Chronic Stress Induces Rapid Occlusion of Angioplasty-Injured Rat Carotid Artery by Activating Neuropeptide Y and Its Y1 Receptors

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Objective—We reported previously that neuropeptide Y (NPY) induces an atherosclerotic-like lesion that is significantly reduced by NPY-Y1 and NPY-Y5 receptor (R) inhibitors. Because antagonists also inhibit neointima induced by angioplasty alone, we now test whether stress-induced endogenous NPY release mimic these changes.

Methods and Results—Rats were nonstressed or stressed (4°C water; 2 hours per day for 14 days) starting immediately before and continuing after carotid artery angioplasty. Stress acutely and chronically increased blood pressure and doubled plasma NPY levels. After 14 days, angioplasty-induced neointima was markedly greater in stressed (than nonstressed) rats, in which most of the vessels became occluded with an atherosclerotic-like lesion containing macrophages, lipids, thrombus, and microvessels that was similar but more inflammatory than the injury in the NPY-treated vessels. Fourteen days after angioplasty combined with stress or NPY, Y1R and Y5R (mRNA and protein) became upregulated in areas of neointima, microvessels, and macrophages in injured carotid arteries. Stress- and NPY-induced changes were completely prevented by a selective Y1R antagonist (0.02 μmol/kg per minute for 14 days), whereas neointima induced by angioplasty alone was reduced by 60%.

Conclusions—Because of sympathetic NPY release, stress may be a less-than-appreciated risk factor for restenosis/atherosclerosis, and Y1R antagonists a potential therapy for these conditions. (Arterioscler Thromb Vasc Biol. 2005;25:2075-2080.)

Key Words: stress ■ neuropeptide Y ■ Y1 receptor antagonist ■ atherosclerosis ■ angioplasty

Atherosclerosis is a multifactorial chronic disease for which many risk factors have been identified. A currently favored view is that it is an inflammatory condition with migration of monocytes/macrophages into arterial wall being an important early event in atherogenesis. Inflammation is also implicated in the development of restenosis after angioplasty. The role of the sympathetic nerves in atherosclerosis and restenosis is acknowledged but believed to be indirectly involved via vasoconstrictive effects, stimulation of platelet aggregation, or insulin resistance and are attributed primarily to adrenergic transmitters. However, sympathetic nerves also contain nonadrenergic comediators that sometime amplify or antagonize these catecholamines.

One of these cotransmitters is neuropeptide Y (NPY). It cooperates with norepinephrine (NE) in regulation of vascular tone as a vasoconstrictor and an amplifier of NE-induced actions; but unlike NE, NPY is also a potent vascular mitogen and an angiogenic factor. Unlike NE, the release, action, and inactivation of which are rapid, NPY is released after more intense and prolonged nerve activation and causes a slow-onset, long-lasting vasoconstriction via its Y1Rs. The same Y1 receptor in cooperation with Y5R mediates mitogenic activities in vascular smooth muscle cells. Previously, we reported that a low physiological dose of NPY induces a rapid occlusion of the vessel with an neointimal lesion containing macrophages, lipids, thrombus, matrix, and microvessels after angioplasty. These lesions resembled advanced atherosclerotic plaques despite being formed in rats devoid of any lipid or metabolic abnormalities, and were prevented by continuous infusion of either Y1 or Y5R antagonists, each of which also inhibited neointima induced by angioplasty alone by 50%. Interestingly, our experimental data corroborated findings in humans of an association of a NPY signal peptide gene polymorphism, which leads to more stress-releasable peptide, with accelerated atherosclerosis in a Northern European population. These observations led us to hypothesize that endogenous nerve-released NPY plays a major role in vascular remodeling after angioplasty. Thus, stress, by releasing NPY, may be a risk factor for accelerated restenosis. This notion, although well supported by epidemiological and clinical evidence suggesting a link between stress and sudden cardiovascular events, has not been scrutinized because of the fact that psychological stress is poorly defined and quantifiable. In our studies, we used our established model of stress with rats...
standing in 1-cm ice-cold water, mimicking natural stress within their habitat (eg, in the northern hemisphere). Using this paradigm, we found for the first time that chronic stress is a powerful stimulus accelerating rat carotid artery restenosis after balloon angioplasty, and this effect is prevented by an antagonist to the NPY-Y1 receptor.

**Methods**

**Balloon Angioplasty and Chronic Cold Stress Model**

Male Wistar rats (350 g) were subjected to angioplasty and were either stressed (group 1; n=18) or nonstressed (group 2; n=36). Stress consisted of placing 1 rat per cage in 1-cm 4°C water on the floor for 2 hours. The first stress started 2 hours before angioplasty and then was repeated daily for 14 days. Group 2 was a nonstressed control, subjected to angioplasty alone and angioplasty plus NPY. Rats were anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg IP), and balloon angioplasty (2F Fogarty; Baxter) in the left common carotid artery was performed. All stressed (n=18) and some nonstressed (n=18) rats received a slow-release pellet containing placebo while the remaining nonstressed rats received NPY pellet (10 µg/14 days; n=18), each placed near the injured artery immediately after angioplasty. One third of the rats from both groups were treated with a specific Y1 receptor antagonist (H409/22) and the remaining with vehicle (60% polyethylene glycol-400) at 0.02 µmol/kg per minute for 14 days by osmotic minipump (Alzet) connected via a catheter in the jugular vein during angioplasty. Arterial blood pressure was recorded (Buxco Electronics, Inc), and blood samples were collected from the femoral artery during anesthesia before and after the experiment. In the stressed rats, these measurements were made immediately after the last stress session. For the effects of an acute vs chronic stress, an additional group of rats (n=12) was stressed once, and blood pressure and blood samples for NPY were taken as described. All procedures were approved by the animal care and use committees of Georgetown University.

**Histology, Morphometry, and Immunohistochemistry**

Blood vessels were perfused <120 mm Hg pressure with 0.9% normal heparinized (50 U/mL) saline. Both common carotid arteries were harvested and fixed in 4% paraformaldehyde or 10% neutral buffered formalin for paraffin sections or acetic acid for frozen sections with Tissue-Tek embedding and cut into 3 segments. For morphometry, 5-µm-thick carotid artery cross-sections were stained with hematoxylin-eosin or Masson’s trichrome. The areas of the neointima and media were measured using NIH Image software. The results were averaged from 15 segments in 3 segments for each vessel (n=6 each). To identify lipid deposition, arterial sections were stained with Oil Red O (Fisher Biotech BP112–10). Immunostaining was used to identify macrophages using CD68 antibody (Dako Cytomation), microvessels using an endothelial marker CD31 (platelet-endothelial cell adhesion molecule-1; BD Pharmingen), and Y1 and Y5 receptor antibodies (gift from AstraZeneca, and the NPY pellets were purchased from the Innovative Research of America).

**Platelet and Plasma Levels of NPY Immunoreactivity**

NPY immunoreactivity (NPY-ir) levels were measured by ELISA (Peninsula Labs Inc.) in blood separated into 2 plasma fractions, as described: platelet-rich plasma (PRP) and platelet-poor plasma (PPP). PRP and PPP were prepared by sequential centrifugation and used to prepare platelet-poor plasma (PPP). PRP and PPP were prepared by sequential centrifugation and used to prepare platelet-poor plasma (PPP). PRP and PPP were prepared by sequential centrifugation and used to prepare platelet-poor plasma (PPP). PRP and PPP were prepared by sequential centrifugation and used to prepare platelet-poor plasma (PPP). PRP and PPP were prepared by sequential centrifugation and used to prepare platelet-poor plasma (PPP).

**RNA Extraction and RT-PCR**

Both common carotid arteries were harvested, snap-frozen, and stored at −80°C for RNA analysis using semiquantitative RT-PCR. Total RNA was extracted from injured and uninjured carotid arteries from all groups (n=6 each) using TRI-Reagent (Molecular Research Center, Inc). cDNA was synthesized with random hexamer by reverse transcriptase (MMLV-RT; Perkin–Elmer) and amplified with TaqDNA polymerase (Promega) for 40 cycles. The 18s rRNA primers (Ambion, Inc.) served as an internal control. NPY, Y1, and Y5 receptor and dipeptidyl peptidase IV (DPP IV) primer sequences were provided online and published. PCR products were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining.

**Results**

**Plasma NPY-ir Responses to Angioplasty and Stress**

As reported, angioplasty alone doubled NPY-ir levels in PRP. In the present study, the angioplasty alone, in nonstressed rats, significantly increased plasma NPY-ir levels compared with time before angioplasty in both fractions of plasma, in PRP (from 12.1±1.1 to 28.5±2.04 ng/mL; P<0.001) and PPP (from 1.8±0.21 to 6.96±0.38 ng/mL; P<0.001; Figure IA, available online at http://atvb.ahajournals.org). Addition of the NPY pellet did not further augment the angioplasty-mediated changes in NPY-ir in PPP but increased it in PRP (to 39.8±4.9 ng/mL; P<0.05), whereas stress increased NPY-ir levels in PRP (to 64.6±7.7 ng/mL; P<0.001; Figure IA). Continuous infusion of the Y1 antagonist into rats subjected to stress and angioplasty decreased NPY-ir levels in PRP (to 34.7±4.7 ng/mL; P<0.01) and PPP (to 3.02±0.78 ng/mL; P<0.01; Figure IA).

**Angioplasty- and Stress-Induced Pressor Changes**

To determine the effect of acute stress alone, a separate group of rats was subjected to a single exposure to cold water stress before angioplasty. Acute cold stress increased mean arterial pressure (MAP) to 137±4.16 mm Hg (P<0.01; Figure IB) compared with that of the nonstressed rats (106.3±4.6 mm Hg). Angioplasty alone had no effect on blood pressure. However, in angioplasty-treated rats, chronic stress increased MAP even higher (151±5.0 mm Hg) compared with that of nonstressed (122±13 mm Hg; P<0.01; Figure IB). Infusion of the Y1 receptor antagonist did not significantly lower MAP increase induced by stress and angioplasty (Figure IB).

**Angioplasty- and Stress-Induced Vascular Remodeling**

A marked neointima was formed in the carotid arteries 14 days after angioplasty (0.089±0.01 mm²), which was significantly reduced (P<0.05; Figure 1) by the Y1 receptor antagonist. Treatment with the NPY pellet increased the angioplasty effect on neointima formation to 0.35±0.02 mm² (P<0.001; Figure 1), and chronic stress had a similar effect (P<0.001; Figure 1). NPY- and stress-induced increases in neointimal areas were similarly inhibited by the Y1R antagonist to 0.08±0.005 mm² and 0.043±0.004 mm², respec-
tively ($P<0.001$ both, compared with their vehicle-treated counterparts; Figure 1, top).

In the placebo-treated rats subjected to angioplasty, media thickness of the injured arteries increased compared with uninjured contralateral arteries (0.070±0.007 and 0.050±0.002 mm², respectively; $P<0.05$; data not shown in Figure 1). Angioplasty-induced medial hypertrophy was significantly reduced by the Y1 receptor antagonist ($P<0.01$; Figure 1; bottom). Neither NPY treatment nor stress induced any further changes in the media thickness, but in both groups, Y1 receptor antagonist decreased medial area significantly ($P<0.05$ for NPY and stress groups; Figure 1).

Stress-Induced Occlusion of Injured Carotid Artery With an Atherosclerotic-Like Lesion
We next characterized vascular lesions induced by angioplasty, chronic stress, and NPY for the presence of neointima, thrombus, macrophages, lipids, and neovascularization (Figure 2). Vessels injured with angioplasty alone remained patent, had no thrombus, and showed only sporadic CD68(+) cells (macrophages; Figure 2G) inside the neointimal layer and no new microvessels (Figure 2J). In contrast, all NPY-treated or stress-exposed injured arteries were occluded with lesions that contained smooth muscle cells, thrombus, marked lipid deposition, CD68(+) macrophages (Figure 2H and 2I), and multiple CD31(+) neovessels (Figure 2K and 2L). Deposition of lipids (Oil Red O staining; Figure 2E and 2F) localized to areas of macrophage accumulation within the occlusive neointima, and often, CD68(+) macrophages surrounded capillaries (Figure 2H and 2I). Unlike NPY, stress induced greater swelling of the adventitial layer in angioplasty-injured arteries, with increased matrix (blue staining; Masson’s trichrome; Figure 2B and 2C), higher density of CD31(+) microvessels, and increased overall density of immune cells. In some vessels, immune cell would spread through the external lamina, leading to its dissolution and cell transmigration into the media and neointima (Figure 2C).

Changes in NPY Receptor mRNA Expression
Fourteen days after angioplasty, the injured and intact carotid arteries expressed different patterns of NPY receptors. Uninjured contralateral arteries remained patent, showed no thrombus, and had only sporadic CD68(+) cells (macrophages; Figure 2G) inside the neointimal layer and no new microvessels (Figure 2J). In contrast, all NPY-treated or stress-exposed injured arteries were occluded with lesions that contained smooth muscle cells, thrombus, marked lipid deposition, CD68(+) macrophages (Figure 2H and 2I), and multiple CD31(+) neovessels (Figure 2K and 2L). Deposition of lipids (Oil Red O staining; Figure 2E and 2F) localized to areas of macrophage accumulation within the occlusive neointima, and often, CD68(+) macrophages surrounded capillaries (Figure 2H and 2I). Unlike NPY, stress induced greater swelling of the adventitial layer in angioplasty-injured arteries, with increased matrix (blue staining; Masson’s trichrome; Figure 2B and 2C), higher density of CD31(+) microvessels, and increased overall density of immune cells. In some vessels, immune cell would spread through the external lamina, leading to its dissolution and cell transmigration into the media and neointima (Figure 2C).
jured vessels expressed only Y1 receptors, NPY and DPPIV, an endothelial protease that cleaves mature NPY and forms a Y2/Y5-prefering agonist (Figure 3). After angioplasty in all groups, injured carotid arteries showed induction of the Y5 receptor, which was undetectable in uninjured vessels and became even more pronounced in NPY-treated rats. In addition, stress combined with angioplasty upregulated Y1 receptor and NPY expression compared with the intact or angioplasty-injured vessels. NPY and stress appear to upregulate DPPIV mRNA compared with the effect of angioplasty. Additionally, there was a weak induction of the Y2 receptor expression in the angioplasty- and NPY-treated arteries but not in others (Figure 3).

Changes in Y1 and Y5 Receptor Immunostaining
Receptor proteins were studied using specific NPY receptor antibodies. Uninjured rat carotid arteries of nonstressed rats showed most positive immunostaining for the Y1 receptor in the media and less in adventitia (Figure 4A) but had no or very low Y5-positive staining in the media (Figure 4G). In stressed rats, uninjured carotid arteries showed significantly higher Y1 and Y5 immunostaining (Figure 4B and 4H, respectively) compared with nonstressed rats.

Following in all angioplasty-injured vessels, expression of Y1 and Y5 receptor shifted from the media–adventitia layer to be most prominent in the neointima (Figure 4C through 4F and 4l through 4L, respectively). In the placebo-treated vessels, angioplasty (Figure 4C and 4L, respectively) significantly increased the density of Y1 and Y5 receptors in the neointima and media compared with the uninjured arteries. NPY treatment (Figure 4D) and stress (Figure 4E and 4F) alike, when combined with angioplasty, induced marked increase in the density of Y1 immunostaining in the neointima, in the areas of vascular smooth muscle cells and macrophage infiltration, and in the endothelial cells of microvessels. The Y5 receptor expression was also increased in the neointima (in smooth muscle cells and macrophages and microvessels) by NPY (Figure 4J) and stress (Figure 4K and 4L), and these increases were significantly greater than those induced by angioplasty alone (Figure 4I).

Discussion
Our study is the first report of pronounced effects of chronic stress leading to augmentation of postangioplasty neointima formation and vessel occlusion in rats without metabolic abnormalities. Exposure to cold stress caused rapid development of an occlusive neointimal atherosclerotic-like, thrombotic, and inflammatory lesion in carotid arteries after angioplasty. These changes mimic those induced by a local periarterial administration of an NPY slow-release pellet. Most important, NPY- and stress-induced vascular occlusions are prevented by an infusion of a specific Y1 receptor antagonist.

The permissive role of stress on atherosclerosis has been generally acknowledged by the lay public and speculated on by cardiologists and scientists. However, to our knowledge, this notion has not been directly tested in an animal model, undoubtedly because of the fact that stress, particularly psychological, is poorly defined and quantifiable. The view of stress as a risk factor is supported by epidemiological evidence linking stress and sudden cardiovascular events.19,25,26
but whether or not accelerated atherosclerosis and restenosis underlie these mechanisms has not been investigated. There appears to be only 1 report that addressed this issue in an animal model of atherosclerosis: apolipoprotein E–deficient (apoE−/−) mice.27 Kumari et al27 reported that apoE−/− mice stressed for 12 weeks by mild stressors (restraint and exposure to rat odor) doubled their area of aortic atheromas compared with the nonstressed mice, albeit with high variability.27 Also, no stress mediators with putative roles in atherosclerosis were studied except for cortisol, an indicator of the stress itself.

Our11,15,28 and other29 previous work has established that NPY is a mediator of stress in animals and humans. NPY is also a vasoconstrictor and a potent vascular growth and angiogenic factor.13 It stimulates vascular smooth muscle cell contraction and proliferation by activating Y1 and Y5 receptors10,11,30 and endothelial cell proliferation and angiogenesis via Y2 and Y5 receptors in an NO-dependent manner.12,13,31

In humans29,32 and rats,11,15,28 particularly intense or prolonged stress increases circulating NPY levels. In rats, Y1 receptor activation mediates up to 80% of the stress-induced pressor and vasoconstrictive responses, inhibited by a selective Y1 receptor antagonist.8 Stress-induced NPY release is neurogenic because of its release from the sympathetic nerves because it is blocked by a ganglionic blockade.28 However, in rats, a portion of this response is derived from platelets, which express high levels of NPY.21 Both platelet (present data) and neuronal expression of NPY appears to be upregulated by chronic stress, and its hypertensive effect is in part NPY dependent.34 Why Y1 receptor antagonist reduced plasma NPY-ir responses to angioplasty and stress is unclear. Because this antagonist (H409/22) does not cross blood–brain barrier, the action must be peripherally mediated. The possibilities include decreased NPY release attributable to inhibition of facilitatory presynaptic Y1 receptors on sympathetic nerve terminals or increased clearance of NPY (renal?). These actions have been speculated but not proven.

Stress-induced NPY release and vasoconstrictive actions are greater in males, in both humans35 and animals,36 and, in the latter, are strongly upregulated by androgens. Higher treadmill exercise–induced plasma NPY levels were also reported in humans with the Leu7/Pro7 signal peptide polymorphism,17 a frequent mutation occurring in European population.16 This polymorphism also associates with accelerated atherosclerosis18 and hyperlipidemia, independently of other risk factors.16

These human data corroborate our previous5 and current findings, in which stress mimicked vascular effects of exogenous NPY at the site of balloon-injured rat carotid artery. Similar to NPY, chronic stress increased plasma NPY-ir levels not only in plasma but also in the platelet fraction; interestingly, angioplasty alone was already a powerful stimulus for activating NPY release from the sympathetic nerves (elevated plasma levels) and platelets (changes in PRP). Although the relevance of these findings to humans, who reportedly do not express platelet NPY,37 remains unknown, similar association between elevated platelet NPY and propensity for neointima formation has been observed recently by us in several murine strains (Abe et al, unpublished observations, 2005). The notion that expression/induction of platelet NPY is a critical mediator of stress-accelerated atherosclerosis is currently being investigated.

The similarity between stress- and NPY-induced responses also includes upregulation of vascular expression of Y1 and Y5 receptors, at mRNA and protein levels, in the angioplasty-injured vascular wall. The Y1 and Y5 receptor became induced in the area of smooth muscle cells and macrophages in the neointima or adventitia and were often localized in and around neointimal neovessels. This represented a shift in the distribution pattern because in normal vessels, the receptors are not only expressed at low levels (Y1 more than Y5) but are also mainly expressed in the media (Y1 only).

A striking feature of vascular lesions induced by angioplasty in stressed rats, like in the NPY-treated animals,5 is their occlusive nature and atherosclerotic-like character. Although neointima induced by angioplasty alone is concentric, laminar, and free of lipids or neovessels, with sparse CD68-positive macrophages, lesions developing in vessels of stressed or NPY-treated rats are nonlaminar, occulsive, neovascularized, and rich in lipids, macrophages, matrix, and thrombus. This suggests that stress and NPY activate an amplifying mechanism leading to a cascade of accelerated vascular thromboremodeling. We propose that perivascular sympathetic nerves, which form a dense plexus at the adventitial–medial border, are the first elements of this cascade. In nonstressed conditions, vascular injury by angioplasty alone activates sympathetic nerves, releases NPY, and the peptide contributes to neointima formation with its vascular growth-promoting activities. As shown5 and confirmed here, this angioplasty-induced neointima formation is reduced by half by NPY-Y1 receptor antagonist.

The amplification of angioplasty-induced vascular lesions by NPY5 or chronic stress probably also depends on activation of additional cells and mediators, beginning with the adventitial–medial border. This layer, which is the site of neurovascular junctions, appeared marked and swollen/thickened and contained rich infiltrate of immune cells and matrix deposition, suggesting that nerves and NPY induced a neuroinflammatory reaction. This possibility is strongly supported by known effects of sympathetic nerves38 and NPY39 in modulation of immune functions. Importantly, the peptide has been shown to stimulate phagocytic activity40 and chemotaxis of monocytes/macrophages40 and endothelial cells12,41 and induce a shift in cytokine release from the Th1 cell to the Th2 cell mediated.39 In addition, NPY is antilipolytic and increases fat storage in adipocytes by activating lipoprotein lipase.42 The same proinflammatory activities are implicated in formation of atheromas and development of atherosclerosis.1

Thus, we propose that stress is an amplifier of atherosclerosis and restenosis by releasing NPY and activating a neuroinflammatory cascade in angioplasty-injured vessels. This hypothesis is strongly supported by the ability of a specific Y1 receptor antagonist to fully prevent vascular lesions induced by stress or NPY. Inhibition of NPY-Y1 receptor may therefore be an attractive therapy for restenosis and atherosclerosis, particularly in men and in people with NPY gene polymorphism, who respond to stress with exaggerated NPY expression and release.
References

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Figure IA. NPY immunoreactivity (NPY-ir) levels in platelet-rich- (PRP) and platelet-poor- (PPP) plasma before and 14 days after angioplasty, in rats stressed or non-stressed, placebo- or NPY-treated, with or without Y1 receptor antagonist. IB. Pressor responses to a 2 hr-stress, either before angioplasty, immediately after a single stress (1x), or after angioplasty, following repeated daily stress (14x) and immediately after the 14th session, in the presence or absence of Y1 antagonist. Asterisks denote level of statistical significance: *P<0.05; **P<0.01; ***P<0.001 as indicated by brackets.