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Biology of Free Radicals

Hemodynamic and biochemical adaptations to vascular smooth muscle overexpression of p22^{phox} in mice

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transgenic animals; oxidant stress; endothelium; nitric oxide

IN MANY PATHOLOGICAL CONDITIONS associated with vascular lesion formation the production of reactive oxygen species (ROS) is increased in the vessel wall. As a consequence, nitric oxide (NO) bioavailability is decreased due in large part to its rapid reaction with superoxide ($O_2^-\cdot$). In addition, it is now clear that ROS may have important intracellular signaling properties. As an example, H_2O_2 has been shown to modulate endothelial NO synthase (eNOS) protein expression in vitro (6). This upregulation of eNOS by H_2O_2 may occur in pathological conditions such as hypercholesterolemia and hypertension where both increased $O_2^-\cdot$ and increased eNOS expression have been observed (1, 12).

Major sources of ROS in vascular cells are the NAD(P)H oxidases, which are multisubunit enzyme complexes similar to the phagocytic oxidase. In phagocytic cells, NAD(P)H oxidases consist of the catalytic gp91^{phox} and p22^{phox} subunits. Recently, it has been recognized that gp91^{phox} is a member of a larger family of Nox proteins, and it has been renamed Nox2. The vascular smooth muscle cells (VSMC) NAD(P)H oxidases differ from the phagocytic enzyme in that they utilize Nox1 and Nox4, and larger vessels do not contain Nox2 (16). However, all NAD(P)H oxidases utilize p22^{phox}, which plays an important role as a docking protein for the cytoplasmic subunits and as a stabilizer of Nox protein expression (15). The vascular NAD(P)H oxidases can be activated by a variety of pathophysiological stimuli, including angiotensin II (ANG II) (16). In addition, over several days, ANG II increases expression of p22^{phox}, Nox1, and Nox4. Previously, we have found that during ANG II-induced hypertension, the time course of increased NAD(P)H oxidase activity and blood pressure paralleled that of an increase in p22^{phox} (18), suggesting that this subunit may have an important role in the modulation of overall enzyme activity. In cultured VSMC, inhibition of p22^{phox} expression using antisense techniques inhibits hypertrophy caused by ANG II (22). Moreover, polymorphisms of p22^{phox} have been variably associated with an increase in cardiovascular events, a reduction in endothelium-dependent vasodilatation, and an increase in coronary atherosclerosis. Intimal VSMC in atherosclerotic lesions contain large amounts of p22^{phox} that colocalizes with sites of $O_2^-\cdot$ production (21).

Given this apparent importance of p22^{phox}, we sought to determine whether increasing its expression in vivo would alter vascular ROS production and to understand how this would affect hemodynamics and endothelium-dependent vasodilata-

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tion by directing expression of p22^{phox} to smooth muscle in transgenic mice. Our initial characterization of these mice showed that their vessels indeed produced excessive $O_2^-\cdot$ and H_2O_2, but their endothelium-dependent vasodilatation and blood pressure were normal. Additional studies indicated that expression of eNOS and extracellular superoxide dismutase (ecSOD) was increased. We propose that compensatory responses such as an increase in expression of eNOS and ecSOD are critical in the setting of oxidative stress and that overt vascular dysfunction does not occur until these fail.

METHODS

Animals. Mice overexpressing the NAD(P)H oxidase p22^{phox} subunit in VSMC (Tg^{p22smc} mice) were created by cloning the p22^{phox} cDNA downstream of the smooth muscle cell-targeted smooth muscle α-actin (SMP-8) promoter and upstream of a sarcovirus 40 (SV40) polyA fragment (Fig. 1*A*). The Sph1-Kpn1 fragment was used for oocyte injection. Several founder Tg^{p22smc} mice were obtained that were able to transmit the transgene to offspring. They were backcrossed at least 10 times to the C57BL/6J background. C57BL/6J mice were used as controls. Blood pressure was measured using telemetry (Data Sciences International) as described previously (4). In some experiments, the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) was given in the drinking water (100 mg·kg^{-1}·day^{-1}). In other experiments, the glutathione peroxidase mimetic ebselen (10 mg·kg^{-1}·day^{-1} in 50% DMSO) was infused chronically via an osmotic minipumps (Alzet; Durect, CA) implanted subcutaneously. As an alternative approach for H_2O_2 scavenging, we crossed Tg^{p22smc} mice with mice overexpressing catalase driven by the Tie-2 promoter (Tg$^{p22phox/catalase}$). On the day of study, the animals were killed with CO_2 inhalation, and the thoracic aortas were removed and dissected free of adherent tissues. All animal experiments were approved by the Emory University Animal Care and Use Committee.

Vascular reactivity. Thoracic aortas were cut into 3- to 4-mm ring segments and mounted for isometric tension recording in a water-jacketed organ bath (14). Passive tension was adjusted to 1 g, and vessels were preconstricted with 2 μM $PGF_{2\alpha}$. Relaxations to cumulative concentrations of acetylcholine were examined.

Determination of aortic $O_2^-\cdot$ and H_2O_2. Extracellular $O_2^-\cdot$ release was measured using the SOD-inhibitable cytochrome *c* reduction assay as previously described (14). To evaluate intracellular $O_2^-\cdot$, we measured the formation of oxyethidium from dihydroethidium using HPLC as recently reported (7). H_2O_2 was measured using a fluorometric HRP-linked assay (Amplex red assay, Molecular Probes) as previously described (23).

Determination of aortic NO production. NO production was measured by electron-spin resonance (ESR) using the specific colloid probe Fe^{2+} diethyldithiocarbamate in the absence or presence of 10 μM A23187 (13). ESR settings were the following: microwave frequency, 9.431 GHz; modulation frequency, 100 kHz; modulation amplitude, 5 G; field sweep, 50 G; microwave power, 10 mW; conversion time, 1,312 ms; time constant, 5,248 ms; and receiver gain, 1×10^5.

Determination of aortic p22^{phox}, Nox1, eNOS, and SOD protein levels. Western blot analyses were performed on aortic homogenates. Antibodies used were the following: a polyclonal antibody against Nox1, an eNOS monoclonal antibody (BD Transduction Laboratories), Mn-SOD and Cu/Zn-SOD polyclonal antibodies (Stressgen), a monoclonal antibody generated against the mouse p22^{phox} protein, and a previously generated ecSOD polyclonal antibody.

Statistical analysis. All values are means ± SE. Data were compared between groups of animals by *t*-test when one comparison was performed and by ANOVA for multiple comparisons. When significance was indicated by ANOVA, the Tukey post hoc test was used to specify between group differences.

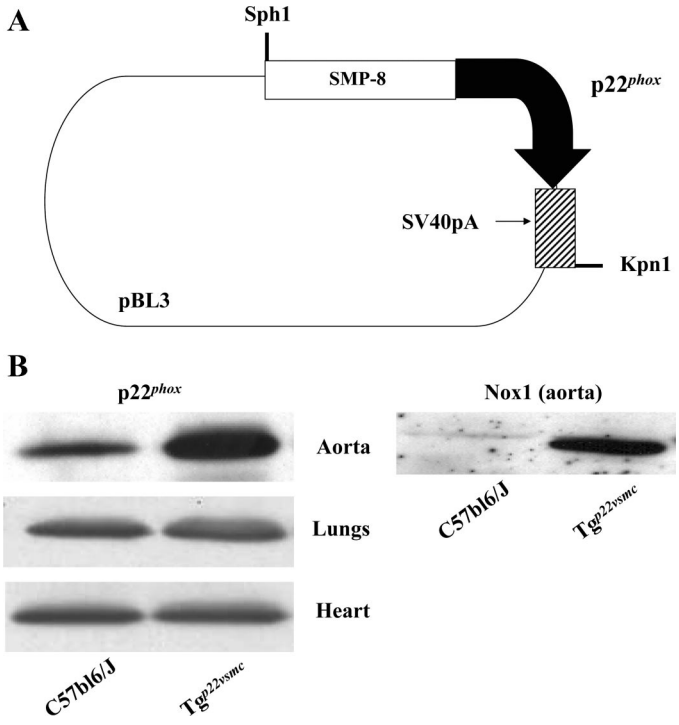

Fig. 1. Creation of transgenic (Tg^{p22smc}) mice. *A*: plasmid construction used for targeting of p22^{phox} overexpression in smooth muscle cells (SMC). *B*: Western blot analysis of p22^{phox} protein in aorta, heart, and lungs homogenates (*left*) and aortic Nox1 protein expression (*right*). Data are representative of 3 separate experiments.

RESULTS

Characterization of the Tg^{p22smc} mice. Compared with their C57BL/6J background, the Tg^{p22smc} mice were overtly normal with normal litter sizes and growth rates. p22^{phox} protein expression was increased in the aorta but not in other tissues such as the heart and lungs (Fig. 1*B*), confirming specific targeting of gene overexpression.

Recent studies have demonstrated that p22^{phox} and Nox2 stabilize each other in granulocytes. We sought to determine whether overexpression of p22^{phox} increased expression of one or more of the Nox proteins. Western blot analysis demonstrated no difference in either Nox2 (gp91^{phox}) or Nox4 (data not shown). In contrast, expression of Nox1 was markedly increased in aortas of Tg^{p22smc} mice (Fig. 1*B*).

Production of $O_2^-\cdot$ and H_2O_2. HPLC analysis of conversion of dihydroethidium to oxyethidium revealed that the intracellular production of $O_2^-\cdot$ was increased in aortas from Tg^{p22smc} mice (Fig. 2*A*). In contrast, extracellular $O_2^-\cdot$ production detected by SOD-inhibitable cytochrome *c* reduction was not increased in Tg^{p22smc} aortas (Fig. 2*B*). H_2O_2 production was significantly increased in aortas from Tg^{p22smc} mice compared with control (Fig. 2*C*).

We also measured the expression of SOD proteins by Western blot analysis. Of the three SOD isoforms, ecSOD was increased in Tg^{p22smc} mice by twofold (Fig. 2*D*).

Effect of p22^{phox} overexpression on vascular function and hemodynamics. To determine whether the increased aortic $O_2^-\cdot$ and H_2O_2 affected vascular function, we measured blood

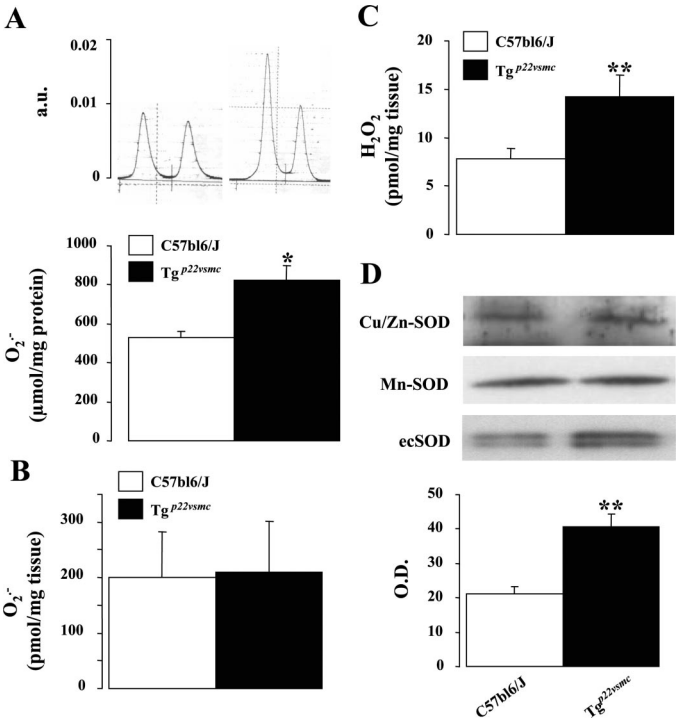

Fig. 2. Aortic $O_2^-\cdot$, H_2O_2, and SOD protein isoforms. *A*: intracellular superoxide ($O_2^-\cdot$) measured by HPLC. *Top*, representative HPLC graph. *Bottom*, grouped data ($n = 6–8$ per group). au, arbitrary units. *B*: extracellular $O_2^-\cdot$ measured by the cytochrome *c* reduction assay ($n = 6–8$ per group). *C*: extracellular H_2O_2 (Amplex red assay, $n = 6–8$ per group). *D*: Western blots of the 3 SOD isoforms. ecSOD, extracellular SOD. *Top*, representative blot of 3 separate experiments. *Bottom*, grouped data for ecSOD. O.D., optical density. Significant differences: $^*P < 0.05$ and $^{**}P < 0.01$, *t*-test.

pressure and aortic endothelium-dependent relaxation. Surprisingly, blood pressure was identical in Tg^{p22smc} mice and in controls (110 ± 1 vs. 116 ± 1 mmHg in control; Fig. 3*A*), and endothelium-dependent vasodilatation to acetylcholine was not altered (Fig. 3*B*).

Effect of p22^{phox} *overexpression on NO production and eNOS protein expression.* Whereas Tg^{p22smc} mice have an increased $O_2^-\cdot$ and H_2O_2 production, there is no alteration in blood pressure or endothelium-dependent vasodilatation. We hypothesized that compensatory mechanisms may develop, preventing increased blood pressure and vascular dysfunction. Recent studies have shown that H_2O_2 potently stimulates eNOS expression and function in cultured endothelial cells (2). Because H_2O_2 production was increased in Tg^{p22smc} mice, we examined eNOS expression and NO production in these animals. NO production was increased by twofold in aortas of Tg^{p22smc} mice under basal conditions and by more than sevenfold in response to the calcium ionophore A23187 (10 μM, Fig. 4*A*). eNOS protein was twofold higher in Tg^{p22smc} mice (Fig. 4*B*).

To further investigate a link between H_2O_2 and eNOS protein upregulation, we treated mice for 7 days with ebselen to scavenge H_2O_2. Ebselen treatment decreased aortic H_2O_2 production in both control and Tg^{p22smc} mice (Fig. 5*A*). Moreover, ebselen infusion decreased eNOS protein expression in both the Tg^{p22smc} and C57BL/6J mice (Fig. 5*B*). Because these results obtained by a pharmacological approach suggest that H_2O_2 upregulates eNOS protein expression in vivo, we also examined eNOS protein in $Tg^{p22phox/catalase}$ mice. Expression of eNOS was normalized in these mice, clearly demonstrating that H_2O_2 is responsible for upregulation of eNOS protein in vivo (Fig. 5*C*).

Effect of NO on ecSOD protein expression. The above data indicate that overexpression of $p22^{phox}$ in smooth muscle increased eNOS expression, NO production, and ecSOD protein expression. Previously, we (9) have found that NO is a potent stimulus for ecSOD protein expression in vitro. We next determined whether increased NO production in Tg^{p22smc} mice underlies the increased ecSOD expression. L-NAME treatment (100 $mg \cdot kg^{-1} \cdot day^{-1}$) for 14 days significantly increased blood pressure in C57BL/6J and Tg^{p22smc} mice (Fig. 6*A*) and abolished basal and stimulated NO production (Fig. 6*B*). L-NAME also significantly decreased ecSOD protein expression in Tg^{p22smc} mice but not in C57BL/6J mice (Fig. 6*C*).

DISCUSSION

In this study, we sought to determine whether increasing smooth muscle expression of $p22^{phox}$ would enhance vascular ROS production and to examine the effect this has on endothelium-dependent vasodilatation and hemodynamics. We found that aortas of Tg^{p22smc} mice produce increased $O_2^-\cdot$ and H_2O_2 but have no change in endothelium-dependent vasodilatation or hemodynamics. $Tg^{p22/smc}$ mice also had a striking increase in vascular NO production and eNOS expression and an increase in ecSOD expression. From studies using L-NAME, an inhibitor of NO production, ebselen, a glutathione peroxidase mimetic to scavenge H_2O_2, and mice overexpressing both $p22^{phox}$ and catalase, we propose that chronic overproduction of $O_2^-\cdot$ and H_2O_2 leads to a compensatory pathway similar to that illustrated in Fig. 7.

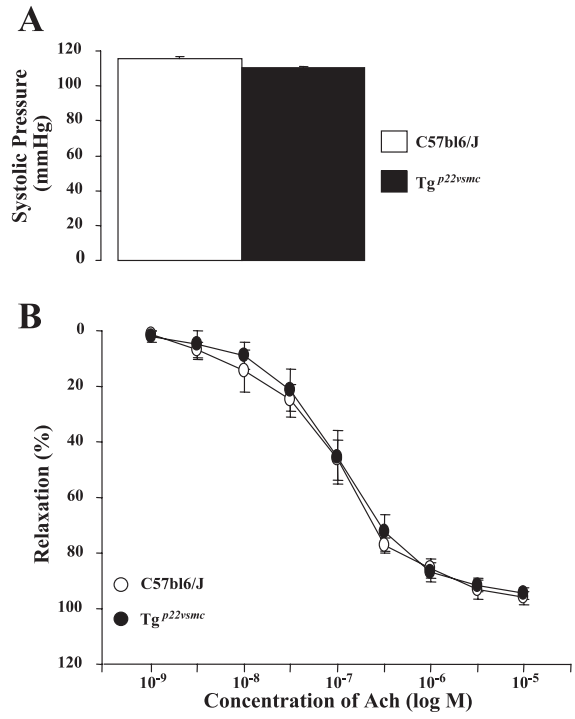

Fig. 3. Hemodynamics and endothelial function. *A*: systolic arterial pressure (telemetry). *B*: aortic endothelium-dependent relaxation to acetylcholine (ACh) after precontraction with 2 μM $PGF_{2\alpha}$ ($n = 5–6$ per group).

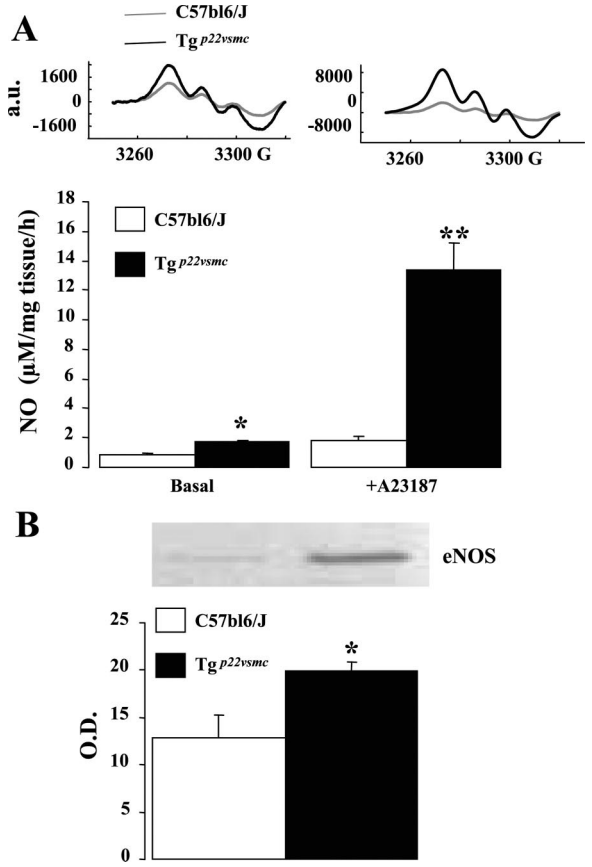

Fig. 4. Aortic nitric oxide (NO) production and endothelial NO synthase (eNOS) protein expression. *A*: basal and stimulated NO production (electron-spin resonance, ESR). *Top*, representative spectra. *Bottom*, grouped data (n = 5–6 per group). *B*: Western blots of eNOS protein. *Top*, representative blot of 3 separate experiments. *Bottom*, grouped data. Significant differences: $*P < 0.05$, $**P < 0.01$, *t*-test.

One interesting finding in this study is that overexpression of $p22^{phox}$ was associated with an increase in expression of the $gp91^{phox}$ homolog Nox1. Whereas the mechanism for this remains unclear, it is possible that increased levels of $p22^{phox}$ stabilize Nox1 protein. This is analogous to the situation in phagocytes where processing of Nox2 is dependent on the presence of $p22^{phox}$ (5). In the neutrophil, $p22^{phox}$ and Nox2 are not targeted to the plasma membrane individually but require one another to avoid degradation in the proteosome. Whether $p22^{phox}$ and Nox1 interact in this fashion in VSMC remains unclear. Nevertheless, the increase in Nox1 probably plays a critical role in the increased production of ROS in vessels of Tg^{p22smc} mice, because this represents the catalytic subunit responsible for electron transfer to oxygen.

NAD(P)H oxidases produce $O_2^-\cdot$ based on the one-electron reduction of oxygen by its Fe^{2+} center, but activation of this system can also lead to the formation of H_2O_2, either via spontaneous dismutation or after enzymatic dismutation by one of the SOD isozymes. The mechanism responsible for the preferential production of H_2O_2 in vessels of Tg^{p22smc} mice remains unclear but may be related to the increased ecSOD protein expression. Indeed, the increased intracellular $O_2^-\cdot$ production we found in vessels of $Tg^{p22vsmc}$ mice was not reflected in extracellular release of $O_2^-\cdot$. Intracellularly produced $O_2^-\cdot$ can diffuse from the cell in the form of uncharged hydroperoxy radical, and we have shown that the cytochrome *c* assay can detect an increase in $O_2^-\cdot$ produced by uncoupled eNOS, which releases $O_2^-\cdot$ intracellularly (14). Therefore, the inability to detect increased $O_2^-\cdot$ in these experiments is un-

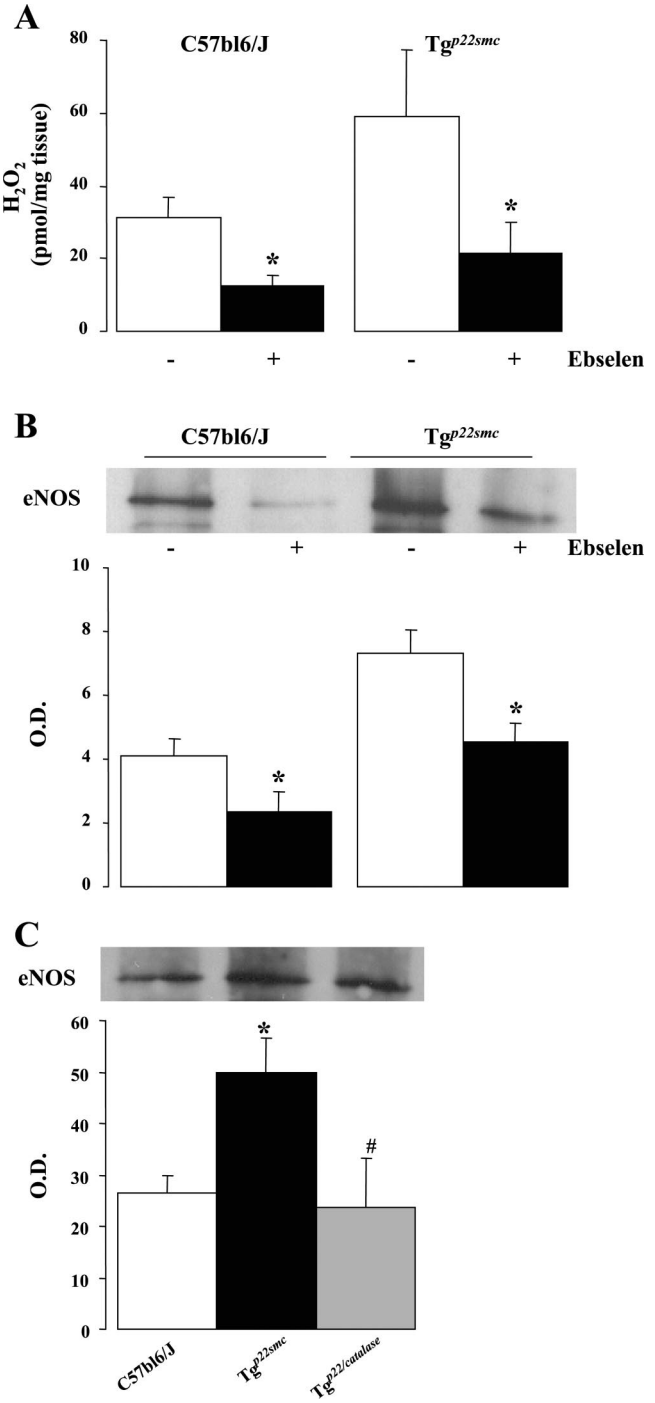

Fig. 5. Effect of H_2O_2 scavenging on eNOS protein expression. *A*: extracellular H_2O_2 after ebselen (n = 6–8 per group). *B*: Western blot analysis of eNOS protein after ebselen. *Top*, representative blot of 3 separate experiments. *Bottom*, grouped data. *C*: Western blot analysis of eNOS protein in $Tg^{p22phox/catalase}$ mice. *Top*, representative blot of 3 separate experiments. *Bottom*, grouped data. Significant differences: $*P \leq 0.05$ vs. C57BL/6J; $\#P \leq 0.05$ vs. Tg^{p22smc}.

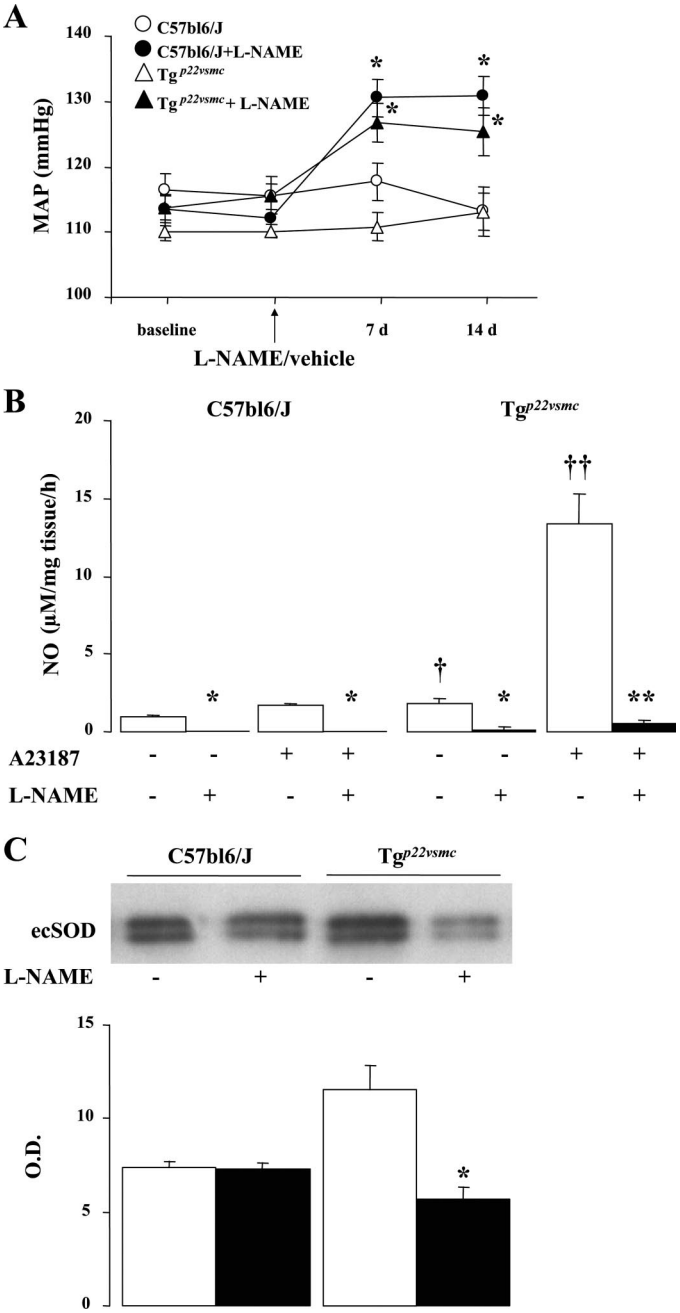

Fig. 6. Effect of NO inhibition on extracellular SOD (ecSOD) protein expression. *A*: mean arterial pressure (MAP) monitored by telemetry ($n = 5–6$ per group). *B*: basal and stimulated NO production (ESR, $n = 6–8$ per group). L-NAME, N^G-nitro-L-arginine methyl ester. *C*: Western blot analysis of ecSOD protein after L-NAME. *Top*, representative blot of 3 separate experiments. *Bottom*, grouped data. Significant differences: $*P < 0.05$ and $**P < 0.01$ vs. untreated; $\dagger P < 0.05$ and $\dagger\dagger P < 0.01$ vs. C57BL/6J.

likely due solely to the fact that $O_2^-\cdot$ was only produced intracellularly.

We found that Tg^{p22smc} mice have a twofold increase in the vascular levels of ecSOD. This enzyme resides in the extracellular space surrounding VSMC and endothelial cells and rapidly catalyzes dismutation of $O_2^-\cdot$ to H_2O_2 (8). It is therefore reasonable to conclude that increased ecSOD diminish detection of $O_2^-\cdot$ from vessels of the Tg^{p22smc} mice. We have previously shown that NO is a potent stimulus for ecSOD production in VSMC via a cGMP-dependent pathway (9). It is likely that increased NO production in the Tg^{p22smc} mice is in part responsible for the increased ecSOD production because L-NAME treatment completely abrogated this increase while having no effects on control mice. In our previous study (9), we showed that ecSOD levels are reduced and not increased by exercise training in eNOS-deficient mice. The present study demonstrates that a chronic increase in NO can have the opposite effect, i.e., stimulating ecSOD expression.

One of the major findings of this study is that the resulting increase in H_2O_2 observed in Tg^{p22smc} mice is associated with an increased eNOS protein expression and NO production. Our group (2) has shown that H_2O_2 upregulates eNOS mRNA and protein expression, as well as eNOS protein activity (3, 6), through a Ca^{2+}/calmodulin kinase II and Janus kinase 2-dependent mechanism. These previous experiments were performed in cultured endothelial cells and involved administration of high concentrations of H_2O_2. The present study is the first to demonstrate that H_2O_2 can stimulate eNOS expression in vivo.

In these experiments, we used the glutathione peroxidase mimetic ebselen to reduce H_2O_2. Ebselen has been shown to inhibit lipoxygenases, NOSs, and protein kinase C and to prevent apoptosis (19). These effects of ebselen are inhibited by thiols and therefore less likely to account for its effects in vivo. Furthermore, our results with mice overexpressing catalase support the concept that H_2O_2 is likely responsible for some of the effects we observed in the Tg^{p22smc} mice.

In additon to the twofold increase in NOS expression, we found an even greater increase in NO production by vessels of Tg^{p22smc} mice, particularly in response to calcium stimulation. These results may help explain why Tg^{p22smc} mice had no alteration in endothelium-dependent vasodilatation or hemodynamics despite an increase in vascular $O_2^-\cdot$ production. In addition to being a potent stimulus for NO production by endothelial cells, H_2O_2 can stimulate GTP-cyclohydrolase mRNA and protein expression in endothelial cells, leading to an increase in tetrahydrobiopterin levels and promoting NO production (20). The increase in ecSOD could also protect NO from degradation by $O_2^-\cdot$, making more NO available for

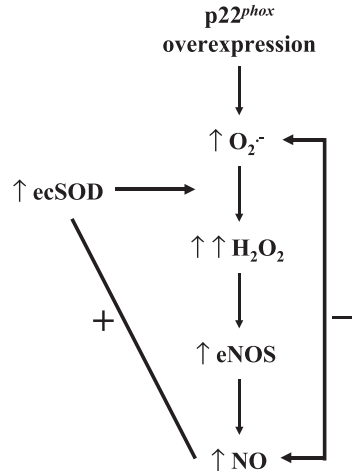

Fig. 7. Proposed compensatory pathway in response to chronic vascular oxidative stress.

trapping by Fe^{2+} diethyldithiocarbamate. It is of interest that the $p22^{phox}$ transgene was targeted to smooth muscle, and thus the source of H_2O_2 was clearly the VSMC oxidase, likely responsible for stimulating endothelial NO production. There are many instances where paracrine factors from the endothelium, such as NO, prostacyclin, or endothelium-derived hyperpolarizing factor, regulate VSMC tone (11). In this instance, we have demonstrated a novel function of H_2O_2 from the VSMC exerting a paracrine effect on endothelial cells, modulating endothelial eNOS expression and NO production.

In summary, our studies in Tg^{p22smc} mice provide insight into how vessels may respond to a prolonged increase in H_2O_2 production. Overexpression of the NAD(P)H oxidase $p22^{phox}$ subunit in VSMC is associated with increased expression of the Nox1 catalytic subunit and an increase in VSMC H_2O_2 production. This is associated with a series of compensatory mechanisms that preserve normal endothelial function. It is interesting to speculate that this compensatory pathway is operative in numerous conditions where oxidative stress is increased. As examples, ANG II can stimulate both increased ecSOD and NO production (3, 10), and, at least in some models of hypercholesterolemia (17), eNOS expression is increased. In many instances, these compensatory mechanisms may preserve normal vascular function, as in Tg^{p22smc} mice. The failure of these compensatory mechanisms are therefore likely responsible for development of vascular dysfunction and initiation of disease.

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