Novel NAD(P)H Oxidase Inhibitor Suppresses Angioplasty-Induced Superoxide and Neointimal Hyperplasia of Rat Carotid Artery


Abstract—Neointimal proliferation occurring after vascular or endovascular procedures is a major complication leading to end-organ or limb ischemia. In experimental models, balloon injury has been shown to induce NAD(P)H oxidase to produce vascular superoxide anion (O$_2^-$) production, which has been implicated in cell proliferation, but a direct link is still unclear. We postulated that inhibition of arterial NAD(P)H oxidase, resulting in decreased O$_2^-$, would lessen the neointimal hyperplasia caused by balloon injury to the common carotid artery (CCA). Sprague-Dawley rats were implanted with osmotic minipumps containing either vehicle, a cell-permeant peptide that inhibits NAD(P)H oxidase (gp91ds-tat, 10 mg/kg per day), or a scrambled peptide control (scrmb-tat). Two days after pump implantation, the left CCA was injured using an intravascular balloon embolectomy catheter (2F Fogarty). Systolic blood pressure was monitored by tail cuff. Fourteen days after injury, CCAs were harvested and analyzed by digital morphometry. Rats in both groups remained normotensive, with no significant differences in systolic blood pressure. Reactive oxygen species measurements after injury indicated a significant reduction in vascular O$_2^-$ in rats infused with gp91ds-tat, and the neointima/media area and thickness ratios were significantly lower in their arteries compared with control. On the contrary, no significant change in overall CCA diameter was observed in any group. Our data indicate that in response to balloon injury of the rat carotid artery, NAD(P)H oxidase activity contributes to neointimal hyperplasia and is involved in vascular cell proliferation and migration during restenosis. (Circ Res. 2003;92:637-643.)

Key Words: superoxide • NAD(P)H oxidase • balloon angioplasty • neointima formation

Proliferation and migration of vascular smooth muscle cells, and more recently fibroblasts, have been implicated in narrowing of the arterial lumen in response to injury, mimicking some of the hallmark characteristics in the pathogenesis of atherosclerosis.1–5 Although the mediators are not fully understood, they include angiotensin II (Ang II), growth factors, and proto-oncogenes; indeed, in the rat both ACE inhibitors and Ang II receptor antagonists have been shown to prevent neointima formation in response to balloon injury.6–10 One factor stimulated by Ang II that is involved in the process of vascular cell proliferation and neointimal growth appears to be superoxide anion (O$_2^-$) derived from vascular NAD(P)H oxidase.11–17 Neointimal proliferation and stenosis after vascular or endovascular procedures in animal models coincide with elevated levels of reactive oxygen species (ROS) implicated in a variety of growth-related signaling pathways18,19 and smooth muscle cell and fibroblast proliferation and migration.20,21

Recent reports have suggested that activation of various isoforms of NAD(P)H oxidase leads to increased O$_2^-$ in the vascular wall in response to injury.18,21,22 However, the lack of specific and effective in vivo inhibitors of NAD(P)H oxidase has prevented determination of the functional involvement of NAD(P)H oxidase in this process. Recently, we reported on a chimeric peptide inhibitor (gp91ds-tat) that interferes with the assembly of vascular NAD(P)H oxidase components, and showed that this chimera abolished Ang II–induced aortic O$_2^-$ generation in vitro and in vivo, whereas its scrambled control did not.23 In the present study, we tested the hypothesis that inhibition of vascular NAD(P)H oxidase would decrease neointimal hyperplasia caused by balloon injury of the rat common carotid artery (CCA).

Materials and Methods

Animals and Pump Implantation

Male Sprague-Dawley rats (Charles River, Wilmington, Mass) were anesthetized with ketamine (80 mg/kg IP) and xylazine (7 mg/kg IP),...
and 14-day intraperitoneal osmotic minipumps (Alzet/Durect) were implanted using aseptic techniques. The pumps contained either vehicle (0.01N acetic acid in 0.9% normal saline, n=8), gp91ds-tat peptide (10 mg/kg per day, n=11), or a scrambled control (scrmb-tat, n=7) dissolved in vehicle as described previously. Chromatographic analysis of peptide preparations confirmed no change in the purity of gp91ds-tat within osmotic minipumps after 7 days in vivo. Animals were given free access to water and rat chow. All protocols were approved by the Institutional Animal Care and Use Committee of Henry Ford Hospital and are consistent with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Balloon Injury
Two days after pump implantation, the animals were anesthetized again, and the left external carotid artery (ECA) was dissected via a midline cervical incision. A 4-0 silk ligature was tied around the distal ECA, and another was passed around the proximal ECA but was not tied. A small transverse arteriotomy was made between ligatures, and a 2F Fogarty embolectomy catheter (Baxter) was passed through the left CCA into the thoracic aorta. The balloon was distended with 0.9% saline to increase CCA diameter by ~100% and withdrawn through the CCA to effect arterial injury. This was repeated four times. Finally, the balloon catheter was removed, and the proximal ECA suture was tied. The animals were allowed to recover and were given free access to water and rat chow.

Blood Pressure Monitoring
Rats were maintained under normal conditions and underwent periodic blood pressure measurement using an automated tail cuff (ITC/Life Science Instruments).

Morphology/Morphometry
Fourteen days after carotid injury, the rats were anesthetized and transcardially perfused with 10 mL PBS and then 10% formaldehyde in PBS under pressure (120 mm Hg). CCAs were harvested from their origin to the carotid bifurcation; care was taken not to interrupt the adventitia. Each artery was embedded in paraffin, and the middle section was examined several times, and the values were averaged. The injured carotid artery was expressed as the ratio of neointimal area and thickness to medial area and thickness, respectively. Each vessel circumference and averaged. Myointimal proliferation of the luminal area from that defined by the intima-external elastic lamina (IEL). The intimal area was tabulated by subtracting the adventitia. Each artery was examined using image analysis software (Spot Diagnostics 3.4.2) by an examiner with no knowledge of the treatment group. When the cross-sectional area was measured, analysis of each segment was performed using image analysis software (ITC/Life Science Instruments).

ROS Measurements
Chemiluminescence Measurement of Injury-Induced \( \cdot O_2^- \) Production
Rats were infused for 2 days with vehicle or gp91ds-tat (10 mg/kg per day IP by minipump) and anesthetized, and CCAs were cleared of adherent adipose and loose connective tissue in situ. Because there was no effect of scrmb-tat on neointimal proliferation, we focused our efforts on examining ROS levels in vehicle-treated and gp91ds-tat-treated rats. CCAs were harvested in ice-cold modified Krebs-HEPES buffer of the following composition (in mmol/L): NaCl 119, HEPES 20, KCl 4.6, MgSO4 1.0, NaHPO4 0.15, KH2PO4 0.4, NaHCO3 5, CaCl2 1.2, and glucose 5.5 (pH 7.4). CCAs were gently flushed with cold buffer to remove blood from the lumen, cut into equal halves, equilibrated in gently bubbled buffer (95% O2/5% CO2) at 37°C for 45 minutes, and then transferred to buffer at 37°C and either (1) distended with a 2F catheter to increase diameter by 100% for a sustained 60 seconds or (2) not distended and left untouched for 60 seconds. Segments were then transferred to a luminometer tube containing lucigenin (5 μmol/L) in buffer at 37°C, and luminescence was integrated over 10 minutes (20 cycles) using a Turner Designs 20e luminometer. Tiron (10 mmol/L) was added, and 10 more cycles were read, taking an average of the final three values. Differences between averaged values of the first 20 readings (in the absence of Tiron) and the last three readings (with Tiron) were expressed as change in chemiluminescence units per minute per milligram blotted tissue weight.

Immunohistochemistry of Nitrotyrosine Accumulation in Injured Rat Carotid Arteries
To determine the location of carotid ROS production in response to injury and to confirm the effect of oxidase inhibition at 0 days, nitrotyrosine accumulation, a footprint marker of peroxynitrite (formed by the reaction of elevated \( \cdot O_2^- \) with NO) was examined in CCA cross sections. At 1 day after injury, left CCAs from a subset of vehicle-treated and gp91ds-tat-treated rats were cleaned of adherent adipose and loose connective tissue in situ and placed in cold PBS; they were then immediately mounted and frozen in OCT fixative (Tissue-Tek) using Shur-freeze (Triangular BS) and stored at −70°C until they were cryocut into 6-μm sections. Sections were allowed to thaw for 30 minutes at room temperature and then fixed in precooled (4°C) 100% acetone, which was incubated at −20°C for 10 minutes. Sections were rinsed in PBS twice for 5 minutes each, preincubated with 0.3% hydrogen peroxide in 80% methanol for 30 minutes, and then washed two or three times in PBS, and nonspecific binding was blocked with 10% goat serum in PBS for 30 minutes in a humidified chamber. Samples were incubated with 5 μg/mL rabbit anti-3-nitrotyrosine antibody (Upstate Biochemicals) overnight at 4°C and rinsed twice in PBS for 5 minutes. Secondary antibody was added (Dako Universal Kit) for 30 minutes, and the slides were rinsed twice for 5 minutes each. Streptavidin-conjugated horseradish peroxidase (S-HRP) was added for 30 minutes, and the slides were rinsed twice in PBS for 5 minutes. DAB reagent was added as needed in fixed increments for all treatment groups; this procedure involved 2-minute incubations until adequate staining was observed in positive controls and minimal staining was observed in negative controls. Separate negative controls omitting (1) primary antibody, (2) primary and secondary antibodies, and (3) primary and secondary antibodies and S-HRP were performed. Staining was analyzed by three blinded observers who scored the intensity of positive staining in four sections from each CCA using an arbitrary grading system from 1 to 4 (lowest to highest). Scores were averaged for each CCA and within treatment groups.

Statistical Analysis
Data are expressed as mean±SEM. Morpometric results and ROS levels were compared by t test as indicated in the legends. Blood pressure was analyzed by 2-way ANOVA, followed by a Student-Newman-Keuls test to identify differences. A value of \( P<0.05 \) was considered significant.

Results
Systolic blood pressure (SBP) was monitored at days −2, 7, and 14 in all groups. As seen in the Table, there was no difference in SBP at any time point among the vehicle, gp91ds-tat, and scrmb-tat groups. Body weight was also similar between vehicle-treated and gp91ds-tat–treated rats, but it was significantly lower in the scrmb-tat group at the beginning and end of the study.

Effect of gp91ds-tat on Morphometric Changes in Carotid Artery After Balloon Injury
Figure 1 shows representative cross sections of injured carotid arteries taken from rats infused with vehicle, scrmb-tat, and gp91ds-tat 14 days after injury. Injured CCAs
exhibited pronounced neointima formation (Figure 1A), whereas no neointimal growth was observed in uninjured sham-operated contralateral CCAs (not shown). Intriguingly, we observed markedly reduced neointimal proliferation in arteries from gp91ds-tat–treated rats (Figure 1B), but no reduction was observed in arteries from scrmb-tat–treated rats (Figure 1C). Digitally enlarged photographs showed intact elastic laminae in all three groups (please refer to the online data supplement, available at http://www.circresaha.org, for online Figures IA through IC). Cumulative data showed that compared with vehicle control, gp91ds-tat infusion significantly attenuated the ratio of neointima to media by 62% at 14 days after the injury (Figure 2A). Moreover, CCAs from rats treated with scrmb-tat control did not exhibit a significant reduction in the neointimal/medial area ratio. When radial thicknesses of each segment were examined, the same trends were observed; ie, compared with vehicle, gp91ds-tat significantly reduced the neointimal/medial thickness ratio (69%), whereas scrmb-tat did not (Figure 2B). There was a tendency for gp91ds-tat, but not scrmb-tat, to increase the luminal area (Figure 3). gp91ds-tat did not significantly affect areas defined by the IEL or EEL, which was consistent with no change observed in the overall CCA diameter (not shown). Online Figure II confirms intracellular access of the peptide into rat fibroblasts.

Effect of gp91ds-tat Versus Vehicle on Injury-Induced Carotid Artery O$_2^·$/H$_2$O$_2$ Production

Previous studies in rabbits demonstrated that balloon injury causes an immediate and large increase in arterial O$_2^·$/H$_2$O$_2$. Our present study confirms that balloon distension of the rat CCA causes a similar rise in O$_2^·$ levels (Figure 4). Infusion of

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Initial Body Weight, g</th>
<th>Final Body Weight, g</th>
<th>Systolic Blood Pressure, mm Hg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day −2</td>
<td>Day 7</td>
<td>Day 14</td>
</tr>
<tr>
<td>Vehicle</td>
<td>377±15</td>
<td>411±15</td>
<td>127±3</td>
</tr>
<tr>
<td>gp91ds-tat</td>
<td>381±15</td>
<td>415±14</td>
<td>127±4</td>
</tr>
<tr>
<td>scrmb-tat</td>
<td>285±2*</td>
<td>366±6†</td>
<td>126±2</td>
</tr>
</tbody>
</table>

Values are mean±SEM. *P<0.001 vs vehicle; †P<0.05 vs vehicle.
gp91ds-tat caused marked inhibition of balloon angioplasty–induced $O_2^-$ but had no effect on $O_2^-$ production in nondistended CCAs.

**Effect of gp91ds-tat Versus Vehicle on Carotid Artery Nitrotyrosine Detection 1 Day After Injury**

We examined nitrotyrosine levels, a marker of peroxynitrite, in cross sections of injured carotid arteries 1 day after injury. 3-Nitrotyrosine (brown DAB staining) was detectable across the vessel wall, evident primarily at the neointimal border and in the adventitia (Figure 5A) and was reduced in CCAs from gp91ds-tat–treated rats compared with vehicle-treated rats (Figure 5B). Negative control omitting primary antibody exhibited no staining (see online Figure III). On analysis of 3-nitrotyrosine staining, CCAs from gp91ds-tat–treated rats exhibited 41% less staining than did those from vehicle-treated rats (Figure 5C).

**Discussion**

Our present findings provide evidence that vascular NAD(P)H oxidase activity is functionally involved in rat carotid stenosis in response to balloon angioplasty. Development of an NAD(P)H oxidase inhibitor that is effective in vivo has allowed us to target endogenous NAD(P)H oxidase and determine for the first time whether it contributes substantially to the progression of neointimal proliferation and stenosis. Morphometry showed large reductions in the CCA neointimal/medial area ratio and thickness ratio in rats infused with the NAD(P)H oxidase inhibitor gp91ds-tat but not with a scrambled peptide or vehicle control. Moreover, ROS measurements immediately after balloon injury and at 1 day confirmed the elevation of vascular $O_2^-$, which was blocked by gp91ds-tat, consistent with NAD(P)H oxidase involvement. These findings appear to support our hypothesis that a gp91 containing NAD(P)H oxidase is involved in neointimal hyperplasia.

We recently reported that gp91ds-tat is able to suppress subcellular oxidase activity and whole aortic $O_2^-$ production. In the present study, we confirmed its ability to suppress $O_2^-$ levels in injured rat CCAs. Because this peptide inhibitor was designed to competitively block a specific 9–amino-acid sequence in gp91 (nox2) that interacts with p47phox, the inhibitor was intended to target the gp91nox-based oxidase functional in the vascular adventitia and endothelium. Balloon injury caused the appearance by 1 day of nitrotyrosine accumulation, primarily in the adventitia and lumen-media interface. Moreover, it would appear that our immunocytochemical data demonstrate inhibition in both layers of the CCA and corroborate findings reported by Szöcs et al, who showed that balloon injury causes increased $O_2^-$ levels in pluripotent adventitial and neointimal cells. However, spatial diffusion of peroxynitrite, once it is formed, does not allow us to definitively localize ROS production in these studies. Most important, our present experiments revealed that at 1 day after injury, total nitrotyrosine staining was substantially reduced by gp91ds-tat, supporting the ability of the peptide to suppress oxidase activity in the vascular wall. At present, we also cannot rule out the contribution of neutrophils and macrophages that contain related NADPH oxidase at this and later time points. Although Szöcs et al have addressed the role of leukocytes in this model and found a minimal contribution, and although examination of hematoxylin-stained cross sections of injured CCAs in the present study has confirmed this at later time points (see online Figure IV), it is plausible that leukocytes play a permissive or indirect role in this process at some point early in the development of neointimal hyperplasia. More careful analysis of a role for leukocytes, including the use of oxidase-targeting strategies, is necessary.

Our data show that neointimal hyperplasia is markedly reduced in rats treated with an NAD(P)H oxidase inhibitor. CCAs from rats infused with gp91ds-tat showed reduced neointimal/medial ratios, whereas rats treated with scrambled peptide did not, consistent with NAD(P)H oxidase–dependent $O_2^-$ production being functionally involved in neointimal growth. Interestingly, the luminal area tended to increase in oxidase-inhibited CCAs, indicating the potential for increased patency of the carotid artery. Other reports have...
suggested that NAD(P)H oxidase is involved in the proliferation of vascular cells and their migration to the neointima. More specifically, NAD(P)H oxidase has recently been implicated in angioplasty-induced neointimal proliferation on observing the activation of smooth muscle and fibroblast oxidase activity after balloon injury in rats, rabbits, and pigs.18,22,26 Those studies show that induction of oxidase subunits and activity is associated with neointimal hyperplasia. The advantage of our present findings is that they suggest that NADPH oxidase activation is functionally involved in vascular cell hyperplasia and neointimal growth.

Previous reports have shown that gp91ds prevents the assembly of NAD(P)H oxidase and reduces $O_2^-$ generation.23,27,28 Souza et al18 have shown that stretching arteries with a balloon catheter causes an immediate and profound increase in ROS that is largely NAD(P)H oxidase–derived. For this reason, we assessed changes in $O_2^-$ and peroxynitrite levels occurring early in the vascular response to angioplasty that are known to coincide with the development of neointimal growth. In fact, our preliminary data suggested that at later time points, whereas there was a slight difference in ROS measurements between gp91ds-tat and vehicle groups, these differences were not nearly as definitive. In contrast, immediately after stretch we observed a rapid and large increase in $O_2^-$ that was inhibited 81% by the infusion of gp91ds-tat. The fact that CCAs from rats infused with gp91ds-tat exhibited significantly lower nitrotyrosine accumulation at day 1 is consistent with the continuing contribution of NAD(P)H oxidase to $O_2^-$ elevations 1 day after balloon angioplasty. Therefore, we have confirmed by two independent techniques that gp91ds-tat is effective at lowering ROS in these vessels. Cell proliferation occurs early in response to arterial injury, with medial smooth muscle cells entering the cell cycle between 1 and 3 days.29 Relevant to this point, studies have shown that $O_2^-$ can rapidly activate signaling pathways, leading to increased transcription factor expression and growth response.18,30 For example, it is noteworthy that balloon angioplasty rapidly induces p38 mitogen–activated protein kinase, which is also known to be activated by ROS and appears to be involved in smooth muscle cell hypertrophy and neointimal hyperplasia.31,32 Thus, the elevations in NAD(P)H oxidase–derived $O_2^-$ that we observed are likely to contribute to the activation of early redox-sensitive signaling pathways, leading to vascular cell proliferation and migration. Although the present study does not dissociate early and late NAD(P)H oxidase activation, it may suggest that inhibition of early rises in oxidase-derived $O_2^-$ in response to balloon angioplasty can largely prevent neointimal hyperplasia in this model.

Despite the potential implication from the present study that the early increase in ROS predominates at the innermost medial layer or intimal surface and the adventitia, it is almost certain that smooth muscle cells play a significant role in the hyperplasia. For one thing, it appears plausible that medial smooth muscle cells closest to the site of injury initiate the oxidative response,22 which would then emanate throughout the media. In rat smooth muscle oxidase, nox1 and nox4 appear to be surrogates for nox2 and are upregulated during restenosis in this model.22 We recently reported that conserved sequences exist in nox1 and nox4 that correspond to the site of p47phox binding and predicted that the peptide may be useful in blocking the involvement of oxidases containing these homologues,23 suggesting the possible effectiveness of gp91ds-tat or related peptides as inhibitors. Moreover, access of gp91ds-tat to medial smooth muscle cells is likely, because tat-linked moieties are expected to penetrate the vascular wall.33 Indeed, inasmuch as previous reports have shown a functional requirement of p47phox translocation to plasma
membranes in smooth muscle cells, which have been shown to contain primarily noxl and nox4, interaction of p47phox with anchoring gp91phox-like components appears to be fundamental to this family of enzymes. Thus, we initially hypothesized that gp91ds-tat would be effective at inhibiting vascular smooth muscle oxidase activity. However, preliminary data suggest that gp91ds-tat is ineffective at lowering oxidase activity in rat aortic smooth muscle cells in vitro (written communication, K.K. Griendling, PhD, March 2002). Thus, we postulate that ROS-mediated crosstalk may exist between the endothelium and adventitia (which contain higher amounts of gp91phox) and smooth muscle layers. Our recent findings suggest such an interaction, in that adventitial O2·−can negatively affect endothelium-dependent relaxation, and are consistent with a report by Wang et al showing that endothelium-dependent tone of the rat aorta is increased by adventitial oxidase.

In summary, our findings suggest that a vascular gp91phox-like NAD(P)H oxidase is critically involved in early vascular signaling, contributing to neointimal hyperplasia. Early induction of arterial O2·−in response to balloon angioplasty and marked inhibition by the oxidase inhibitor gp91ds-tat, leading to reduced hyperplasia, appear to support a fundamental role of this oxidase in neointimal growth and stenosis. Further studies are necessary to determine whether this and other specific inhibitors that suppress other isoforms of NAD(P)H oxidase, including noxl and nox4, will prove beneficial in preventing vascular complications arising from injury.

Acknowledgments

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Online Figure 1A: Digital enlargement of Figure 1A, showing intact elastic laminae in an injured carotid artery from a vehicle-infused rat. I.E.L. = internal elastic lamina; E.E.L. = external elastic lamina.
Online Figure 1B: Digital enlargement of Figure 1B, showing intact elastic laminae in an injured carotid artery from a gp91ds-tat-infused rat. I.E.L. = internal elastic lamina; E.E.L. = external elastic lamina.
Online Figure 1C: Digital enlargement of Figure 1C, showing intact elastic laminae in an injured carotid artery from a scrmb-tat-infused rat. I.E.L. = internal elastic lamina; E.E.L. = external elastic lamina.
Online Figure 2: Confirmation of intracellular access of gp91ds-tat. Quiescent rat cardiac fibroblasts were incubated with FITC-labeled gp91ds-tat for 3 hr and then washed 3 times with PBS. Representative confocal micrograph (Biorad confocal MRC 1024) shows accumulation of fluorescence in fibroblast cytosol but not nuclei.
Online Figure 3: Negative control for nitrotyrosine immunostaining. Cross-section of a carotid artery taken from an injured vehicle-treated rat. The primary antibody was omitted from the immunohistochemistry protocol as described in the Methods. Original magnification ×400.
Online Figure 4: Hematoxylin-stained cross-section of an injured CCA from a vehicle-infused rat at 14 days confirming the lack of leukocytes in the vascular wall. Original magnification, X 400.