Inhibition of Apoptotic Signaling and Neointimal Hyperplasia by Tempol and Nitric Oxide Synthase following Vascular Injury

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Key Words

Apoptosis · Gene transfer · Nitric oxide · Oxidative stress · Restenosis

Abstract

Objectives: We hypothesized that redox-mediated apoptosis of medial smooth muscle cells (SMC) during the acute phase of vascular injury contributes to the pathophysiology of vascular disease. **Methods:** Apoptosis of medial SMC (1–14 days following balloon injury) was identified in rat carotid arteries by in situ DNA labeling. NADPH-derived superoxide and expression of Bcl-xL, Bax, caspase-3 and caspase-9 were assessed. The antioxidant tempol was administered in drinking water throughout the experimental period, and local adenoviral-mediated gene transfer of eNOS was performed prior to vascular injury. **Results:** Balloon injury increased NADPH-dependent superoxide production, medial SMC apoptosis, Bax-positive medial SMC index, Bax/Bcl-xL ratio, and caspase-3 and caspase-9 expression in the injured arteries. Treatment with tempol or eNOS gene transfer decreased superoxide levels and medial SMC apoptosis, with a concomitant increase in medial SMC density. Inhibition of superoxide was associated with a decreased Bax/Bcl-xL ratio, and caspase-3 and -9 expression. Tempol treatment and eNOS gene therapy significantly reduced neointima formation. **Conclusion:** Vascular generation of reactive oxygen species participates in Bax

activation and medial SMC apoptosis. These effects likely contribute to the shedding of cell-cell adhesion molecules and promote medial SMC migration and proliferation responsible for neointimal hyperplasia.

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Introduction

Reactive oxygen species (ROS) generated by blood vessels following injury mediate intracellular signaling and cellular growth, contributing to the pathobiology of vascular disease [1]. We and others have shown that ROS levels are increased in vascular smooth muscle cells (SMC) from atherosclerotic arteries [2] and following balloon injury [3–5]. Increased ROS production is implicated in medial SMC apoptosis following injury [6], and this increase in ROS lasts for at least 14 days [3, 7–9]. It has also been shown that the source of ROS following vascular injury is not infiltration of inflammatory cells, but instead, an increase in NADPH oxidase activity in vascular SMC and fibroblasts [10]. These studies have suggested that ROS may have a critical role in the responses associated with vascular remodeling, including apoptosis, and migration and proliferation of SMC. We have recently demonstrated that tempol treatment inhibits neointima formation in balloon-injured rat carotid artery [11]. Although numerous studies have demonstrated

the usefulness of anti-oxidant therapy to inhibit neointimal hyperplasia [9, 12–15], the mechanisms by which antioxidants inhibit SMC proliferation following angioplasty are not fully understood.

Studies have provided evidence for the role of SMC apoptosis in the progression of atherosclerosis, as well as in the development of neointimal hyperplasia after balloon injury [2, 6, 16]. It has been postulated that apoptosis of a subset of medial SMC during the acute phase following injury may trigger a different subset of SMC to migrate and proliferate to form neointima [17]. Although the primary ROS-dependent determinants of medial SMC apoptosis have not been fully elucidated, emerging evidence suggests a role for the Bcl-2 protein Bax. Bcl-2 is critical to apoptosis in response to a variety of stimuli [18, 19], and its response is mediated by dimerization with other pro-apoptotic Bcl-2-related proteins [19]. Anti-apoptotic members of the same family, e.g. Bcl-xL, prevent apoptosis by heterodimerizing with pro-apoptotic molecules [20], and it is the balance between pro- and anti-apoptotic signaling that determines the fate of the medial SMC in response to injury.

We and others have demonstrated that nitric oxide (NO) inhibits SMC proliferation and migration, both *in vivo* and *in vitro* [21–27]. The anti-proliferative properties of eNOS gene therapy may be mediated in part by its antioxidant effects. The temporal changes in ROS generation and the biochemical changes occurring in the vascular wall remain poorly defined. Furthermore, it is unclear whether different antioxidant therapies reduce neointima formation by inhibition of similar signaling pathways. The aims of the present study were to investigate the effects of the antioxidant tempol on the temporal expression of Bcl-xL, Bax and caspases-3 and -9 in balloon-injured rat carotid artery, and to determine the link between medial SMC apoptosis and neointima formation.

Materials and Methods

Animal Preparation

Adult male Sprague-Dawley rats weighing 300–325 g were used for this study. Animals were housed in a room with a 12-hour light/12-hour dark cycle and ambient temperature. All experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all protocols were approved by the Animal Care and Use Committee at the University of Iowa.

Surgical Procedures and Drug Treatment Protocol

Balloon catheter injury of the left common carotid artery of adult rats was performed as described previously [11]. In brief, the

rats were pre-anesthetized with halothane (Halocarbon, River Edge, N.J., USA) and anesthetized by intraperitoneal injection of ketamine (10 mg/kg). To produce balloon injury, the bifurcation of the left common carotid artery was exposed through a midline incision in the cervical region, and the left common and internal carotid arteries were temporarily ligated. A 2-french embolectomy balloon catheter (Baxter Healthcare, Deerfield, Ill., USA) was introduced into the isolated common carotid artery through an arteriotomy site in the external carotid artery, and was advanced to the distal ligation of the common carotid artery. The balloon was inflated with air and drawn toward the arteriotomy 3 times to produce a distending, de-endothelializing injury. After withdrawal of the catheter, the proximal end of the external carotid artery was ligated, and blood flow was restored by relieving the ligation of the common carotid artery. The surgical incision was closed and the rats were allowed to recover from anesthesia. The right, uninjured artery was used as a control. For one group of animals, tempol (1 mM; freshly dissolved in drinking water) was administered orally. Tempol administration was initiated 1 day prior to surgery and was continued throughout the experiment. A second group was subjected to eNOS gene transfer, as detailed below.

In vivo eNOS Gene Transfer

Replication-deficient recombinant adenovirus Ad5/RSVeNOS was obtained from the Vector Core at the University of Iowa Carver College of Medicine, Iowa City. The E1A, E1B, and E3 regions of the adenovirus were deleted to impair the ability of the viral construct to replicate in nonpermissive cells. *In vivo* gene transfer to catheter balloon-injured carotid arteries was performed essentially as described earlier [21]. In brief, rats were anesthetized by intraperitoneal injection of ketamine, and 60 μ l of 10^{10} plaque-forming units per milliliter of Ad5/RSVeNOS or Ad5/RSVLacZ (control) adenovirus were instilled into the injured arterial segment, which became distended and remained so for 30 min. Blood flow was restored after the syringe was withdrawn. The animals were killed either 3 days or 14 days after the surgery, at which point uninjured and injured arteries were processed for immunostaining or histological examination.

Immunohistochemistry for Bax

Formalin-fixed and paraffin-embedded tissue sections (6 μ m thick) were mounted on poly-L-lysine-coated glass slides. After deparaffinization and rehydration, endogenous peroxidase activity in the sections was quenched using 3% H₂O₂ for 10 min, and this was followed by washing with PBS. For antigen retrieval, the samples were boiled in citrate buffer, pH 6.0, in a microwave oven for 5 min, and then allowed to stand at room temperature for 20 min. Blocking serum was applied for 30 min, followed by overnight incubation at 4°C with a primary anti-Bax antibody (1:50, Santa Cruz Biotechnology, Santa Cruz, Calif., USA). The sections were then incubated with the biotinylated secondary antibody and the peroxidase-labeled ABC solution (Vectastain) for 30 min each. All dilutions were made in phosphate-buffered saline (PBS; pH 7.2), and all incubations were performed in humid chambers at room temperature. Between each step of the staining procedure, slides were rinsed three times in PBS. Peroxidase activity was demonstrated by exposing sections to a substrate, 3,3'-diaminobenzidine tetrahydrochloride. For negative control sections, PBS was substituted for the primary antibody. The index of Bax-

positive cells was calculated as the percentage of positive cells relative to the total number of cells.

Evaluation of Apoptosis in Medial SMC

Apoptotic cells from the medial arterial layer were visualized using the Apop Tag in situ detection kit (Trevigen, Gaithersburg, Md., USA). The staining procedure was performed according to the manufacturer's instructions. Briefly, formalin-fixed, paraffin-embedded sections (6 μ m) were deparaffinized, rehydrated and washed with PBS buffer. The slides were immersed in 3% H₂O₂ for 10 min and then washed with PBS. Slides were then exposed to equilibration buffer for 10 min, after which the terminal deoxynucleotidyl transferase reaction mixture was added to the samples, and incubation at 37°C in a humidified chamber proceeded for 1 h. The reaction was terminated by immersion of the slides into stop/wash buffer. After washing of the slides, anti-digoxigenin-peroxidase was added and samples were incubated in a humidified chamber at 37°C for 30 min, then washed, stained with diaminobenzidine substrate and counterstained with 1% methyl green. For negative controls, terminal deoxynucleotidyl transferase was substituted by PBS. Sections treated with DNase I served as positive controls.

Western Blot Analysis

Tissue samples were snap-frozen in liquid nitrogen and stored at -80°C. The samples were homogenized in lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 2 mmol/l EDTA, 10 μ g/ml leupeptin, 1 μ g/ml aprotinin and 0.02% NaN₃). Homogenates were then centrifuged (10,000 g for 10 min at 4°C), and subsequently protein extracts were aliquoted for further experiments and stored at -80°C. Protein extracts (35 μ g) were separated on 12% SDS-PAGE gels and transferred to Immobilon-P membranes (Millipore, Billerica, Mass., USA). Before blocking in skimmed milk (5%), protein loading was checked by Ponceau S (Sigma, St. Louis, Mo., USA) staining. Blots were incubated overnight at 4°C, with mouse monoclonal anti-Bcl-xL antibodies at a 1:1,000 dilution, mouse monoclonal anti-Bax antibody at a 1:1,000 dilution, mouse anti-caspase-9 at a 1:1,000 dilution, or anticaspase-3 at a 1:1,000 dilution (all from Santa Cruz). The membrane was washed and then incubated with secondary antibody conjugated with horseradish peroxidase (Amersham, Little Chalfont, UK), in 1% non-fat dry milk in TBS-T buffer for 1 h. Enhanced chemiluminescence reagent (Amersham Biosciences) served as substrate solution and was used according to the manufacturer's instructions. Horizontal scanning densitometry of films was performed using the Quantity One GS-710 Densitometry kit (Bio-Rad, Hercules, Calif., USA). Quantitative analysis of Bax/Bcl-xL protein expression was performed using a scanning densitometer and Multianalyst Software (Quantity One). Detection of Bax and Bcl-xL was performed separately on the same membrane. For each sample, the ratio of Bax/Bcl-xL expression was estimated from the same filter.

Caspase-3 and -9 Analysis

Arterial lysate was prepared using buffer containing (in mM) sodium pyrophosphate 50, NaF 50, NaCl 50, EDTA 5, EGTA 5, Na₃VO₄ 2, HEPES 10, pH 7.4, with a mixture of protease inhibitors (0.1 mg/ml phenylmethylsulfonyl fluoride, 2 μ g/ml leupeptin, and 1 μ g/ml pepstatin A). Protein (25 μ g) was separated

by electrophoresis on 12% polyacrylamide gel and transferred onto a PVDF membrane in a cooling system at 100 V for 1 h. The membranes were sequentially incubated with primary antibody that recognizes the active form only (catalogue No. SC-7148 and SC-7885 for caspase-3 and -9, respectively; Santa Cruz). Unspecific binding was blocked by incubation of the blotting membranes with 1% bovine serum albumin in incubation buffer [50 mM Tris, 150 mM NaCl, 0.1% (vol/vol) Tween 20, pH 8.0]. The membrane was washed and incubated with secondary antibody conjugated with horseradish peroxidase in 1% non-fat dry milk in TBS-T buffer for 1 h. The samples were subsequently developed with ECL Western blotting detection reagents (Amersham Biosciences), served as substrate solution and were used according to the manufacturer's instructions. Horizontal scanning densitometry of films was performed using the Quantity One GS-710 Densitometry kit (Bio-Rad).

Assessment of Medial SMC Density

Cryosections (7 μ m) were stained for 2 h at 37°C with Hoechst 33258 (5 μ g/ml) (Molecular Probes, Carlsbad, Calif., USA). Images were captured with a $\times 40$ objective on an epifluorescence microscope in 3–4 different fields, and the area was calculated by a computerized program. The cellularity in the medial layer was expressed as the Hoechst index, which is defined as the positive nuclear staining averaged for one fixed area (0.1 mm²). Histological samples from different treatment groups were examined in random order by an independent investigator who was blinded to the treatment protocol.

Superoxide Measurement by Lucigenin-Enhanced Chemiluminescence

ROS were measured using lucigenin-enhanced chemiluminescence. Briefly, frozen sections (40 μ m thick) of carotid arteries were incubated for 10 min in the dark, in 1 ml of PBS containing 5 μ M lucigenin (bis-N-methylacridinium). Samples were placed in a luminometer (Monolight 2010) for the measurement of relative light units emitted every 30 s over a period of 4 min. The values obtained between 2 and 4 min were averaged to determine basal emission levels. NADPH (50 μ M, Sigma) was then added, and luminescence was measured every 30 s over a period of 4 min. The values obtained between 2 and 4 min were averaged and the basal emission level was subtracted.

Cytochrome c Reduction Assay

Tissue homogenate was adjusted to a final protein concentration of 1 mg/ml using Dulbecco's modified Eagle's medium without phenol red, and was distributed into 96-well plates in a final volume of 200 μ l/well. Cytochrome c (500 μ mol/l) and NADPH (100 μ mol/l) were added to the reaction mixture in the presence or absence of SOD (200 U/ml), and incubated at room temperature for 30 min. Reduction of cytochrome c was measured by reading absorbance at 550 nm on a microplate reader (Titertek multiscan plus; Flow Laboratories, McLean, Va., USA).

Histomorphometric Measurement

Fourteen days after angioplasty, animals were anesthetized as described above. The neck and thorax were opened to expose the carotid arteries, the heart and the great vessels. Carotid arteries were pressure fixed by the perfusion of first 200 ml of pure saline and then 500 ml of saline containing 4% paraformaldehyde through

a large cannula placed in the left ventricle. Perfusion was carried out over a 20-min period at a pressure of 100 mm Hg. After perfusion fixation, the right and left carotid arteries were excised, excess periaortal tissue was trimmed, and arteries were placed in 4% paraformaldehyde for 48 h before paraffin embedding. Cross-sections (6 μ m) were obtained from uninjured control carotid arteries, injured carotid arteries from the control group, and injured carotid arteries from the tempol-treated group and eNOS gene transduced group, and these were stained with hematoxylin and eosin. Morphometric analysis of these arterial sections was performed as described previously [15]. The images were digitized using an Olympus BX 51 microscope (Leeds precision Instruments, Minneapolis, Minn., USA) and photographs were captured using a digital camera. For each arterial cross-section, the intimal and medial areas were measured, and the ratio was calculated. Morphometric analysis was performed using NIH Image Analysis Software.

Statistical Analysis

Comparisons between different treatment groups were made at various time intervals. Data are expressed as means $\pm$ SEM. Statistical comparisons, including the type $\times$ time relations, were performed by two-way analysis of variance followed by a Bonferroni test. The comparisons were also estimated by paired t test. A value of $p < 0.05$ was considered significant.

Results

As we have previously reported [11, 21], concentric intimal hyperplasia develops in carotid arteries within 2 weeks following balloon injury (fig. 1). Immunostaining for smooth muscle α -actin confirmed that the neointima consisted primarily of SMC (data not shown). Development of neointima, measured as the intima/media ratio, was markedly attenuated by two distinct antioxidant treatments: the systemic administration of tempol, or by overexpression of eNOS at the site of injury (fig. 1). Tempol treatment reduced the intima/media ratio of the injured arteries by 58%, whereas eNOS gene transfer reduced the intima/media ratio by 38% (fig. 1). We have previously shown by immunostaining, immunoblotting, and activity assay that adenoviral-mediated eNOS gene transfer at the site of injury results in increased expression of functional NOS in injured arteries [21].

NADPH-dependent superoxide levels reached maximal levels within 24 h after injury and remained elevated 14 days after injury as measured by lucigenin-enhanced chemiluminescence and SOD-inhibitable reduction in cytochrome c (fig. 2a, b). Tempol treatment significantly decreased superoxide levels at each time point in the carotid artery after injury (fig. 2a, b). Similarly, eNOS gene transfer led to a reduction in NADPH-dependent superoxide levels, and the extent of inhibition was similar between two antioxidants (fig. 2c).

Fig. 1. Effects of tempol and eNOS gene transfer on intimal hyperplasia in balloon-injured arteries. Histological sections of carotid arteries were obtained 14 days after balloon injury and stained with hematoxylin and eosin. Note the intima formation in the injured artery (b) compared to the noninjured artery (a). Tempol administration in drinking water (c) or local adenoviral-mediated gene transfer of eNOS (d) markedly reduced intima formation in injured arteries. **e** Summary data of the intima-media ratios are shown (means $\pm$ SEM, $n = 4$). * $p < 0.05$ vs. uninjured; ** $p < 0.05$ vs. injured.

To assess the proportion of medial SMC expressing Bax protein, we performed immunohistochemistry on arterial sections from sham, injured and tempol-treated injured carotid arteries at different time intervals (days 1–14) and 3 days after injury for the arteries receiving

Fig. 2. Effects of tempol and eNOS gene transfer on superoxide levels in balloon-injured rat arteries. NADPH oxidase-dependent superoxide production was measured by lucigenin-enhanced chemiluminescence (a) or reduction in cytochrome c (b) between 1 and 14 days after balloon injury in the uninjured (○), injured (●), and tempol-treated (▼) carotid arteries. c NADPH oxidase-dependent superoxide production measured by lucigenin-enhanced chemiluminescence 3 days after injury in tempol-treated and eNOS gene-transferred arteries. RLU = Mean relative light units per second obtained between 2 and 4 min of measurement (means $\pm$ SEM, n = 4). * p < 0.05 vs. uninjured; ** p < 0.05 vs. injured.

eNOS gene transfer. The Bax-positive cell index (Bax positive/total cells $\times$ 100) within the media of injured carotid arteries was more than 30-fold greater than that in uninjured arteries 1 day following balloon injury, with decreases observed over the next 14 days (fig. 3a). Tempol treatment significantly decreased the proportion of Bax-positive cells at all time intervals examined (fig. 3a). Gene transfer of eNOS resulted in a 40% reduction of Bax-positive medial SMC after injury (fig. 3b). Figure 3c shows a representative photomicrograph demonstrating immunoreactive Bax-positive SMC in injured arteries, which is reduced when the vessels were overexpressing eNOS.

Compared to control arteries, Bax protein expression was increased in injured arteries with maximal differences 3 days after injury, and a decline by 7 days after injury (fig. 4a). Tempol treatment reduced Bax expression in the injured arteries (fig. 4a). In contrast, Bcl-xL expression in injured and uninjured arteries was unaffected at all time points between 1 and 14 days following

injury, and tempol treatment had no effect on Bcl-xL levels in injured vessels. A Bax/Bcl-xL ratio of 0.5 was observed in uninjured arteries and increased to 3.0 in injured arteries 1 day after balloon injury, declining to a Bax/Bcl-xL ratio of 1.2 by 7 days after injury. Tempol treatment significantly reduced the Bax/Bcl-xL ratio in injured vessels. Similarly to tempol treatment, eNOS gene transfer resulted in 47% reduction in Bax protein expression 3 days following injury (data not shown).

Having observed an inhibition in Bax expression in response to antioxidant therapy, we next determined whether these changes in the pro-apoptotic state are associated with similar changes in the activation of caspases, an event central to apoptosis [28]. Indeed, arterial lysates prepared 1 day after balloon injury showed a significant increase in the 17-kDa fragment cleaved from caspase-3 expression, with levels peaking 3 days after injury, which was inhibited by tempol treatment (fig. 4b). Changes in caspase-9 expression levels paralleled those

Fig. 3. Effects of tempol and eNOS gene transfer on expression of pro-apoptotic proteins in balloon-injured arteries. Immunostaining for Bax protein in carotid arteries between 1 and 14 days after vascular injury in animals treated with tempol (**a**) and 3 days after injury in animals receiving eNOS gene transfer (**b**). The percentage of Bax-positive medial SMC was calculated as positive cells per total number of cells $\times 100$. The micrographs (**c–e**) show immunohistochemistry for Bax-positive SMC (brown stain) in uninjured (**c**), injured (**d**), and injured arteries after eNOS gene transfer (**e**). * $p < 0.05$ vs. uninjured; ** $p < 0.05$ vs. injured.

observed for caspase-3, with a peak observed 3 days after balloon injury and a significant reduction observed after tempol treatment (fig. 4c; online suppl. fig. 1, www.karger.com/doi/10.1159/000151444).

In arterial sections sequential to those used for Bax immunostaining, medial SMC apoptosis was assessed by in situ DNA labeling (TUNEL assay). The medial SMC apoptotic index increased $>15\%$ 1 day after injury. Tempol treatment inhibited medial SMC apoptosis in the injured arteries by day 1, and this effect persisted at subsequent time intervals (fig. 5a). The apoptotic index was also estimated by immunostaining for activated caspase-3 (fig. 5b), and the results were similar to those obtained by the TUNEL assay. eNOS gene transfer reduced the apoptotic index by 64% 3 days after injury. Finally, we measured SMC density in the arterial samples as an indicator of the sum effect of apoptosis and proliferation in the medial layer. SMC density was decreased in injured arteries compared to uninjured control arteries, and tempol treatment significantly ameliorated this effect (fig. 6).

Discussion

The major findings of this study are that (i) NADPH oxidase-derived ROS generation increases in injured arteries; (ii) temporal changes in ROS levels are paralleled by activation of the redox-sensitive Bcl-2 family protein Bax and activated caspase-3 and -9; (iii) changes in ROS and Bax expression parallel the apoptosis of medial SMC and associated reduction in medial SMC density; (iv) inhibition of ROS by two distinct antioxidants, tempol and eNOS, have similar effects on reducing Bax, caspase-3 and -9, and medial SMC apoptosis, and (v) increasing antioxidant capacity of the blood vessel restored medial SMC density and decreased neointima formation following injury. Collectively, these findings indicate that ROS generation at the site of vascular injury plays a role in mediating apoptosis and in the development of neointimal hyperplasia.

Although a role for ROS in the development of intimal formation following injury has been demonstrated [3–5, 11], the mechanisms that couple proliferative response to injury via ROS generation remain unclear. In the current study, we present novel data showing the temporal changes in ROS levels from day 1 to 14 and correlate these changes with biochemical alterations in the vessel wall. Although we did not attempt to define the cellular source of ROS generation following carotid injury, consistent with previous observations [10], the absence of infiltrat-

Fig. 4. Effects of tempol on expression of Bax and caspase protein expression in balloon-injured arteries. Expression of Bax protein (a), caspase-3 (b), and caspase-9 (c) in carotid arteries were measured by Western blotting between 1 and 14 days after balloon injury. Results are densitometry data presented as means $\pm$ SEM, n = 4. * $p < 0.05$ vs. uninjured; ** $p < 0.05$ vs. injured.

Fig. 5. Effects of tempol on apoptotic activity in balloon-injured rat arteries. TUNEL-positive SMC (a) and caspase-3-positive SMC (b) were detected in carotid artery sections by immunostaining 1–14 days after balloon injury (means $\pm$ SEM, n = 4). * $p < 0.05$ vs. uninjured; ** $p < 0.05$ vs. injured.

Fig. 6. Effects of tempol on medial SMC density in balloon-injured arteries. Cell density was measured in carotid artery sections after staining with Hoechst 33258 at three different time points following balloon injury. The number of cells was averaged for a fixed area (0.1 mm²) of the medial layer (means $\pm$ SEM, n = 5). * p < 0.05 vs. uninjured; ** p < 0.05 vs. injured.

ing inflammatory cells suggests the ROS are derived from vascular SMC and fibroblasts.

Recent studies have shown that NADPH oxidase is the major source of the superoxide produced in the balloon-injured artery [29, 30], and the inhibition of NADPH oxidase reduces superoxide levels. Potential mechanisms by which NADPH oxidases increase ROS levels in response to vascular injury may include increased activation of NADPH oxidase and/or an increased expression of NADPH oxidase. To identify the role of NADPH oxidase-derived ROS in neointima formation, it will be desirable to evaluate the response to vascular injury in genetically modified animals with varying expression of the different catalytic subunits of NADPH oxidase.

Redox-dependent apoptosis may be one of the mechanisms by which vascular ROS contribute to neointimal hyperplasia. The observation that inhibiting medial SMC apoptosis by local delivery of the caspase inhibitor ZVAD-fmk during balloon-mediated injury results in a significant reduction in neointimal proliferation 4 weeks after injury [20] supports our proposal that inhibiting medial SMC apoptosis – and thus increasing medial SMC density – may reduce neointima formation following vascular injury. In contrast, induction of medial SMC apoptosis in transgenic mice expressing human diphtheria toxin receptor did not result in neointima formation [31]. The lat-

ter data suggest that apoptosis alone is not sufficient to induce neointimal hyperplasia. Observations in the current and in previous studies [3, 6, 11, 16, 32, 33] utilizing a rat carotid injury model suggest that loss of medial SMC contributes to neointimal hyperplasia. Although this discrepancy in the arterial response between these two animal models may be attributed to species differences (mice vs. rats), it is more likely that the signals responsible in initiating apoptosis, or the concurrent increase in ROS accompanying injury, are necessary to activate medial SMC to migrate and proliferate, thereby forming neointima.

Further, the apoptotic response to injury is mediated by the oxidative stress which in addition to producing medial SMC apoptosis may also induce shedding of cell-cell and cell-matrix connections favoring cell migration and proliferation. Nonetheless, our data do not conclusively demonstrate a primary causal role for apoptosis in initiating neointimal hyperplasia.

ROS-mediated mechanisms associated with SMC apoptosis are not well defined. In the present study, we found that Bax expression and Bax immunoreactivity in medial SMC occurred maximally 3 days after injury and these levels returned to near basal values by day 14, with minimal changes observed in Bcl-xL expression. On the contrary, Shibata et al. [34] observed that balloon injury resulted in a small increase in Bax expression and a significant increase in Bcl-xL expression estimated 14 days after balloon injury. Inhibition of Rho-kinase enhanced Bax expression and reduced neointima formation [34]. Whereas we showed that inhibition of Bax by tempol was associated with reduced apoptosis and neointima formation, they found that inhibition of Rho-kinase increased Bax and reduced neointima formation. These apparent discordant observations may be explained in that intimal formation can be reduced either by limiting migration of SMC from the media with subsequent proliferation in the intima (consistent with our observations), or by increasing intimal SMC apoptosis (consistent with Shibata et al. [34]).

We demonstrated that inhibition of ROS by two different antioxidants inhibited medial SMC apoptosis, and the effects of antioxidants on ROS levels paralleled inhibition of Bax and activated caspase expression. Similarly, in atherosclerotic lesions, SMC have been shown to express Bax at high levels [35]. Bax immunoreactivity in SMC was diminished as cholesterol levels were reduced and these changes were associated with a decrease in SMC apoptosis [35]. Furthermore, inhibiting Bcl-xL expression has been shown to induce SMC apoptosis [32], whereas overexpressing Bcl-2 has been shown to inhibit injury-induced apoptosis in these cells [33]. Our findings

regarding the temporal association of ROS and Bax-positive medial SMC by immunoreactivity and immunoblotting suggest that activation of NADPH oxidase may be an important upstream regulator of the signaling pathway leading to Bax activation. An increased Bax/Bcl-xL ratio in the injured arteries, combined with the fact that inhibiting ROS by tempol administration or eNOS gene transfer can change the balance of Bax/Bcl-xL to favor survival of medial SMC, supports a role for Bax in inducing ROS-mediated apoptosis of medial SMC [36].

It has been postulated that apoptosis during the early stages after balloon injury stimulates restenosis by provoking a wound-healing process characterized by increased migration and proliferation of the remaining SMC [37]. Previous studies suggest that dismantling of cadherin junctions between SMC and the release of β -catenin modulate SMC apoptosis, migration and proliferation [38–43]. It is of note that in our preliminary data, thrombin-induced SMC migration depended on an NADPH oxidase-dependent shedding of the extracellular domain of N-cadherin [unpubl. observ.]. These data suggest that NADPH oxidase-derived ROS may stimulate medial SMC migration and proliferation to the subendothelial space by dismantling cadherin junctions and cell-cell contacts. In the present study, we measured SMC density as a surrogate marker of cell-cell contacts and found a decrease in medial SMC density in the injured arteries. Inhibition of ROS increased medial SMC density and correlated with the extent of intimal hyperplasia (fig. 1, 6). The role of ROS-mediated dismantling of N-cadherin and cell-cell contacts in the migration and proliferation of SMC requires further investigation.

The results of the current study suggest that the anti-proliferative effects of eNOS are due, at least in part, to the antioxidant properties of NO. An increase in superoxide levels will result in a reduction in the bioavailability of NO and the subsequent formation of peroxynitrite.

In this way, an increase in NADPH oxidase activity and superoxide generation not only has the potential to reduce levels of the protective compound NO but also increases levels of the toxic compound peroxynitrite. Antioxidants have served as useful tools in demonstrating a causal role for ROS in the formation of neointima. Whereas previous animal studies using the antioxidant probucol have highlighted the benefits of antioxidant therapy with respect to the inhibition of neointima formation [9, 13, 14], probucol had to be administered as much as 4 weeks prior to vascular injury in order to be effective. The data presented here demonstrate that, in contrast, tempol administration 1 day prior to injury and continued during the experimental period is sufficient to decrease ROS levels, inhibit the initial phase of medial SMC apoptosis, and attenuate neointima formation.

In summary, we show that ROS differentially alters Bcl-2 family protein expression following vascular injury, resulting in a pro-apoptotic state of medial SMC that may contribute to intimal hyperplasia. We provide novel observations of the temporal changes in ROS levels and biochemical alterations following balloon injury, and have provided a link between superoxide levels, Bax expression, medial SMC apoptosis, and neointima formation. These findings suggest that increasing antioxidant capacity of the blood vessel may attenuate vascular proliferative processes and subsequent neointima formation in part by inhibiting medial SMC apoptosis.

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