Hemodynamic and biochemical adaptations to vascular smooth muscle overexpression of p22<sup>phox</sup> in mice

Karine Laude,1 Hua Cai,1 Bruno Fink,1 Nyssa Hoch,1 David S. Weber,1 Louise McCann,1 Georg Kojda,2 Tohru Fukai,1 Harald H. H. W. Schmidt,1 Sergey Dikalov,1 Santhini Ramasamy,1 Graciela Gamez,1 Kathy K. Griendling,1 and David G. Harrison1

1Division of Cardiology, Emory University, Atlanta, Georgia; and 2Institut für Pharmakologie und Klinische Pharmakologie, Duesseldorf, and 3Rudolf-Bucheim Institut für Pharmakologie, Justus-Liebig-Universitat, Giessen, Germany

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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<sup>phox</sup> and p22<sup>phox</sup> subunits. Recently, it has been recognized that gp91<sup>phox</sup> 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. However, all NAD(P)H oxidases utilize p22<sup>phox</sup>, 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 pathophysiologival stimuli, including angiotensin II (ANG II) (16). In addition, over several days, ANG II increases expression of p22<sup>phox</sup>, 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<sup>phox</sup> (18), suggesting that this subunit may have an important role in the modulation of overall enzyme activity. In cultured VSMC, inhibition of p22<sup>phox</sup> expression using antisense techniques inhibits hypertrophy caused by ANG II (22). Moreover, polymorphisms of p22<sup>phox</sup> 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<sup>phox</sup> that colocalizes with sites of O<sub>2</sub>· production (21).

Given this apparent importance of p22<sup>phox</sup>, 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-
tion by directing expression of p22\textsuperscript{phox} to smooth muscle in transgenic mice. Our initial characterization of these mice showed that their vessels indeed produced excessive \(\text{O}_2^-\) and \(\text{H}_2\text{O}_2\), 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\textsuperscript{phox} subunit in VSMC (Tg\textsuperscript{p22\textsuperscript{phox}} mice) were created by cloning the p22\textsuperscript{phox} cDNA downstream of the smooth muscle cell-targeted smooth muscle \(\alpha\)-actin (SMP-8) promoter and upstream of a sarcovirus 40 (SV40) polyA fragment (Fig. 1A). The Sph1-Kpn1 fragment was used for oocyte injection. Several founder Tg\textsuperscript{p22\textsuperscript{phox}} 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\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME) was given in the drinking water (100 mg/kg/day). Expression of one of the Nox proteins, Nox1, was also increased in aortas of Tg\textsuperscript{p22\textsuperscript{phox}} mice (Fig. 1B). In contrast, expression of Nox2, which is also increased in Tg\textsuperscript{p22\textsuperscript{phox}} mice, was decreased in aortas of Tg\textsuperscript{p22\textsuperscript{phox}} mice (Fig. 1B). Recent studies have demonstrated that p22\textsuperscript{phox} and Nox2 stabilize each other in granulocytes. We sought to determine whether overexpression of p22\textsuperscript{phox} increased expression of one or more of the Nox proteins. Western blot analysis demonstrated no difference in either Nox2 (gp91\textsuperscript{phox}) or Nox4 (data not shown). In contrast, expression of Nox1 was markedly increased in aortas of Tg\textsuperscript{p22\textsuperscript{phox}} mice (Fig. 1B).

**Production of \(\text{O}_2^-\) and \(\text{H}_2\text{O}_2\).** HPLC analysis of conversion of dihydroethidium to oxyethidium revealed that the intracellular production of \(\text{O}_2^-\) was increased in aortas from Tg\textsuperscript{p22\textsuperscript{phox}} mice (Fig. 2A). In contrast, extracellular \(\text{O}_2^-\) production detected by SOD-inhibitable cytochrome c reduction was not increased in Tg\textsuperscript{p22\textsuperscript{phox}} aortas (Fig. 2B). \(\text{H}_2\text{O}_2\) production was significantly increased in aortas from Tg\textsuperscript{p22\textsuperscript{phox}} mice compared with control (Fig. 2C).

We also measured the expression of SOD proteins by Western blot analysis. Of the three SOD isoforms, ecSOD was increased in Tg\textsuperscript{p22\textsuperscript{phox}} mice by twofold (Fig. 2D).

**Effect of p22\textsuperscript{phox} overexpression on vascular function and hemodynamics.** To determine whether the increased aortic \(\text{O}_2^-\) and \(\text{H}_2\text{O}_2\) affected vascular function, we measured blood...
extracellular H$_2$O$_2$ (Amplex red assay, Recent studies have shown that H$_2$O$_2$ potently stimulates preventing increased blood pressure and vascular dysfunction. We hypothesized that compensatory mechanisms may develop, blood pressure or endothelium-dependent vasodilatation. We next determined whether increased NO production in Tg(p22phox) mice underlies the increased ecSOD expression. l-NAME treatment (100 mg·kg$^{-1}$·day$^{-1}$) for 14 days significantly increased blood pressure in C57BL/6J and Tg(p22phox) mice (Fig. 6A) and abolished basal and stimulated NO production (Fig. 6B). l-NAME also significantly decreased ecSOD protein expression in Tg(p22phox) mice but not in C57BL/6J mice (Fig. 6C).

**DISCUSSION**

In this study, we sought to determine whether increasing smooth muscle expression of p22phox would enhance vascular ROS production and to examine the effect this has on endothelium-dependent vasodilatation and hemodynamics. We found that aortas of Tg(p22phox) mice produce increased O$_2^·$ and H$_2$O$_2$ but have no change in endothelium-dependent vasodilatation or hemodynamics. Tg(p22phox) 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$_2$O$_2$, and mice overexpressing both p22phox and catalase, we propose that chronic overproduction of O$_2^·$ and H$_2$O$_2$ leads to a compensatory pathway similar to that illustrated in Fig. 7.

![Fig. 2. Aortic O$_2^·$: H$_2$O$_2$, and SOD protein isoforms. A: intracellular superoxide (O$_2^·$) measured by HPLC. Top, representative HPLC graph. Bottom, grouped data ($n = 6$–8 per group). au, arbitrary units. B: extracellular O$_2^·$ measured by the cytochrome c reduction assay ($n = 6$–8 per group). C: extracellular H$_2$O$_2$ (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.](http://ajpheart.physiology.org/)
One interesting finding in this study is that overexpression of p22phox was associated with an increase in expression of the gp91phox homolog Nox1. Whereas the mechanism for this remains unclear, it is possible that increased levels of p22phox stabilize Nox1 protein. This is analogous to the situation in phagocytes where processing of Nox2 is dependent on the presence of p22phox (5). In the neutrophil, p22phox and Nox2 are not targeted to the plasma membrane individually but require one another to avoid degradation in the proteosome. Whether p22phox 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;p22vsmc 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$_2$O$_2$, either via spontaneous dismutation or after enzymatic dismutation by one of the SOD isoforms. The mechanism responsible for the preferential production of H$_2$O$_2$ in vessels of Tg;p22vsmc 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 pro-

duced 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. 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.](http://ajpheart.physiology.org/)

![Fig. 5. Effect of H$_2$O$_2$ scavenging on eNOS protein expression. A: extracellular H$_2$O$_2$ 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 ≤ 0.05 vs. C57BL/6J; #P ≤ 0.05 vs. Tg;p22vsmc.](http://ajpheart.physiology.org/)
likely due solely to the fact that $O_2^-$ 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 $O_2^-$ to $H_2O_2$ (8). It is therefore reasonable to conclude that increased ecSOD diminishes detection of $O_2^-$ 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 addition 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 vasodilation or hemodynamics despite an increase in vascular $O_2^-$ 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 $O_2^-$, making more NO available for

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**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$^\omega$-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; †P < 0.05 and ††P < 0.01 vs. C57BL/6J.

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trapping by Fe²⁺ diethyldithiocarbamate. It is of interest that the p22phox transgene was targeted to smooth muscle, and thus the source of H₂O₂ 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 H₂O₂ from the VSMC exerting a paracrine effect on endothelial cells, modulating endothelial eNOS expression and NO production.

In summary, our studies in Tg²²⁺ mice provide insight into how vessels may respond to a prolonged increase in H₂O₂ production. Overexpression of the NAD(P)H oxidase p22phox subunit in VSMC is associated with increased expression of the Nox1 catalytic subunit and an increase in VSMC H₂O₂ 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 Tg²²⁺ mice. The failure of these compensatory mechanisms are therefore likely responsible for development of vascular dysfunction and initiation of disease.

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Present address of H. Cai: Section of Cardiology, Dept. of Medicine, The Univ. of Chicago, Chicago, IL 60637.

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