Regression of Atherosclerosis in Monkeys Reduces Vascular Superoxide Levels

Christopher A. Hathaway, Donald D. Heistad, Donald J. Piegors, Francis J. Miller, Jr

Abstract—Superoxide (O$_2^-$) in arteries may contribute to atherosclerosis in part by inactivation of nitric oxide. We hypothesized that regression of atherosclerosis in nonhuman primates is associated with a decrease in vascular NAD(P)H oxidase, decreased O$_2^-$ levels, and improved endothelium-dependent relaxation. Cynomolgus monkeys (n=28) were fed an atherogenic diet for 47±10 (mean±SE) months. In carotid arteries (containing advanced lesions), femoral arteries (moderate lesions), and saphena arteries (minimal lesions), we examined O$_2^-$ levels and vasomotor function. Compared with vessels from normal monkeys (n=8), O$_2^-$ levels (measured by lucigenin-enhanced chemiluminescence) were 3.3-fold higher in carotid, 1.7-fold higher in femoral, and not different in saphena arteries from atherosclerotic monkeys. Dihydroethidium staining also demonstrated increased O$_2^-$ levels throughout the vessel wall in femoral and carotid arteries from atherosclerotic monkeys. Components of the NAD(P)H oxidase (p22$^{phox}$ and p47$^{phox}$) were increased in atherosclerotic arteries, and immunohistochemistry demonstrated colocalization primarily to areas of macrophage infiltration. Relaxation to acetylcholine was impaired in carotid and femoral, but not saphena, arteries from atherosclerotic monkeys. After 8 months of regression diet (n=9), serum cholesterol decreased to normal, and O$_2^-$ levels (basal and NAD(P)H-stimulated), as well as expression of NAD(P)H oxidase, returned toward normal. Relaxation to acetylcholine improved in femoral arteries, but not in the more diseased carotid arteries. We conclude that, in a primate model of moderately severe atherosclerosis and regression of atherosclerosis, changes in endothelial function are inversely related to O$_2^-$ and NAD(P)H oxidase levels. Reduction in vascular O$_2^-$ during regression of atherosclerosis may contribute to improvement in vasomotor function. (Circ Res. 2002;90:277-283.)

Key Words: oxidative stress ■ vascular reactivity ■ blood vessels ■ macrophages ■ endothelium

Vascular levels of reactive oxygen species increase during hypercholesterolemia and may contribute to the pathophysiology of atherosclerosis. For example, reaction with superoxide (O$_2^-$) reduces the bioavailability of nitric oxide (NO), which impairs vasomotor function,¹ and increases platelet aggregation and monocyte adhesion. Superoxide can also activate matrix metalloproteinases and produce apoptosis, which may contribute to instability of atherosclerotic lesions.²-⁴

Reduction of cholesterol levels in patients with established coronary artery disease markedly reduces the risk of major coronary events.⁵-⁸ Improvement in vasomotor function and clinical benefits, however, occur much more rapidly than structural improvement during regression, possibly through an increase in the bioavailability of NO.⁸,⁹ This observation is compatible with the hypothesis that regression of atherosclerosis is associated with reduction of vascular reactive oxygen species (ROS) levels.

Macrophages, smooth muscle, fibroblasts, and endothelium all are potential sources of O$_2^-$ in blood vessels.²,¹⁰-¹² Reduction of endothelial O$_2^-$ by a gene transfer approach is not sufficient to restore NO-mediated relaxation in atherosclerotic aorta,¹⁰ suggesting that O$_2^-$ generation by other cell types may contribute importantly to inactivation of NO. The effect of regression of atherosclerosis on O$_2^-$ levels in different cellular components of the vessel wall has not been studied.

The primary goal of this study was to examine effects of regression of atherosclerosis on vascular O$_2^-$ levels. We used a nonhuman primate model of regression to examine O$_2^-$ levels and endothelium-dependent relaxation in arteries that vary in the severity of atherosclerosis (carotid, severe lesion; femoral, moderate lesion; saphena, minimal lesion). We tested the hypothesis that regression of chronic atherosclerosis would decrease levels of O$_2^-$ in diseased blood vessels and result in improvement of vascular function. We also hypothesized that during regression, reduction of vascular O$_2^-$ levels would be associated with a decrease in NAD(P)H oxidase activity, a major source of O$_2^-$ in vascular cells.¹²

Materials and Methods

Preparation

The Institutional Care and Use Committee of the University of Iowa approved experiments in this study. Adult male Cynomolgus mon-
keys were obtained from Biomedical Research Foundation (Houston, Tex) and fed a normal diet (Purina monkey chow,Ralston Purina; n=8) or an atherogenic diet (cholesterol 1 mg/calorie; fat, 43% of total calories, n=29). After 45±1 months (mean±SE) on an atherogenic diet, segments of common carotid, femoral, and saphena arteries were harvested (atherosclerotic) from anesthetized monkeys (ketamine, 20 mg/kg IM; sodium pentobarbital, 20 mg/kg IV and supplemented when needed). Some monkeys were allowed to recover and place on a normal diet (regression group, n=8). After 8 months of regression, monkeys were sedated (ketamine, 20 mg/kg IM), euthanized with an intravenous injection sodium pentobarbital (200 mg/kg), and contralateral segments of common carotid, femoral, and saphena arteries were removed. Loosely adhering adventitia was removed, vessels were cut into 3- to 4-mm segments and kept in Kreb’s buffer (4°C) until their use. Kreb’s buffer contained (in mmol/L) NaCl 118, KCl 4.7, CaCl2 2.5, MgSO4 1.2, NaHCO3 23, KH2PO4 1.2, and D-glucose 11. Blood samples were taken at the termination of the study for the measurement of plasma lipids.

Measurement of Isometric Tension
Vascular rings were mounted on stainless steel hooks and placed in an organ chamber bath that contained warm (37°C), aerated (95% O2, 5% CO2) Kreb’s buffer. Preliminary experiments were used to determine optimal resting tension of carotid, femoral, and saphena arteries (3 g, for all vessels), which was used in subsequent experiments. Rings were contracted twice with 60 mmol/L KCl and allowed to relax. Treatment groups were generated for acetylcholine (10-5 to 10-3 mol/L) after precontraction with PGF2α (60% to 85% of maximum contraction with KCl). Levels of precontraction with PGF2α were similar between groups. Relaxation to sodium nitroprusside (10-4 to 10-3 mol/L) was examined in each vessel segment as a measure of endothelium-independent relaxation.

Measurement of Superoxide
Lucigenin-enhanced chemiluminescence was used to assess levels of O2·− in vascular rings.10,11 Samples were added to polypropylene cuvettes containing phosphate-buffered saline (PBS; NaCl 137 mmol/L; KCl 2.7 mmol/L; Na2HPO4 4.3 mmol/L; KH2PO4 1.5 mmol/L) and lucigenin (0.005 mmol/L), and chemiluminescence was measured using a luminometer (Zylux, FB12). After dark adaptation, basal chemiluminescence was measured for 5 minutes. Cumulative concentrations of NADH (0.2 and 1 mmol/L) or NADPH (0.2 and 1 mmol/L) were added and luminescence was measured for an additional 5 minutes for each concentration. In some samples, the flavin inhibitor diphenylenediiodonitrosoamide (DPI, 0.1 mmol/L) was added after the final dose of NAD(P)H, and a 5-minute measurement of chemiluminescence performed. Other samples were pretreated with the O2·− scavenger Tiron (10 mmol/L), or inhibitors of nitric oxide synthase (NOS)-nitro-L-arginine, L-NNA, 0.01 mmol/L) or xanthine oxidase (oxypurinol, 0.1 mmol/L) for 30 minutes prior to chemiluminescence measurements as described. Relative light unit values were normalized to the surface area of the vessel.

Dihydroethidium (DHE), an oxidative fluorescent dye, was used to localize O2·− in vessel segments in situ as previously described.10 Briefly, fresh, unfixed vessel segments were frozen in OCT compound until the detection of O2·−. Transverse sections (30 mm thick) were cut in a cryostat and placed on glass slides. Samples were then incubated at room temperature for 30 minutes with DHE (0.002 mmol/L) and protected from light. Images were obtained using a Bio-Rad MRC-1024 laser (krypton/argon) scanning confocal microscope. The fluorescence excitation/emission spectrum for ethidium bromide was used during the imaging process (488 and 610 nm, respectively). Fluorescence was detected with a 585-nm long-pass filter.

Immunohistochemistry
Immunohistochemistry for macrophages and subunits of the NAD(P)H oxidase (p22phox and p47phox) were examined using a previously described protocol.14 Briefly, sections of fresh-frozen blood vessels (8 μm thick) were placed on slides and air dried overnight (≈20°C). Normal horse serum (1 hour) was used as a blocking agent. Primary antibodies against p22phox (monoclonal antibody 44.1, obtained from Dr A.J. Jesaitis, Dept of Microbiology, Montana State University), p47phox (Santa Cruz Biotechnology), or macrophages (HAM 56) were added to the tissue sample for 1 hour and then washed 3 times with PBS. The secondary antibody, biotinylated anti-rabbit (p22phox and HAM 56) or anti-goat (p47phox), was added to the tissue for 30 minutes, washed, and strep-avidin was added for another 30 minutes. Vector Blue was used as a chromogenic substrate, and the tissue sections were fixed in paraformaldehyde, washed, and counterstained with nuclear fast red. Tissue sections not treated with a primary antibody showed no staining after treatment with the secondary antibody.

Data Analysis
Data were tested for normality and homogeneity of variance. If either of these assumptions were not satisfied, the data were log transformed and tested again. Data from the vascular reactivity experiments were analyzed using a 2-way ANOVA. All other data were analyzed using 1-way ANOVA followed by the Dunnett’s method for multiple comparisons. All data are presented as mean±SE. Differences were considered to be significant when P<0.05.

Results
Physiological Parameters
The atherogenic diet produced increases in plasma low-density lipoproteins from 32±8 to 326±24 mg/dL and total cholesterol from 90±7 in normals to 373±23 mg/dL in atherosclerotic monkeys. Eight months of regression (normal) diet reduced levels of low-density lipoproteins to 24±3 mg/dL and total cholesterol to 64±4 mg/dL. There were no significant differences in body weight, plasma triglyceride levels, or hematocrit (data not shown) between normal, atherosclerotic, and regression groups of monkeys.

Morphometric Data
We chose to examine common carotid, femoral, and saphena arteries because we anticipated that the atherogenic diet would produce different severity of disease in these vessels. In normal monkeys, there was little or no neointimal formation in carotid, femoral, and saphena arteries. In atherosclerotic monkeys, intimal area and the ratio of intima to media were increased in the carotid and femoral arteries, but not in the saphena artery (Figure 1). The medial area of carotid, femoral, and saphena arteries was not altered by the atherogenic diet (data not shown). There was no significant decrease in the intimal area or intima to media ratio of carotid, femoral, or saphena arteries after 8 months of the regression diet (Figure 1).

Vasomotor Responses
Maximal contractions of femoral and saphena arteries to KCl were not altered in atherosclerotic or regression monkeys, but contraction of carotid arteries was impaired (5.1±0.3 g for normal versus 2.5±0.3 g for atherosclerotic, P<0.05). Contraction of carotid arteries to KCl did not improve after regression.

After precontraction to similar levels in all groups, relaxation of carotid and femoral arteries to acetylcholine was significantly attenuated in monkeys from the atherosclerotic group when compared with normal (Figure 2). There was no
significant impairment of relaxation to acetylcholine in saphena arteries. After 8 months on a regression diet, relaxation of carotid arteries to acetylcholine was not improved (Figure 2). In contrast, after regression in femoral arteries, relaxation to acetylcholine was normal. Maximal relaxation of carotid, femoral, and saphena arteries to sodium nitroprusside did not differ between groups.

**Superoxide Levels**

Basal levels of O$_2^-$, as detected with lucigenin-enhanced chemiluminescence, were similar in carotid, femoral, and saphena arteries harvested from normal animals. Levels of O$_2^-$ were elevated in femoral and carotid arteries of atherosclerotic monkeys, compared with normals (Figure 3A). Despite prolonged hypercholesterolemia, the saphena artery did not develop significant atherosclerotic lesions (Figure 1).

Addition of NADH to vascular segments increased O$_2^-$ to similar levels in carotid, femoral, and saphena arteries from normal animals (Figure 5A). NADH-stimulated chemiluminescence was increased in the atherosclerotic group and returned to normal levels in the regression group in all vessels examined (Figure 5A). NADPH stimulation produced similar

**Figure 1.** Cross-sectional area of intima (A) and ratio of intima to media (B) in saphena, femoral, and carotid arteries from monkeys on an atherogenic (n=28) or regression (n=9) diet. Normal vessels had no detectable intima and are not included in this figure. *P<0.05 compared with the normal group.

**Figure 2.** Relaxation to acetylcholine of arteries from normal (n=7 to 9), atherosclerotic (n=16 to 29), and regression (n=8 to 9) monkeys. *P<0.01 atherosclerotic vs normal; †P<0.01 regression vs atherosclerotic.

**Figure 3.** Basal levels of superoxide in vessel segments from normal (n=8 to 9), atherosclerotic (n=20 to 22), and regression (n=8 to 9) monkeys. Superoxide levels were measured with lucigenin-enhanced chemiluminescence (A), and data are presented as relative light units (RLU) normalized for surface area of the vessel section or by dihydroethidium staining (B). Representative laser-scanning fluorescent confocal micrographs of cross-sections of carotid arteries from normal (left), atherosclerotic (middle), and regression (right) monkeys. Red fluorescence represents dihydroethidium staining for superoxide; Yellow-green fluorescence represents autofluorescence of the blood vessel. IEL indicates internal elastic laminae. *P<0.05 vs atherosclerotic group; †P<0.05 vs normal group.

**Figure 4.** The flavin inhibitor DPI and the radical scavenger Tiron markedly inhibited O$_2^-$ in femoral arteries from atherosclerotic monkeys, whereas inhibition of nitric oxide synthase (L-NNA) and xanthine oxidase (oxypurinol) had no effect (Figure 4), suggesting an NAD(P)H oxidase as a source of O$_2^-$. Addition of NADH to vascular segments increased O$_2^-$ to similar levels in carotid, femoral, and saphena arteries from normal animals (Figure 5A). NADH-stimulated chemiluminescence was increased in the atherosclerotic group and returned to normal levels in the regression group in all vessels examined (Figure 5A). NADPH stimulation produced similar
increases in levels of O$_2^·$ of normal carotid, femoral, and saphena arteries (Figure 5B). In atherosclerotic vessels, in contrast to NADH stimulation, NADPH-stimulated O$_2^·$ levels differed in arteries with different amounts of lesion (Figure 5B). For example, the carotid artery, with the most severe atherosclerosis, had the greatest level of O$_2^·$ after NADPH treatment, and the saphena, the artery with the least disease, had the lowest levels of O$_2^·$ after NADPH. The flavoenzyme inhibitor DPI reduced NADPH-stimulated O$_2^·$ levels in atherosclerotic vessels by approximately 70%. After regression, levels of NADPH-stimulated O$_2^·$ were similar to those of normal vessels (Figure 5B).

Expression of the NAD(P)H oxidase was examined by immunostaining for both the membrane-associated component p22phox and the cytosolic component p47phox. Staining for p22phox and p47phox were increased in carotid and femoral arteries with the greatest staining localized to areas that also stained for macrophages (Figure 6). There was a decrease in immunostaining for p22phox and p47phox in arteries from regression animals, again primarily associated with intimal areas that also stained positive for macrophages (Figure 6). Expression of p22phox and p47phox was also increased in the smooth muscle cells of the medial layer of femoral arteries from atherosclerotic monkeys and decreased toward normal in arteries from regression animals (Figure 7). Similar changes in expression of p22phox and p47phox were seen in carotid arteries (not shown).

**Macrophage Infiltration**

There were few, if any, macrophages detected by immunohistochemistry in normal arteries. In contrast, many macrophages were found in the intima and adventitia of atherosclerotic vessels by approximately 70%. After regression, levels of NADPH-stimulated O$_2^·$ were similar to those of normal vessels (Figure 5B).

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rotic femoral arteries (Figure 6). After regression, there were fewer macrophages in the intima and persistence of many macrophages in the adventitia of femoral arteries (Figure 6). Similar results were obtained in carotid arteries. Macrophage infiltration was limited to the adventitia in saphena arteries, in which intimal lesions were not observed (data not shown).

**Time Controls**

Three atherosclerotic monkeys were maintained on a high cholesterol diet after removal of the carotid, femoral, and saphena arteries. Eight months later the contralateral arteries were studied as a time control for the regression animals. In these vessels, intimal lesion size in the femoral and carotid vessels did not change noticeably with the additional 8 months of atherosclerotic diet. The saphena artery contained no intimal thickening. In contrast to regression monkeys, relaxation of carotid and femoral arteries to acetycholine (10−6 mol/L) tended to be more impaired in vessels from the time control group compared with atherosclerotic vessels (28±18% and 51±22% in the carotid and femoral time controls, respectively). Dihydroethidium staining for O2− and immunostaining for macrophages and p47phox and p22phox were similar in the time control vessels compared with atherosclerotic vessels.

**Discussion**

The major findings in this model of prolonged atherosclerosis followed by a short duration of regression in primates are as follows. (1) In atherosclerotic animals, lesion severity is related to O2− levels and magnitude of impaired relaxation to acetycholine. (2) After a relatively short period of reduction of cholesterol, O2− levels decreased in atherosclerotic femoral and carotid artery, but endothelium-mediated relaxation improved only in the less diseased femoral artery. (3) After regression of atherosclerosis, decreases in O2− levels are associated with decreases in NAD(P)H oxidase activity and decreased expression of oxidase subunits p22phox and p47phox. (4) Reduction in O2− levels and oxidase expression in the vessel wall following regression of atherosclerosis is associated with reduction in intimal macrophages, but not lesion size.

In animal models, vascular O2− levels are increased after a short period of hypercholesterolemia and associated with abnormal nitric oxide–mediated relaxation.1 Vasomotor function and O2− levels are restored to normal after a short period of normocholesterolemia.15 Although this observation suggests that regression of atherosclerosis is associated with decreased vascular ROS, lesions in short-term models of hypercholesterolemia are not representative of lesions found in humans. For example, in hypercholesterolemic rabbits, the majority of aorta examined contained no macrophages,15 an important potential source of O2−. The current study used a primate model of chronic hypercholesterolemia in which moderate to severe lesions developed in the femoral and carotid arteries.

The saphena artery did not develop significant intimal thickening or impaired endothelium-dependent relaxation, despite more than 4 years of hypercholesterolemia. It is not clear why saphena arteries are protected during hypercholesterolemia. It is of interest that the absence of atherosclerotic lesions in saphena arteries was associated with normal relaxation to acetycholine because others have found impairment of endothelium-dependent relaxation during hypercholesterolemia without atherosclerotic lesions.15–17 We did not observe a significant increase in basal levels of O2− in the saphena, which could contribute to preservation of normal endothelium-dependent relaxation.

In contrast to minimal atherosclerosis in saphena arteries, moderately severe atherosclerosis was observed in carotid arteries. Despite reduction in O2− levels, relaxation of carotid artery to acetycholine did not improve after reduction of plasma cholesterol. Thus, reduction in O2− levels is not sufficient to improve endothelium-dependent relaxation in the presence of moderately severe atherosclerosis. However, O2− levels in carotid arteries from the regression group remained higher than O2− levels in carotid arteries from normal monkeys (Figure 3A). This persistent increase in O2− may contribute to impaired relaxation of carotid arteries after regression. Our previous studies of regression of atherosclerosis in monkeys demonstrated improved endothelial function in carotid arteries.18,19 Differences in the duration of atherogenic diet, duration of regression, and branch of the carotid studied may account for differences in results between these studies and the current study.

Hyperhomocysteinemia may produce vascular dysfunction through increased levels of O2−.20 In addition to hypercholesterolemia, the atherogenic diet used in this study also produces moderate homocysteinemia.21 It is unlikely, however, that changes in homocysteine contributed importantly to vascular dysfunction and increase in O2− associated with atherosclerosis in this study because reduction of homocysteine alone does not restore endothelial function.21 We cannot completely exclude, however, the possibility that reduction of homocysteine during regression contributed to the reduction of O2− levels and improvement in vascular function.

The conclusion that regression of atherosclerosis is associated with reduction in vascular NAD(P)H oxidase activity is supported by the finding of decreased O2− generation in response to NADPH and NADH, and by decreased expression of the oxidase subunits p22phox and p47phox, in vessels after regression. Because inflammatory cells are present in atherosclerotic arteries, however, it is difficult to distinguish whether changes in NAD(P)H oxidase activity reflect changes in macrophage number or oxidase activity in vascular cells. Reduction in levels of O2− and oxidase subunit expression is associated with a decrease in the number of intimal macrophages. Two findings, however, suggest that regression of atherosclerosis also decreases vascular NAD(P)H oxidase function. First, fluorescence of DHE and expression of p22phox and p47phox decrease throughout the vessel wall after regression, suggesting a reduction in NAD(P)H activity in nonphagocytic cells. Second, NADH-stimulated O2− levels increase to similar levels in the 3 types of vessels examined, despite different degrees of lesion size and macrophage content, and return to normal with regression of atherosclerosis. If NADH-stimulated O2− in atherosclerotic vessels were derived only from macrophages, levels of O2− would correlate with lesion size. Furthermore, both
NADH and NADPH are used as substrate for the vascular NAD(P)H oxidase, whereas the phagocyte NADPH oxidase uses NADPH almost exclusively. These observations suggest that a reduction in both phagocyte and nonphagocyte (vascular cell) NAD(P)H oxidase function contribute to reduction of levels of $O_2^-$ during regression of atherosclerosis.

It is controversial whether addition of NADH and NADPH to intact vessels accurately reflects NAD(P)H oxidase activity. Others have demonstrated convincingly that addition of NADH or NADPH to intact vessel segments produces increases in levels of $O_2^-$. In intact vessel segments, addition of NADH or NADPH increases levels of $O_2^-$ detected by electron paramagnetic resonance spectroscopy, HE staining, or a sensitive bioassay that measures the ability of $O_2^-$ to scavenge NO. NAD(P)H oxidase activity measured in intact vessel segments correlates with measurements made in vessel homogenate. In addition, our conclusions, based on the chemiluminescence data, are supported by the findings of increased immunostaining of oxidase components p22phox and p47phox in human atherosclerotic coronary arteries, expression of p22phox was increased throughout the vessel wall, especially within the intima. Our results are consistent with these findings.

In clinical trials, reduction of plasma cholesterol in patients with coronary atherosclerosis reduces major coronary events, including death. Angiographic studies have shown that reduction of levels of $O_2^-$ in atherosclerotic vessels accurately reflects NAD(P)H oxidase activity of intimal phagocytes. Others have demonstrated convincingly that addition of NADH and NADPH to intact vessels uniformly reflects NAD(P)H oxidase activity. NAD(P)H oxidase activity is therefore inherently limited. Two experimental approaches, however, strengthen the conclusions. First, we studied changes in 3 types of arteries that were exposed to the same level of hypercholesterolemia (and hyperhomocysteinemia), but demonstrated profoundly different severity and consequences of atherosclerosis. This approach allowed us to discriminate between changes produced by atherosclerosis versus hypercholesterolemia. Second, we used an intervention (regression of atherosclerosis) that is timely and of interest per se, and also may be of value in discriminating between associations and causal relationships. Nevertheless, it is important to acknowledge that the changes that we have observed are associations and may or may not be causally related.

In summary, our findings suggest that $O_2^-$ levels are increased and endothelium-dependent relaxation is impaired in large arteries of monkeys with moderate atherosclerosis, and that these effects are due at least in part to increased expression and activation of NAD(P)H oxidase within the vessel wall. Moreover, $O_2^-$ levels and vascular function in these arteries return toward normal within a few months of regression of atherosclerosis. Increases in expression of the p47phox and p22phox subunits of the NADPH oxidase largely colocalize with macrophages in diseased vessels and disappear from the intima after regression. NAD(P)H oxidase activity of intimal phagocytes appears to be important in vascular dysfunction that occurs with atherosclerosis. Furthermore, improvement of vascular function is associated with reduction of macrophages and NAD(P)H oxidase components in atherosclerotic vessels.

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