Vascular Oxidant Stress Enhances Progression and Angiogenesis of Experimental Atheroma

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Background—Although multiple pathologic processes have been associated with oxidative stress, the causative relation between oxidative stress and arterial lesion progression remains unclear.

Methods and Results—To test the effect of creating arterial wall oxidative stress, we compared progression of mouse carotid lesions induced by flow cessation in the wild-type (WT) versus transgenic mice (Tg22vsmc), in which overexpression of p22phox, a critical component of NAD(P)H oxidase was targeted to smooth muscle cell (SMC). Compared with WT mice, arterial lesions grew significantly larger in Tg22vsmc (P<0.001) and demonstrated elevated hydrogen peroxide (H2O2) and vascular endothelial growth factor (VEGF) levels at all time points examined (P<0.001, n=4 animals per time point), probably related to increased expression of hypoxia inducible factor (HIF)-1α via SMC oxidative stress in the Tg22vsmc arteries, both basally (203±12% versus WT, P<0.001, n=3) and after lesion formation. Interestingly, Tg22vsmc lesions were complicated by extensive neointimal angiogenesis. In vitro experiments confirmed SMCs isolated from Tg22vsmc to be the source for increased H2O2, VEGF, and HIF-1α and their capacity to induce angiogenic cord-like structures when cocultured with endothelial cells. The antioxidant ebselen inhibited SMC activities in vitro and intralosomal angiogenesis and lesion progression in vivo.

Conclusions—We have demonstrated a novel pathway by which oxidative stress can trigger in vivo an angiogenic switch associated with experimental plaque progression and angiogenesis. This pathway may be related to human atheroma progression and destabilization through intraplaque hemorrhage. (Circulation. 2004;109:520-525.)

Key Words: angiogenesis | atherosclerosis | free radicals

In vitro and in vivo experimental models have demonstrated that atherosclerotic lesions are characterized by oxidative stress caused by inflammatory and vascular cell production of reactive oxygen species (ROS).1 Because the causative relation remains unclear, we investigated the hypothesis that oxidative stress drives arterial lesion progression. On the basis of findings that membrane-associated NAD(P)H oxidase activity is a major vascular source of ROS and that the level of p22phox expression can modulate vascular smooth muscle cell (SMC) NAD(P)H oxidase activity,2,3 we developed a transgenic mouse with targeted SMC overexpression of the p22phox subunit of NAD(P)H oxidase (Tg22vsmc). Clinical significance is supported by a reported association of a specific polymorphism of vascular p22phox with progression of coronary artery disease as determined by serial angiography.4 We then evaluated the effects of elevated vascular ROS production via upregulation of p22phox on carotid artery lesion progression in vivo in Tg22vsmc and wild-type (WT) mice and confirmed the contribution of transgenic SMCs by performing in vitro analyses of their angiogenesis-related activities. The causal role of oxidative stress was further confirmed by the effects of in vitro and in vivo antioxidant treatment.

Methods

Mouse Carotid Injury Model
Tg22vsmc mice were generated by cloning the p22phox and a SV40 poly A sequence immediately 3’ to the SMC actin promoter SMP8 in a C57/BL/6J background (Jackson Laboratories, Bar Harbor, Maine). RNase protection assays and Western blotting confirmed arterial overexpression of p22phox. Experimental lesions were induced by ligation of the left common carotid artery.6 Groups of 10 to 12 WT or Tg22vsmc animals were killed at 0, 7, 14, and 28 days after surgery. Carotid arteries were collected fresh or after perfusion with zinc fixative at physiological pressure. Fixed paraffin-embedded specimens were used for histological analysis and morphometry. Frozen sections of O.C.T. (Sakura)–embedded tissues were used for in situ ROS detection. In addition, groups of 3 Tg22vsmc animals underwent infusion of ebselen (Cayman; 50% vol/vol in DMSO, 10 mg · kg⁻¹ · d⁻¹) versus saline (50% vol/vol in DMSO) during the first 14 days after lesion initiation via subcutaneous osmotic minipumps (Durect). Carotid arteries from

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these mice were collected fresh and assayed for ROS levels and angiogenesis. The Institutional Animal Care and Use Committee of Emory University School of Medicine approved all animal protocols.

**Carotid Artery Histological Analyses**

Morphometric analysis was performed at the level of maximum lesion.6 Neointimal angiogenesis was visualized by immunohistochemistry after blocking for endogenous peroxidase activity (3% H2O2 solution) and nonspecific binding (rabbit serum) by use of rat monoclonal antibody against murine CD31 (BD PharMingen) and biotinylated rabbit anti-rat IgG followed by chromogenic detection (Vector). For in situ localization of ROS, we incubated carotid artery frozen sections with fluorophores sensitive to superoxide (O2−), DHE (10 μmol/L), or hydrogen peroxide (H2O2). DCFDA (5 μmol/L, Molecular Probes).7 H2O2 detection was confirmed by simultaneously treating consecutive sections with polyethylene glycol (PEG)-catalase (Sigma, 350 U/mL). Sections incubated with vehicle served as negative controls. All images were acquired at identical settings with a Zeiss Axioskop microscope.

**Carotid Artery Biochemical Analysis**

Fresh tissue harvested from 4 animals for each time point was processed for total protein and mRNA with TriPure Isolation Reagent (Roche). We determined carotid artery vascular endothelial growth factor (VEGF) mRNA levels using real-time PCR (Roche Lightcycler) with the following primers: +559 (5′-AGACGTGTAAATGTTCTGCAAA-3′) and +806 (5′-CCTTCTCCCTATCATGGTTTC-3′), normalized by use of 18S rRNA–derived cDNA amplified with QuantumRNA Classic II 18S primers (Ambion). Samples without cDNA served as negative controls. VEGF and hypoxia-inducible factor-1α (HIF-1α) protein levels were determined in individual carotid artery lysates by Western blotting using rabbit polyclonal antibodies against human VEGF and rabbit polyclonal antibodies against human HIF-1α (Santa Cruz) and peroxidase-conjugated donkey anti-rabbit IgG and chemiluminescence (Amersham). Bands were quantified by optical densitometry (BioRad). Additional carotid artery lysates were assayed for gelatinolytic activity by zymography.6

**In Vitro SMC Experiments**

Monolayers of SMCs obtained from aortic explants harvested from Tg222smc and WT mice+ were made quiescent in serum-free medium (24 hours) followed by 24-hour treatments with CoCl2 (100 μmol/L, Sigma), a chemical mimic of hypoxia,8 or various concentrations (10, 20, and 40 μmol/L) of ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one, Cayman; 50% vol/vol in DMSO) to confirm the role of H2O2. Potential effects on SMC viability were measured with the Live/Dead viability/cytotoxicity kit (Molecular Probes) and a fluorescence multwell plate reader (Cytofluor Series 4000, Perceptive Biosystems). Conditioned media were assayed for VEGF expression by Western blotting and ELISA (Oncogene) and normalized for total protein amounts determined by Bradford assay (BioRad). Gelatinolytic activity was assessed by SDS-PAGE zymography. HIF-1α expression was determined in SMC lysates by Western blotting and in monolayers by immunofluorescence using the same primary antibody followed by Alexa568-conjugated goat anti-rabbit IgG (Molecular Probes).

**Analysis of ROS in Isolated SMCs**

Levels of O2− and H2O2 were determined by incubation of quiescent SMC monolayers with DHE or DCFDA fluorophores10 and analyzed by fluorescent microscopy (DHE) or fluorescent spectroscopy (Perceptive Biosystems). Results were normalized for cell number determined by nuclear counterstaining with Hoechst (Sigma). SMC membrane fractions11 were subjected to electron spin resonance (ESR) spectroscopy (Bruker) to quantitatively determine NAD(P)/H oxidase–derived ROS production. O2− was detected by incubating 10 μg of protein with the spin probe 1-hydroxy-3-carboxy-2,2,5,5-tetramethyl-pyrroline hydrochloride (CPh; 1 mmol/L, Alexis) and DTPA (0.1 mmol/L, Sigma) in the presence or absence of NADPH (200 μmol/L, Sigma)12 and confirmed by inhibition with manganese superoxide dismutase (25 U/mL, Sigma). CPh does not react with H2O2 directly. H2O2 was detectable by co-oxidation of CPh in a horse-radish peroxidase (1 U/mL, Sigma)-acetalaminophenol (1 mmol/L, Sigma) reaction only in the presence of manganese superoxide dismutase. NADPH-dependent H2O2 production was confirmed by inhibition of the ESR signal with catalase (0.1 mg/mL, Roche).

**Murine Endothelial Cell–SMC Coculture Assay**

Tg222smc or WT SMCs (3.5×105 to 7×105 cells/gel) were seeded on collagen gels (BD Bioscience) containing WT mouse aortic endothelial cells (ECs)13 (3.5×105 cells/mL). Cocultures were maintained in the presence or absence of ebselen (Cayman; 50% vol/vol in DMSO) for 7 days, then freeze-embedded in O.C.T. with liquid nitrogen, sectioned on a cryotome, and stained with anti-CD31 after cold acetone fixation.

**Statistical Analysis**

All results are expressed as mean±SEM. Statistical analysis was performed with an unpaired Student’s t test and ANOVA using the Tukey method for multiple comparisons. Values of P<0.05 and P<0.001 were considered statistically significant in the 2 tests.

**Results**

**Vascular p22phox Overexpression Increases ROS In Situ and Is Associated With Enhanced Lesion Progression and Intraläsional Angiogenesis**

In situ detection of ROS indicated that H2O2-associated signal was higher in both normal carotid arteries and fully developed experimental lesions6 (28 days after ligation) in the Tg222smc versus WT (Figure 1). Signal was inhibited by PEG-catalase, a specific H2O2 scavenger. In contrast, O2−-associated signal seemed similar in normal and injured carotid arteries of either mouse strain.

Detailed morphological examination of the carotid arteries revealed a surprisingly robust angiogenic response in the Tg222smc but not in the WT lesions, confirmed by consistent detection of numerous structures positive for CD31, an EC-specific antigen (Figure 1c).

Morphometric measurements taken throughout lesion development and analyzed with the Tukey simultaneous comparison ANOVA indicated that intimal area of fully developed lesions was significantly larger in Tg222smc than WT carotid arteries, after lesion induction, p22vsmc or WT carotid arteries, after lesion induction, Tg222smc carotid arteries expressed significantly greater VEGF during lesion progression. ANOVA showed that although there was no difference between the levels expressed by the normal Tg222smc or WT carotid arteries, after lesion induction, Tg222smc carotid arteries expressed significantly greater VEGF levels at all time points examined (P<0.001, n=4 animals per group). To explore a potential mechanism for enhanced VEGF expression, we next examined carotid artery levels of HIF-1α, the major VEGF transcription factor.14 Tg222smc carotid arteries contained significantly greater HIF-1α levels...
than WT both before injury (203 ± 12%, P < 0.001, n = 3) and after lesion formation (overall P < 0.001) (Figure 2f).

Oxidant Stress Is Associated With Induction of VEGF Expression in SMCs

To confirm the role of oxidative stress in the induction of angiogenesis and specifically transgenic SMC ROS contribution in our experimental model, we compared SMCs isolated from the Tg\(^{p22vsmc}\) and WT aortas in vitro (Figure 3). ESR spectroscopy of purified SMC plasma membranes confirmed significantly greater NADPH-dependent H\(_2\)O\(_2\) production by Tg\(^{p22vsmc}\) (643 ± 16 nmol/L · min\(^{-1}\) · g protein\(^{-1}\)) versus WT (385 ± 37 nmol/L · min\(^{-1}\) · g protein\(^{-1}\)) SMCs (P < 0.01, n = 4, Figure 3a). Conversely, ESR confirmed no significant difference in O\(_2\)· production in either preparation (Tg\(^{p22vsmc}\), 77 ± 4 nmol/L · min\(^{-1}\) · g protein\(^{-1}\); WT, 74 ± 8 nmol/L · min\(^{-1}\) · g protein\(^{-1}\); n = 3).

**Figure 1.** Overexpression of p22phox in SMCs Tg\(^{p22vsmc}\) increases H\(_2\)O\(_2\) arterial levels and leads to robust intralesion angiogenesis. **a**, Tg\(^{p22vsmc}\) carotid arteries display high H\(_2\)O\(_2\)-associated signal compared with WT lesions at baseline (day 0) and at day 28 after ligation as detected by DCFDA staining (n = 3). Insets illustrate controls: sections incubated with PEG-catalase or in absence of fluorophores. **b**, No difference was detected in O\(_2\)·-associated signal in Tg\(^{p22vsmc}\) and WT arterial lesions (DHE staining, n = 3). Each image is representative of results obtained from 3 different animals. **c**, Detection of EC using anti-CD31 immunostaining in WT and Tg\(^{p22vsmc}\) carotid arteries illustrates extensive angiogenesis in an advanced Tg\(^{p22vsmc}\) lesion (day 28). Lower right inset illustrates a higher magnification of one of numerous CD31-positive structures (boxed) detected within neointimal lesions (arrows point to some of these). Lower-left inset illustrates a negative control (Neg ctrl) for immunocytochemistry obtained in absence of primary antibody.

**Figure 2.** Overexpression of p22phox in SMCs enhances progression of carotid artery lesions and expression of VEGF and HIF-1α. Morphological analysis of major parameters indicates (a) significantly greater intimal area, (b) lumen loss despite (c) significantly greater expansive remodeling (external elastic lamina perimeter) in Tg\(^{p22vsmc}\) vs WT arteries (\(^*\)P < 0.05, Tukey simultaneous-comparison ANOVA). Analysis of carotid artery VEGF expression by (d) real-time PCR and (e) Western blotting indicates greater levels in Tg\(^{p22vsmc}\) vs WT samples. **f**, Quantification of HIF-1α expression obtained by Western blot illustrates that levels are significantly elevated in Tg\(^{p22vsmc}\) vs WT arteries both at baseline (day 0) and in advanced lesions (day 28). ArbU indicates arbitrary unit. **\(^{**}\)P < 0.001 (ANOVA).
We also found that Tg\(^{p22vsmc}\) SMCs expressed significantly more HIF-1\(\alpha\) than WT (361±16\%, \(P<0.001, n=3\)). CoCl\(_2\), an inducer of HIF-1\(\alpha\),\(^9\) was able to upregulate its expression in WT (159±10\%, \(P<0.05, n=3\), Figure 3b) but not in Tg\(^{p22vsmc}\) SMCs. Immunocytochemical analysis of HIF-1\(\alpha\) expression in SMC monolayers (not illustrated) supported these observations. CoCl\(_2\) similarly increased VEGF levels produced by WT (24±6\%, \(P<0.05, n=4\)) but not by Tg\(^{p22vsmc}\) SMCs (not shown), suggesting maximal baseline upregulation of VEGF in Tg\(^{p22vsmc}\) SMCs.

Treatment with ebselen, a glutathione peroxidase–mimetic antioxidant,\(^{15}\) at concentrations that significantly decreased H\(_2\)O\(_2\) levels in SMCs (Figure 3c) without affecting their viability (not shown), also decreased VEGF expression (Figure 3d), further supporting the causal role of H\(_2\)O\(_2\).

**Scavenging of H\(_2\)O\(_2\) Inhibits Lesion Progression and Angiogenesis**

To confirm the causative role of increased endogenous H\(_2\)O\(_2\) production in vivo, we administered ebselen during lesion development (Figure 4a). Systemic delivery of ebselen markedly reduced arterial H\(_2\)O\(_2\) levels, lesion size (\(P<0.01\)), and expansive remodeling (\(P<0.05\)) of Tg\(^{p22vsmc}\) carotid arteries compared with vehicle-treated controls (n=3 per group). Importantly, ebselen treatment markedly reduced intralesion angiogenesis (Figure 4a).

The direct functional contribution of Tg\(^{p22vsmc}\) SMCs on angiogenesis was further confirmed in an EC-SMC in vitro coculture system. We found that ECs cocultured with Tg\(^{p22vsmc}\) SMCs organized into elongated cord-like structures (Figure 4b). In contrast, no organization was observed in ECs cocultured with WT SMCs or when cocultures were treated with ebselen (Figure 4b), as well as when no SMCs were present (not shown). Gels lacking ECs remained acellular throughout the incubation period (data not shown), indicating minimal migration of SMCs into the gels. These results supported the notion that EC organization was mediated via SMC H\(_2\)O\(_2\) and other secreted factors and confirmed the functional angiogenic effect of oxidative stress.

**Discussion**

Our results provide in vivo and in vitro evidence to support an important causative role for oxidative stress in atherosclerotic lesion progression. We also demonstrate a novel pathway by which ROS can trigger an angiogenic switch within lesions. Although hypoxia is regarded as the classic angiogenesis inducer, other potential stimuli, including the presence of oxidative stress, associated with the pathogenesis of a broad spectrum of inflammatory and angiogenic disease processes,\(^{16}\) have recently come under investigation. In the present studies, we were able to demonstrate the causal effect of oxidative stress on carotid lesion progression by using an in vivo experimental model.

We created oxidative stress by genetic manipulation of SMCs in a SMC-driven model of arterial lesion development.\(^8\) It is worth noting that experimental lesions induced in either WT\(^{8,17}\) or Tg\(^{p22vsmc}\) mice lack inflammatory cells, specifically macrophages (data not shown), probably a major source of oxidative stress in human atheroma. However, we suggest that the enhanced arterial oxidative stress we created through experimental manipulation serves as a good model for the environment of human lesions, characterized by oxidative stress, including the contribution of inflammatory cell–derived ROS.

We chose to create experimentally vascular oxidative stress through p22phox subunit overexpression on the basis of several lines of evidence. (1) Regulation of p22phox gene transcription can modulate NAD(P)H oxidase activity\(^{17}\); (2) antisense p22phox transfected into SMCs results in decreased NAD(P)H oxidase activity\(^8\) and inhibits ROS generation as well as HIF-1\(\alpha\) and VEGF expression\(^{18}\); and (3) analysis of human coronary arteries reveals low p22phox expression in...
normal arteries, upregulation in atherosclerosis, and colocalization with ROS production. However, other subunits of the vascular NAD(P)H oxidase may also be involved in ROS-driven atheroma progression. The catalytic subunit (Nox1) has been implicated in in vitro angiogenic switching. In addition, the p47phox subunit seems to be required for plaque progression in hypercholesterolemic mice.

The predominant ROS detected in our in vivo model was H$_2$O$_2$, consistent with previous studies indicating H$_2$O$_2$ as the predominant NAD(P)H oxidase–derived ROS generated in association with an angiogenic switch. The heme-based catalytic subunit (Nox1/Nox4) of the vascular NAD(P)H oxidases should perform only a 1-electron reduction. Nevertheless, we have demonstrated quantitatively that both Tg$^{p22vsmc}$ and WT SMC membranes produce severalfold more NADPH-dependent H$_2$O$_2$ than O$_2^-$. Potentially, O$_2^-$ release from the catalytic pore of the enzyme is electrostatically hindered, leading to 2 sequential single-electron reductions by heme, thus favoring H$_2$O$_2$.

We found that arteries and SMCs under oxidative stress express high levels of the angiogenic potentiators VEGF and matrix metalloproteinases (MMPs) (not illustrated). In the present experiments, we confirmed our previous observations, associating carotid artery lesion development with increased MMP activity, specifically with induction of MMP-9 (not shown). In addition, we found that MMP-9 present in normal Tg$^{p22vsmc}$ carotid arteries was further increased with arterial lesion development. Similarly, gelatinolytic activity was increased in Tg$^{p22vsmc}$ compared with WT SMCs (183±19%, P<0.05, n=3, not illustrated), further supporting a relationship between increased production of ROS and MMP expression and activation. Functional consequences of enhanced MMP activity include atherosclerotic lesion progression and expansive or outward arterial remodeling, which may explain the enhanced outward remodeling of Tg$^{p22vsmc}$ arteries observed in the present experiments.

The high SMC content of experimental lesions probably contributed to the progressive increase in levels of VEGF and HIF-1α, the major VEGF transcription factor. Others recently demonstrated that multiple nonhypoxic stimuli can increase HIF-1α levels in certain cell types. We found that HIF-1α levels were elevated in both Tg$^{p22vsmc}$ SMCs and arterial tissues. HIF-1α levels were not affected by carotid artery lesion progression. Thus, in our model, induction of HIF-1α was mediated by a ROS-sensitive, hypoxia-independent mechanism, as suggested by some in vitro reports. Previous studies indicate that cellular levels of HIF-1α are determined primarily by the rate of its ubiquitin-proteosome–dependent degradation. Thus, oxidative stress may be associated with inhibition of HIF-1α degradation independently of hypoxia, potentially through the Shc-Ras signaling pathway.

Our model of experimental atheroma demonstrates that inducing plaque neovascularization enhanced lesion progression, as suggested by others. Clinically, there is a higher incidence of intralesion angiogenesis in culprit lesions that result in unstable angina. These findings illustrate the significance of intraplaque angiogenesis and suggest that regulation of this process may be important in the management of atherosclerosis. Our results support the lowering of arterial oxidative stress, potentially through the use of ROS scavengers, as a therapeutic strategy for prevention of intralesion angiogenesis related to atheroma progression and destabilization through intraplaque rupture.
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