Increased NAD(P)H Oxidase and Reactive Oxygen Species in Coronary Arteries After Balloon Injury

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Abstract—Reactive oxygen species (ROS), produced by cellular constituents of the arterial wall, provide a signaling mechanism involved in vascular remodeling. Because adventitial fibroblasts are actively involved in coronary remodeling, we examined whether the changes in the redox state affect their phenotypic characteristics. To this end, superoxide anion production and NAD(P)H oxidase activity were measured in porcine coronary arteries in vivo, and the effect of ROS generation on adventitial fibroblast proliferation was examined in vitro. Superoxide production (SOD- and Tiron-inhibitable nitro blue tetrazolium [NBT] reduction) increased significantly within 24 hours after balloon-induced injury, with the product of NBT reduction present predominantly in adventitial fibroblasts. These changes were NAD(P)H oxidase–dependent, because diphenyleneiodonium (DPI) abolished superoxide generation ($P<0.001$). Furthermore, the injury-induced superoxide production was associated with augmented NAD(P)H oxidase activity and upregulation of $p47^{phox}$ and $p67^{phox}$ in adventitial fibroblasts (immunohistochemistry). Serum stimulation of isolated adventitial fibroblasts produced time-dependent increases in ROS production (peak 3 to 6 hours). The inhibition of ROS generation with NAD(P)H oxidase inhibitor (DPI) or the removal of ROS with antioxidants (Tiron, catalase) abrogated proliferation of adventitial fibroblasts. These results indicate that vascular NAD(P)H oxidase plays a central role in the upregulation of oxidative stress after coronary injury, providing pivotal growth signals for coronary fibroblasts. (Arterioscler Thromb Vasc Biol. 2001;21:739-745.)

Key Words: reactive oxygen species ■ NAD(P)H oxidase ■ coronary remodeling ■ adventitial fibroblast

Oxidative stress has been implicated in several steps leading to the development of vascular disease. Initial observations focused on reactive oxygen species (ROS) derived from invading macrophages and their possible involvement in oxidative lipid modifications in the vessel wall. Subsequently, it has become apparent that ROS are also produced in a controlled fashion by all vascular cells and that they act as “second messengers” regulating various cellular functions. Several extracellular signals, such as growth factors or even physical stimuli, induce ROS and their derivatives in vascular smooth muscle cells (SMCs) and fibroblasts, activating the intracellular growth program. For example, superoxide anion ($O_2^\cdot-$) increases expression of extracellular signal–regulated kinase 1/2 mitogen-activated protein kinase, whereas $H_2O_2$ activates p38 mitogen-activated protein kinase and stress-activated proteins. Furthermore, $H_2O_2$ also stimulates early proto-oncogenes and redox-sensitive transcriptional factors (eg, NF-κB and AP-1). 

In vascular cells, the major enzymatic source of intracellular ROS is NAD(P)H oxidase, which generates $O_2^\cdot-$ by 1-electron reduction of molecular oxygen. Although NADPH oxidase is responsible for the burst of $O_2^\cdot-$ in phagocytic cells, the generation of ROS in vascular cells differs from that in neutrophils. In the former, it occurs over a period of hours (rather than minutes), appears to be mostly intracellular (rather than extracellular and intracellular), and may involve the assembly of different enzymatic subunits of NAD(P)H oxidase. Significant progress has been made toward the identification of NAD(P)H oxidase subunits in normal vascular cells and in atherosclerotic lesions, including both membrane-associated ($p22^{phox}$) and cytoplasmic ($p67^{phox}$, $p47^{phox}$, Rac1) components. The activity of the NAD(P)H oxidase in vascular cells is modulated by extracellular signals known to influence vascular remodeling and lesion development (eg, thrombin and angiotensin II). Furthermore, gene polymorphism affecting at least one of the subunits ($p22^{phox}$) has been linked to the development of atherosclerosis in humans.

The regulation of the redox state appears to be heterogeneous across the vessel wall. Higher expression of NAD(P)H oxidase and $O_2^\cdot-$ production have been reported in normal adventitia than in the media. The importance of this finding initially remained unclear, because the activation of medial SMCs and lipid peroxidation occur in the proximity of the arterial lumen. Several studies from our laboratory and others, however, have suggested active involvement of ad-
ventral fibroblasts in arterial repair. In particular, after severe coronary injury, these cells demonstrate preferential proliferation and migration toward intima. This is not surprising, because coronary SMCs display more advanced differentiation and a limited response to stimulation compared with noncoronary SMCs. In view of our previous findings and the established role of ROS in the regulation of cell proliferation, we hypothesized that the increase in oxidative stress after coronary injury involves adventitial fibroblasts. The results of this study demonstrated the upregulation of NAD(P)H oxidase activity and ROS production in adventitial fibroblasts after coronary injury. In cell culture, ROS is important signals for growth response of coronary fibroblasts. We postulate that phenotypic responsiveness of coronary fibroblasts to stimulation is mediated, in part, by NAD(P)H oxidase–derived oxidative stress.

Methods

Animal Model
Domestic crossbred female pigs (12 to 15 kg) were anesthetized and instrumented as previously described. After the exposure of the right common carotid artery, heparin (5000 U) was administered intravenously. Coronary ostia were cannulated under fluorescent guidance, and intracoronary nitroglycerin was given (100 μg). Coronary injury was carried out in 2 coronary arteries in each animal with an oversized balloon (balloon:artery ratio 1.3 to 1.5) inflated 2 to 3 times for 30 seconds. The third artery was used as control. The animals were euthanized with intravenous Euthanol (Delmarva Laboratory) at times indicated in the text. All experiments were carried out in accordance with institutional guidelines.

Measurement of \( \cdot O_2^- \) Production

The production of \( \cdot O_2^- \) was measured by superoxide dismutase (SOD)– or Tiron-inhibitable conversion of nitro blue tetrazolium (NBT) to formazan.

\( \cdot O_2^- \) Production in Coronary Arteries
Coronary arteries were dissected free from adipose tissue and myocardium, then cut into \( 5 \times 5 \) mm rings and placed in 24-well plates. Tissues were incubated in phenol-free DMEM at 37°C in a CO2 incubator for 30 minutes with or without addition of SOD (1000 U/mL) or Tiron (10 mM). Freshly made NBT (100 mg/L in phenol-free DMEM) was added to tissues with gentle rocking for 3 hours. The reaction was terminated by addition of an equal volume of 0.5N HCl, and tissues were rinsed twice with cold PBS. To extract formazan, tissues were pulverized in liquid nitrogen and dissolved in 100% pyridine at 80°C for 30 minutes. After centrifugation, light absorbance was measured at 540 nm. The NBT reduction to formazan was calculated by the following formula: NBT reduction = A × V(T × E × X/L), where A is absorbance, V is volume of solubilizing solution, T is time of incubation with NBT (minutes), E is extinction coefficient = 0.72 mmol/mm, and L is length of light travel through the solution, 10 mm. Either SOD– or Tiron-inhibitable NBT reduction was calculated as a measure of \( \cdot O_2^- \) production (pmol · min⁻¹ · mg wet wt⁻¹). To determine pathways mediating \( \cdot O_2^- \) production, several inhibitors were used in the experiments, including diphenylletheneonidium (DPI, 100 μmol/L), rotenone (50 μmol/L), oxyypurinol (300 μmol/L), N-o-nitro-L-arginine methyl ester (L-NAME, 1 mmol/L), and diethyldithiocarbamic acid (10 mmol/L). The n value represents the number of vascular rings obtained from \( \geq 3 \) animals per experimental condition.

To assess the location of injury-induced \( \cdot O_2^- \) production, the injured coronary arteries were incubated with NBT and processed to visualize formazan deposits. Briefly, coronary rings were fixed in 10% formalin and embedded in paraffin. They were sectioned into 6-μm-thick sections and deparaffinized by heating at 65°C for 1 hour. To avoid solubilization of NBT in tissue, the sections were rinsed with Clear-Rite 3 solution (Richard-Allan Scientific) and counterstained with nuclear fast red.

Measurement of NAD(P)H Oxidase Activity in Coronary Arteries
NAD(P)H oxidase activity was measured by SOD-inhibitable cytochrome c reduction using NADH or NADPH as substrate. To measure NAD(P)H oxidase activity in injured coronary arteries, the arteries were harvested at 2 days after injury, and the injured segments (including the adventitia and media) were dissected free from adipose tissue and myocardium. The noninstrumented coronary arteries were used as control. After the removal of endothelial cells, tissues were minced in 10 volumes of ice-cold Tris-sucrose buffer (pH 7.1) containing Tris base 10 mmol/L, sucrose 340 mmol/L, PMSF 1 mmol/L, EDTA 1 mmol/L, leupeptin 10 μg/mL, aprotonin 10 μg/mL, and pepstatin 10 μg/mL. Then the tissue homogenates were sonicated for 20 seconds on ice, followed by extraction for 30 minutes. After centrifugation at 15 000 g for 10 minutes, an aliquot (20 μL) of supernatant (50 to 150 μg of protein) was added to the reaction buffer (980 μL) containing cytochrome c (78 μmol/L), NADH, or NADPH (100 μmol/L), with or without SOD (1000 U/mL). The samples were then incubated at 37°C for 1 hour, and the absorbance at 550 nm was measured. There was no measurable activity in absence of NADH. A buffer blank was measured in each assay, and SOD-inhibitable cytochrome c reduction in buffer blank was subtracted from each sample. The activity of NADPH oxidase was calculated as SOD-inhibitable cytochrome c reduction and expressed as \( \cdot O_2^- \) in pmol · mg⁻¹ · min⁻¹.

Measurement of SOD Activity in Coronary Arteries
SOD activity in vascular tissues was measured by SOD-dependent inhibition of cytochrome c reduction catalyzed by xanthine/xanthine oxidase. To assess SOD activity in uninjured coronary arteries, coronary adventitia and media were dissected after the removal of endothelium. SOD activity after coronary injury was measured in arterial segments including the adventitia and media. The samples were minced and homogenized in 10 volumes of 50 mmol/L potassium phosphate (pH 7.4) containing 0.3 mmol/L KBr and a cocktail of protease inhibitors (0.5 mmol/L PMSF, 90 μg/mL aprotonin, 10 μg/mL pepstatin, 10 μg/mL leupeptin). After sonication for 20 minutes, the homogenates were extracted at 4°C for 30 minutes, followed by centrifugation at 15 000 g for 10 minutes. The supernatants were added to the reaction mixture consisting of 0.1 mmol/L EDTA, 0.090 mmol/L xanthine, and 0.018 mmol/L cytochrome c (pH 7.4). SOD activity was assessed by monitoring the inhibition of xanthine oxidase–mediated cytochrome c reduction, with the absorbance measured at 550 nm over 3 minutes.

Immunohistochemistry
The Vectastain Elite ABC system (Vector Laboratories) was used for immunohistochemistry as previously described. Sections were deparaffinized, incubated with 0.6% H2O2 in methanol for 30 minutes, and blocked with 5% horse or rabbit serum. After being washed in PBS, sections were incubated with primary antibodies for 1 hour at room temperature in a moisture chamber. The following primary antibodies were used: polyclonal antibodies against p47phox and p67phox (1:200, Santa Cruz), monoclonal antibody recognizing SM myosin heavy chain (SM-MHC, 1:800, Sigma), and porcine anti-goat antibodies (1:2000, Vector Laboratories) for 1 hour. They were visualized with DAB substrate (Vector Laboratories) followed.
by counterstain with Gill’s hematoxylin (Sigma Diagnostics). Negative controls were carried out with nonimmune serum instead of primary antibody.

Cell Proliferation Assay

Fibroblasts were isolated from the adventitia of porcine coronary arteries as described.26 The cells (passages 2 to 6) were plated in triplicate at 10,000 cells/well in 24-well plates in DMEM supplemented with 10% FBS. At 24 hours later, cells were arrested in DMEM containing 0.5% FBS for 48 hours. They were then stimulated with 10% FBS. At 24 hours later, cells were arrested in triplicate at 10,000 cells/well in 24-well plates in DMEM supplemented with 1% FBS for 3 days with or without addition of indicated inhibitors. Cells were trypsinized at 72 hours after stimulation and counted in a Coulter counter. Values were derived from 3 wells per treatment, and the experiments were repeated ≥3 times on separate occasions.

Statistical Analyses

Data were expressed as mean ± SD. The statistical significance regarding multigroup comparisons was determined by ANOVA with Bonferroni correction. A value of \( P < 0.05 \) was considered significant.

Results

Oxidative Stress in Normal and Injured Coronary Arteries

Normal Coronary Arteries

Normal coronary adventitia exhibited higher basal \( \mathrm{O}_2^- \) generation (4.4 ± 1.2 pmol \cdot mg\(^{-1}\) \cdot min\(^{-1}\) SOD-inhibitable NBT reduction) than did the media (0.4 ± 0.5 pmol \cdot mg\(^{-1}\) \cdot min\(^{-1}\), \( n = 6, P < 0.001 \)). As shown in Figure 1, the preincubation of coronary rings with DPI [NAD(P)H oxidase inhibitor] abolished \( \mathrm{O}_2^- \) generation in coronary adventitia, whereas rotenone (mitochondrial dehydrogenase inhibitor) and oxypurinol (xanthine oxidase inhibitor) had no effect. The difference in basal \( \mathrm{O}_2^- \) generation between coronary adventitia and media is probably due to heterogeneous distribution of endogenous SOD, inasmuch as adventitia exhibited lower SOD activity than did media (101 ± 7 versus 166 ± 9 U/g, \( P < 0.001, n = 5 \)). Subsequently, SOD inhibitor (diethylthiocarbamic acid) augmented more \( \mathrm{O}_2^- \) generation in the media (adventitia 19.6 ± 2.5 and media 36.3 ± 8.2 pmol \cdot mg\(^{-1}\) \cdot min\(^{-1}\), \( P < 0.001 \) versus no diethylthiocarbamic acid).

Injured Coronary Arteries

Because coronary injury induces a short-lived adventitial cell proliferation, the change in oxidative stress during this time period was examined. To this end, the SOD activity and \( \mathrm{O}_2^- \) generation were measured in the entire coronary segments, because precise separation of the adventitia from media is not technically feasible at early time points after injury. SOD activity showed no difference between control and injured coronary segments (not shown). \( \mathrm{O}_2^- \) generation, as measured by SOD- and Tiron-inhibitable NBT reduction, increased significantly within 1 day after injury, and it remained elevated for ≥10 days (Figure 2). Higher values of Tiron-inhibitable NBT reduction were probably due to better cellular permeability of Tiron than SOD. To ascertain the site...
of \( \cdot \text{O}_2^- \) generation in injured vessels, reduced NBT (formazan) was identified in cross sections. Figure 3 demonstrates preferential adventitial localization of intracellular deposits of formazan in injured segments. As in uninjured vessels, NAD(P)H oxidase inhibitor (DPI) almost entirely abolished the production of \( \cdot \text{O}_2^- \) after coronary injury (n=4 per time point, \( P<0.001 \) versus no treatment). Although dynamic changes in inducible nitric oxide synthase expression during coronary repair could contribute to oxidative stress, its inhibitor, L-NAME, showed no effect (Table 1).

### NAD(P)H Oxidase Activity and Expression of Subunits

To ascertain that NAD(P)H oxidase is the major pathway responsible for oxidative stress after coronary injury, NADH/NADPH oxidase activity was measured by SOD-inhibitable cytochrome c reduction using NADH or NADPH as substrates. At baseline, coronary arteries exhibited similar levels of NADH and NADPH oxidase activity. At 2 days after coronary injury, NADH oxidase activity was significantly augmented in the injured and adjacent segments (Table 2), whereas NADPH oxidase activity showed no changes after coronary injury.

### Expression of p47\text{phox} and p67\text{phox}

To localize NAD(P)H oxidase in injured coronary arteries, expression of p47\text{phox} and p67\text{phox} [cytoplasmic subunits of NAD(P)H oxidase] were examined by immunohistochemistry. They were low in normal coronary arteries (not shown) but showed a marked increase in adventitial cells after injury. The expression began at 1 day and peaked at 2 days after injury. Positive cells were adventitial fibroblasts, because they lacked SM differentiation markers (SM-MHC, \( \alpha \)-SM actin, desmin, and caldesmon), and only infrequent cells (<5%) were positive for macrophage immunoreactivity (Figure 4).

### Role of NAD(P)H Oxidase–Derived ROS Production in Coronary Fibroblast Proliferation

#### Serum-Induced Superoxide Production in Adventitial Fibroblasts

To assess the functional importance of increased oxidative stress in coronary adventitia, the \( \cdot \text{O}_2^- \) production was examined in serum-stimulated adventitial fibroblasts. In response to serum stimulation, adventitial fibroblasts demonstrated a time-dependent increase in \( \cdot \text{O}_2^- \) production, reaching maximum levels at 3 to 6 hours (Figure 5). As expected, either the inhibition of NAD(P)H oxidase with DPI (10 \( \mu \)mol/L) or dismutation of \( \cdot \text{O}_2^- \) with exogenous SOD (500 U/mL) produced significant reduction in \( \cdot \text{O}_2^- \) production (Figure 6), whereas L-NAME, rotenone, and oxypurinol showed no effects (not shown).

#### Serum-Induced Superoxide Generation and Adventitial Fibroblast Proliferation

To assess whether altering ROS generation could modulate adventitial fibroblast proliferation in vitro, growth inhibition of serum-stimulated cells was determined either by inhibiting...
Adventitial fibroblasts demonstrated a time-dependent and intracellular accumulation of formazan was measured. The NBT was added to cells for 1 hour, and intracellular accumulation of formazan was measured. Adventitial fibroblasts demonstrated a time-dependent -O$_2^-$ production peaking at 6 hours after stimulation (n=9 per time point). *P<0.001 vs no stimulation.

the generation of ROS (DPI) or facilitating their removal (-O$_2^-$: Tiron, SOD; and H$_2$O$_2$: catalase). The inhibitor of NAD(P)H oxidase (DPI) significantly inhibited fibroblast growth in a concentration-dependent manner (Figure 7, P<0.001). In contrast, L-NAME and oxypurinol produced no significant effects (not shown), consistent with the lack of inhibition of ROS generation by these inhibitors. The removal of either -O$_2^-$ with Tiron or H$_2$O$_2$ with catalase inhibited fibroblast proliferation. In contrast, dismutation of -O$_2^-$ to H$_2$O$_2$ after SOD did not prevent serum-induced cell replication.

**Discussion**

The major findings of this study were that (1) coronary adventitia is an important source of increased oxidative stress after endoluminal coronary injury, (2) the NAD(P)H oxidase is the major pathway for ROS generation in injured coronary arteries and stimulated adventitial fibroblasts, and (3) ROS are involved in the regulation of growth response of coronary adventitial fibroblasts.

Oxidative stress is known to increase after various forms of vascular insult. Although the presence of NAD(P)H oxidase has been shown in normal adventitia, its role in cellular proliferation during arterial repair has not previously been elucidated. In noncoronary vasculature, there is a rapid decrease in glutathione level, an indirect marker of the redox state, after mechanical injury. Others have reported the induction of p47$^{phox}$, thus implicating NAD(P)H oxidase and ROS generation in initial SMC proliferation. Likewise, p22$^{phox}$ expression and oxidative stress are increased in aortic medial SMCs after angiotensin II infusion. Unique characteristics of coronary SMCs, however, raise the question of whether similar events occur during coronary repair. Earlier studies showed an increase in -O$_2^-$ production at 2 weeks after coronary injury, although the presence of the neointima, containing cells of adventitial and medial origin, blood-borne cells, and regenerating endothelial cells, did not allow for the identification of its source. To characterize the mechanism of oxidative stress and its role in cellular proliferation, the present study focused on earlier stages of coronary response to injury, with cellular constituents still remaining at their sites of origin. Predominant increases in ROS generation and vascular NAD(P)H oxidase (p47$^{phox}$ and p67$^{phox}$ subunits) were evident in the adventitia (Figures 3 and 4). In contrast, coronary media exhibited higher levels of SOD and subsequently lower oxidative stress. It remains to be determined whether the degree of SMC differentiation, which differs among vascular beds, contributes to regional differences in the activation of NAD(P)H oxidase and ROS generation after injury. The inhibition of NAD(P)H oxidase with DPI or the removal of ROS (-O$_2^-$ and H$_2$O$_2$ with Tiron or H$_2$O$_2$ with catalase) abrogated serum-induced growth response of isolated coronary fibroblasts in vitro (Figure 7). Not surprisingly, dismutation of -O$_2^-$ to H$_2$O$_2$ after SOD was...
ineffective in preventing cell replication, pointing to the essential role of \( \text{H}_2\text{O}_2 \) in the regulation of vascular cell growth.\(^3\) The above findings suggested the involvement of ROS in a rapid proliferation of adventitial fibroblasts after coronary injury in vivo.\(^{22,24}\) The relatively slow and prolonged ROS production in adventitial fibroblasts (Figure 5) was similar to that in noncoronary SMCs but quite distinct from the faster and greater response previously seen in phagocytes.\(^3\) Preferential utilization of NADH as substrate for NAD(P)H oxidase in injured coronary arteries contrasts with the observations by others that aortic adventitial fibroblasts primarily generate \( \text{O}_2^- \) in response to NADPH.\(^{12,21}\) Several experimental conditions (eg, cell origin and type of stimulation), as well as assay methods (eg, cytochrome c reduction versus lucigenin assay) may be responsible for these differences.\(^3\)

The increase in oxidative stress stimulates cell growth, but ROS can also cause cellular death (reviewed by Griendling and Harrison).\(^3\) It is likely that these opposite results are related to the level and the type of ROS (\( \text{O}_2^- \) versus \( \text{H}_2\text{O}_2 \)).\(^{35,36}\) Much less is known, however, regarding the consequences of oxidative stress in vascular cells with a broad range of differentiation. When terminally differentiated cardiomyocytes and interstitial fibroblasts were exposed to \( \text{H}_2\text{O}_2 \), apoptosis was induced in the former and proliferation in the latter.\(^37\) Although endoluminal injury in a porcine model did not significantly enhance intracellular ROS generation in coronary media, extracellular oxidative stress may impact SMC survival. In chronic intimal lesions, inflammatory cells, particularly active in the generation of oxidative stress, have been shown to contribute to SMC apoptosis.\(^38\) The loss of differentiated coronary SMCs may lead to a decrease of a protective barrier of the intact media, resulting in the expansion of less differentiated fibroblasts and the development of intimal lesions. Our results support the notion that ROS production may serve as an attractive target for therapeutic interventions. Nevertheless, several questions remain unresolved, including the choice of antioxidants, because negative clinical results with vitamin E.\(^{39}\) In contrast, 2 independent clinical studies suggested a reduction in coronary restenosis in patients pretreated with the antioxidant procubol before angioplasty.\(^{40,41}\) The recently published Heart Outcomes Prevention Evaluation (HOPE) trial also provided evidence for the reduction of cardiovascular mortality after chronic administration of the ACE inhibitor ramipril.\(^{42}\) These results are particularly notable because NAD(P)H oxidase activity is regulated by angiotensin II.\(^{10,15,21}\) Undoubtedly, better understanding of the regulation of NAD(P)H oxidase in different vascular cells may provide further insights into the pathogenesis of coronary artery disease and aid the development of therapeutic interventions.

In conclusion, this study demonstrated an increase in NAD(P)H oxidase–derived \( \text{O}_2^- \) production in coronary adventitial fibroblasts after balloon injury. The inhibition of NAD(P)H oxidase and the attenuation of ROS production abrogated proliferative responses of adventitial fibroblasts. The results imply that ROS serve as pivotal signals for growth response of coronary fibroblasts.

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References


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