

**Statistical Analysis**

Results were analyzed by one- and two-way ANOVA, and, where appropriate, a post hoc Dunnett’s t test was used. Data are presented as mean±SEM for indicated number of repetitions and were considered significant at P<0.05.

**Results**

**NPY-ir Levels in Plasma and Carotid Artery**

Fourteen days after angioplasty, plasma NPY-ir levels in PPP remained unchanged compared with intact rats (Figure 1A). In PRP, however, plasma NPY-ir levels were significantly increased in the rats treated with angioplasty+placebo compared with the unoperated rats (P<0.01, Figure 1B). This increase tended to be even greater in the angioplasty+NPY-treated rats (P<0.01 compared with intact, Figure 1A).
NPY-ir levels in PRP in angioplasty + NPY-treated rats were comparable to those attained during 2-hour 4°C cold water stress (Figure 1A). Stress, unlike angioplasty, also increased NPY-ir levels in PPP (Figure 1A).

Angioplasty in the placebo-treated rats did not affect NPY-ir levels in the carotid artery compared with the contralateral uninjured vessels from the same rats. However, angioplasty-injured NPY-treated carotid arteries had significantly elevated NPY-ir levels ($P<0.01$, Figure 1B) compared with intact arteries. NPY-ir content in the muscle around the NPY-treated (0.00427 ± 0.00082 ng/mg), but not placebo-treated, angioplasty-injured artery was significantly higher ($P<0.01$) than in their contralateral muscle surrounding the uninjured right carotid artery (0.00280 ± 0.00081 ng/mg).

NPY-Induced Neointima Formation and Media Thickening

Fourteen days after angioplasty, there was a significant neointima formation in the carotid artery, whereas no changes were seen in the contralateral uninjured vessel (Figures 2A and 2B). NPY (1 to 10 μg for 14 days) dose-dependently increased neointima formation in the angioplasty-injured artery (from 0.048 ± 0.002 mm$^2$ with placebo to 0.112 ± 0.008 mm$^2$ with 1 μg of NPY, $P<0.05$, or 0.347 ± 0.015 mm$^2$ with 10 μg of NPY, $P<0.01$, Figures 2B through 2D). Unlike in the angioplasty + placebo group, the NPY-induced neointima was not laminar, and at the lower NPY dose, neointima grew into the lumen and divided vessels into 2 (Figure 2C).

Angioplasty in the placebo-treated rats also significantly increased media thickness of the injured (0.074 ± 0.003 mm$^2$) compared with those of the contralateral uninjured arteries from the same rats (0.050 ± 0.002 mm$^2$, $P<0.01$) or intact vessels of the sham-operated rats (0.048 ± 0.001 mm$^2$, $P<0.01$). NPY treatment of the uninjured arteries also caused significant media thickening (0.084 ± 0.005 mm$^2$) compared with the untreated vessels (0.050 ± 0.002 mm$^2$, $P<0.01$).

NPY-Induced Formation of an Atherosclerotic-Like Lesion

In addition to neointima, NPY also stimulated formation of thrombus (Figures 2D, 3A, and 3B). NPY-induced lesion was additionally characterized using cell-specific markers and histocytochemistry. Continuous, vWf-positive (endothelial) staining was found in the endothelial layer of the uninjured vessels (Figure 3A). In contrast, in the angioplasty + placebo-treated vessels, vWf-positive staining was patchy, indicating noncontinuous regenerated endothelium (Figure 3A). In response to NPY, massive vWf-positive staining and numerous cd31-positive microvessels were identified within the neointimal lesion and thrombus (Figures 3A and 3C), indicating neovascularization. This was confirmed in Masson’s trichrome staining, which visualized the presence of red blood cell–containing microvessels (red-stained cells, Figure 3B) within neointima of the NPY-treated but not placebo-treated injured arteries. Masson’s trichrome staining also revealed increased matrix deposition (blue staining, Figure 3B), also indicated by increased hyaluronan staining in the NPY-treated but not placebo-treated injured vessels (Figure 3D).

Accumulation of macrophages and lipids in human atherosclerotic plaques has been a key marker for atherosclerosis. Figure 3E shows a significant increase in cd68-positive staining for macrophages in the neointimal lesion of the NPY-treated, but not placebo-treated, injured or uninjured vessels. In the same lesion area, in NPY-treated vessels, there was also a marked increase in the Oil Red O staining, indicating lipid deposition absent in vessels from other groups (Figure 3F).

NPY Receptor Expression

Intact rat carotid arteries showed low Y1 receptor expression by reverse transcriptase–PCR and Western blot analysis and also expressed NPY itself and DPPIV mRNA (Figures 4A and 4B). Six and 24 hours after angioplasty, the Y1, Y2, and, to a lesser extent, Y5 receptor mRNAs and proteins became
upregulated, as compared with sham-operated or intact carotid arteries (Figures 4A and 4B). In contrast, at the same time (6 to 24 hours), DPPIV mRNA became downregulated in the angioplasty-injured placebo-treated arteries (Figure 4A). Expression of Y1 and Y5, but not Y2, receptor expression persisted in the injured vessels until 14 days after angioplasty when, additionally, vessels regained expression of DPPIV mRNA (Figure 4A).

Administration of the NPY pellet did not change the pattern of the NPY receptor and DPPIV expression except that the Y5 mRNA at 6 (not shown) and 24 hours after angioplasty and DPPIV mRNA at 14 days after angioplasty appeared stronger than in the placebo-treated rats (Figure 4A).

Effects of Y1 and Y5 Receptor Antagonist on Neointima and Media Thickening

Angioplasty-induced neointima formation was reduced by 50% by the Y1 receptor antagonist (H409/22 acetate, 0.02 μmol/kg per min for 14 days, IV, \(P<0.01\), Figure 5) and by 40% by the Y5 antagonist (CGP71683A, 0.02 μmol/kg per min for 14 days, IV, \(P<0.01\), Figure 5). These same receptor antagonists, alone or combined, completely prevented the NPY-induced vessel occlusion and neointimal thickening, although the Y1 antagonist was significantly more effective than the Y5 antagonist (\(P<0.05\), Figure 5). Also, only the Y1 receptor antagonist reduced media thickening in the injured vessels in the placebo-treated group (from 0.074±0.003 to 0.046±0.002 mm², \(P<0.01\)).

Discussion

This is the first report that balloon angioplasty causes early and persistent activation of the NPY growth-promoting receptor system in injured rat carotid arteries as well as peptide accumulation in platelets. To our knowledge, our study also provides the first description of an animal model leading to rapid vascular occlusion with an atherosclerotic-like lesion in normal rats without prior genetic or dietary modification of lipid metabolism. The mechanism of NPY-induced formation of this occlusive atherosclerotic-like lesion seems to be multifactorial.

First, NPY has been shown before by us\(^{14}\) and others\(^{29}\) to stimulate VSMC proliferation. In vitro, NPY is a potent mitogen—its activity for rat and human VSMC starts at sub-pM concentrations—and elicits a bimodal response by activating multiple receptors, Y1 and Y5, which seem to exist in high- and low-affinity states.\(^{14}\) The sensitivity of VSMCs to the proliferative effects of NPY is augmented by intense β-adrenergic receptor activation.\(^{14}\) Reciprocally, NPY cooperates synergistically with other sympathetic neurotransmitters, norepinephrine, and ATP, all of which were shown in vitro to potentiate each other’s actions.\(^{29}\)

In the present study, we used a slow-release pellet to administer NPY at the injury site. The 1- to 10-μg pellets,
delivering NPY over 14 days, increased NPY-ir levels in the vessel wall and neighboring muscle (used to support the pellet) 2.5-fold and without altering circulating peptide levels in PPP. In contrast, angioplasty alone or with NPY markedly elevated NPY-ir in PRP to levels similar to those yielded by high-intensity cold stress in unoperated rats. A rise of NPY-ir in platelet-rich, but not platelet-poor, plasma suggests accumulation of NPY in platelets adhering at the site of endothelial injury. The latter is known to augment platelet aggregation attributable to removal of antiaggregatory factors, such as nitric oxide and prostacyclin, and platelet uptake of other circulating peptides, ie, cardiac natriuretic peptide, has also been reported.

Alternatively, the angioplasty-induced rise in platelet NPY may be attributable to upregulation of de novo peptide synthesis in megakaryocytes. Earlier, we and others reported that in rats and some strains of mice, NPY is synthesized in megakaryocytes and released from platelets during secondary aggregation. NPY may be proaggregatory, because it potentiated aggregation to subthreshold doses of collagen in rat platelets, and higher platelet NPY levels were found to associate with greater aggregability in spontaneously hypertensive rats. Because levels similar to those in spontaneously hypertensive rats were found in our study, this may suggest that increased platelet NPY augments platelet aggregability, which in turn promotes thrombus formation.

Thrombus formation per se may additionally accelerate neointima formation by platelet secretion of vascular mitogens such as platelet-derived growth factor. Conceivably, NPY could also act indirectly to promote thrombus formation by changing vessel rheology. However, this mechanism appears insufficient, because thrombus did not form in vessels treated with 1 µg NPY, even though they were more obstructed than the placebo-treated injured vessels. Furthermore, a direct proaggregatory effect of NPY is suggested by the massive secretion of vWF in vessels treated with 10 µg NPY. vWF facilitates binding of platelet surface glycoprotein Ibα to collagen and mediates platelet adhesion to injured vascular wall, and its elevated levels have been linked to atherosclerosis. Thus, NPY-mediated stimulation of vWF expression and thrombus formation within the injured vessel

Figure 4. Expression of NPY, its receptors, and DPPIV in rat carotid artery after angioplasty by RT-PCR (A) and Western blot analysis (B). C, Effect of NPY (10 µg/pellet for 14 days) on the NPY receptor system expression after angioplasty (RT-PCR); representative images from n=6 each group; 18s RNA used as internal control.

Figure 5. Effects of Y1 (H409/22) and Y5 (CGP71683A) receptor antagonists, both at 0.02 µmol/kg per min for 14 days, IV, on neointima formation of rat carotid artery after angioplasty in placebo-treated and NPY-treated (10 µg/pellet for 14 days) vessels. *P<0.05; **P<0.01; ***P<0.001, as indicated; ‡P<0.01 compared with placebo/(-) antagonist group (n=6).
NPY in adipocytes. 45

by marked hyaluronan immunostaining40 in neointima from
storage in adipocytes. 45 The latter action is not only of central
origin 46 but results from the strong antilipolytic action of
Loss of DPPIV may then augment NPY-Y1 receptor
36, which is inactive at Y1 but activates Y2/Y5 receptors. 21
induced by angioplasty. DPPIV converts NPY1-36 to NPY3-
lipid, its disappearance suggests successful deendothelization
of angioplasty-injured vessels. Formation of
matrix, which is a major factor contributing to vascular
occlusion in both experimental and human atherosclerosis,34,41
could represent another mechanism for NPY-induced
atherosclerotic-like lesion.

Although organized, vascularized thrombus is a hallmark
of advanced atherosclerosis, transmigration of monocytes/
macrophages, lipid deposition, and foam cell formation are
prerequisites for its early stages.4 In humans, development of
advanced atherosclerotic plaques usually occurs over many
years of life, and to be reproduced in animals, major di-
etary2,24 or genetic modifications of lipid transport systems
such as apolipoprotein E42 or LDL receptors43 are required.
To our knowledge, no animal model has been described in
which atherosclerotic lesions were formed within normol-
penic environment. Surprisingly, neointimal lesion induced
by NPY, but not angioplasty alone, was highly positive for
cd68-immunostaining, indicating macrophage infiltration. At
similar locations, NPY-treated angioplasty-injured vessels
also showed marked lipid deposition, indicating formation of
foam cells. The mechanisms of the effects of NPY on
macrophages and lipid deposition remain to be determined.
Previously, however, NPY was shown to activate monocyte
migration and phagocytosis44 and also to promote lipid
storage in adipocytes.35 The latter action is not only of central
origin36 but results from the strong antilipolytic action of
NPY in adipocytes.45

Supporting the activities of NPY, the endogenous vascular
NPY receptor system was found to be activated by angioplas-
y. Expression of Y1, Y2, and Y5 receptors increased as
early as 6 to 24 hours after angioplasty (both mRNAs and
proteins), whereas expression of DPPIV became downregu-
lated. Because DPPIV is constitutively expressed in endothe-
lium, its disappearance suggests successful deendothelization
induced by angioplasty. DPPIV converts NPY1-36 to NPY3-
36, which is inactive at Y1 but activates Y2/Y5 receptors.21
Loss of DPPIV may then augment NPY-Y1 receptor–medi-
ated VSMC proliferative activities.14 These changes, both
DPPIV and Y5 mRNA expression, appeared stronger after
NPY treatment, although semiquantitative RT-PCR did not
allow for real quantification.

By 14 days after angioplasty, DPPIV mRNA expression
was regained by injured vessels, suggesting reendothelization
of angioplasty-injured vessels, also indicated by partial rest-
oration of vWF-positive cells of the innermost layer of the
neointima. Finally, angioplasty-induced upregulation of Y2
receptors and DPPIV, particularly strong in the context of
NPY, may contribute to neointima formation by promoting
NPY-mediated neovascularization.16

Alone or combined, Y1 and Y5 receptor antagonists
(H409/22 acetate and CGP71683A, respectively), which
block NPY-mediated VSMC proliferation in vitro,14 reduced
the angioplasty-induced neointima formation by 40% to 50%.
Even more remarkably, the effect of NPY was entirely
prevented when treated with either Y1 or Y5 receptor
antagonist. The Y1 antagonist was more effective than the Y5
antagonist in inhibiting NPY-induced neointima and was the
only one to prevent media thickening. The latter findings
support previous reports of NPY-Y1 receptor involvement
in medial hypertrophy in hypertension.10 Thus, it seems that
the Y1 receptor plays a major role in medial VSMC growth but
both Y1 and Y5 receptors cooperate in NPY-induced neo-
tima formation.

Why the antagonists of either receptor similarly inhibit
angioplasty-induced neointima formation but their effect was
not additive remains to be determined. One possibility is
receptor heterodimerization, as shown for an increasing
group of G-protein–coupled receptors, for example angio-
tensin receptors.47 All of the NPY receptors are G<sub>i/o</sub>-coupled
and share similar signaling, including inhibition of adenylyl
cyclase activity and phosphorylation of mitogen-activated
protein kinase.48 NPY receptor heterodimerization is pres-
ently being investigated.

In summary, angioplasty of the rat carotid artery increases
the platelet content of NPY and activates the vascular
NPY-Y1/Y5 receptor system, which then contributes to
≈50% of neointima formation. Increasing NPY vascular
content within a pathophysiological range such as yielded by
intense stress rapidly occludes angioplasty-injured vessels
with an atherosclerotic-like lesion containing vascularized
neointima, thrombus, matrix, macrophages, and lipids. Thus,
NPY, a neurotransmitter from the sympathetic nerves, is a
new factor that can accelerate atherosclerosis and mediate
restenosis after vascular interventions. Inhibitors of NPY-Y1
and -Y5 receptors may, therefore, be potential new ways to
treat restenosis and ischemic cardiovascular diseases. Such a
treatment may particularly benefit patients with elevated
NPY levels subjected to chronic stress16 or carriers of the
NPY signal peptide polymorphism, which apparently makes
the peptide more releasable and is associated with accelerated
atherosclerosis.16

**Acknowledgments**

This work was supported by grants to Zofia Zukowska from AstraZeneca and NIH-HL55310.

**References**

503–516.


3. Fischman DL, Leon MB, Baim DS, Schatz RA, Savage MP, Penn I, Detre
coronary-stent placement and balloon angioplasty in the treatment of
coronary artery disease. Stent Restenosis Study Investigators. N Engl


