Modulation of Extracellular Superoxide Dismutase Expression by Angiotensin II and Hypertension

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Abstract—Angiotensin II and hypertension increase vascular oxidant stress. We examined how these might affect expression of the extracellular superoxide dismutase (ecSOD), a major form of vascular SOD. In mice, angiotensin II infusion (1.1 mg/kg for 7 days) increased systolic blood pressure from 107±3 to 152±9 mm Hg and caused a 3-fold increase in ecSOD, but there was no change in the cytosolic Cu/Zn SOD protein, as determined by Western blot analysis. This was associated with a similar increase in ecSOD mRNA as assessed by RNase protection assay and was prevented by losartan. Induction of ecSOD by angiotensin II was not due to hypertension alone, because hypertension caused by norepinephrine (5.6 mg·kg⁻¹·d⁻¹) had no effect on ecSOD. Similarly, exposure of mouse aortas to angiotensin II (100 nmol/L) in organoid culture increased ecSOD by ≈2-fold. In the organoid culture, angiotensin II–induced upregulation of ecSOD was prevented by losartan (10 μmol/L) and PD985059 (30 μmol/L), a specific inhibitor of p42/44 MAP kinase kinase. Angiotensin II activates the NADH/NADPH oxidase; however, diphenyleneiodonium chloride (10 μmol/L), an inhibitor of this oxidase, did not prevent p42/44 MAP kinase phosphorylation or ecSOD induction by angiotensin II. Finally, in human aortic smooth muscle cells, angiotensin II moderately increased transcriptional rate (as assessed by nuclear run-on analysis) but markedly increased ecSOD mRNA stability. Thus, angiotensin II increases ecSOD expression independent of hypertension, and this increase involves both an increase in ecSOD transcription and stabilization of ecSOD mRNA. This effect of angiotensin II on ecSOD expression may modulate the oxidative state of the vessel wall in pathological processes in which the renin-angiotensin system is activated.

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Key Words: hypertension ■ angiotensin II ■ norepinephrine ■ superoxide ■ superoxide dismutase

Oxidant stress contributes to vascular diseases by promoting vascular smooth muscle proliferation, monocyte/macrophage infiltration, vascular tone alteration, and matrix metalloproteinases activation. Recent evidence suggests that hypertension, in particular, is associated with increased vascular oxidant stress. A membrane bound NADH/NADPH oxidase, the major source of reactive oxygen species in blood vessels, is activated in rats made hypertensive by prolonged angiotensin II infusion. This increase in superoxide anion production (O₂⁻) contributes to impaired endothelium-dependent relaxation, and the hypertension is ameliorated by treatment with membrane-targeted forms of superoxide dismutase (SOD), which is one of the major cellular defenses against superoxide anion (O₂⁻). Hypertrophy of vascular smooth muscle cells caused by angiotensin II is mediated by reactive oxygen species derived mainly from the membrane-bound NADH/NADPH oxidase. Similarly, a recombinant heparin-binding SOD acutely lowers blood pressure in spontaneously hypertensive rats. Taken together, these findings indicate that oxidant stress critically contributes to the pathogenesis of hypertension and its related vascular disease.

Three isozymes of SOD have been identified, including a copper/zinc-containing form (Cu/ZnSOD), which is primarily cytosolic in location, a mitochondrial manganese form (Mn-SOD), and an extracellular isozyme (ecSOD). In vascular tissue, depending on the species studied, up to one half of the total SOD is the extracellular form. Given the location of ecSOD, it probably plays a critical role in modulation of redox state in the vascular interstitium. Expression of ecSOD is subject to substantial degrees of regulation. In particular, ecSOD levels are increased by cytokines and in atherosclerotic vessels.

As a result of these considerations, we performed the present experiments to determine whether ecSOD expression is regulated by angiotensin II and hypertension. In these studies, we provide evidence that angiotensin II–induced hypertension strongly upregulates ecSOD activity, protein, and mRNA expression. These responses are independent of NADH/NADPH oxidase activation and mediated through p42/44 MAP kinase, which plays a pivotal role in cell growth and differentiation. In addition, we also found that angiotensin II–induced upregulation of ecSOD is due to both transcriptional and posttranscriptional mechanisms. These results may provide insight into the role of ecSOD in the modulation of the oxidative state of the vessel wall in pathological processes in which the renin-angiotensin II system is activated.
Materials and Methods

Animals Studied
Male C57BL/6 mice, which were purchased from Jackson Laboratories (Bar Harbor, Maine), were studied between age 3 to 4 months. For implantation of osmotic minipumps, the mice were anesthetized with intraperitoneal Avertin 2.5% (0.3 mL per 25g of body wt IP). The intrascapular region was shaved, and an osmotic minipump (Alzet model 2002; Alza Corp) that contained angiotensin II was inserted via a 1-cm incision to permit subcutaneous infusion of angiotensin II ([Val][8]Angiotensin II, infusion rate 0.7, 1.1 mg · kg⁻¹ · d⁻¹) or norepinephrine (5.6 mg · kg⁻¹ · d⁻¹). Sham-operated animals underwent an identical surgical procedure, except that neither a pump or an empty osmotic pump was implanted.

Systolic blood pressures were measured by a computerized tail-cuff system (Visitech Systems). Everyday for 5 days before the osmotic pump was implanted, the mice were trained in the blood pressure device to accustom them to the procedure. After the minipumps were harvested for study. In some experiments, the AT₁-receptor antagonist losartan (25 mg · kg⁻¹ · d⁻¹) was associated with an increase in mRNA expression, RNase assays and exposed to various experimental conditions for the times indicated.

Western Blot Analysis and Determination of Aortic SOD Activity
Vessels were homogenized in a 50 mmol/L Tris-HCl buffer that contained 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1% Triton X-100, 0.5 mmol/L PMSF, 10 μg/mL leupeptin, and 10 μg/mL antipain. Western blot analysis was performed to examine the effect of angiotensin II on protein expression of both ecSOD and the cytosolic Cu/ZnSOD as previously described. The primary antibodies used included a rabbit polyclonal antibody against murine ecSOD and a sheep antibody against human Cu/ZnSOD (Biodesign International). SOD activity was assayed by monitoring inhibition of the rate of xanthine/xanthine oxidase–mediated reduction of cytochrome c (pH 7.4) as previously described. Cyanide (3 mmol/L) was used to distinguish between the cyanide-sensitive isozymes Cu/ZnSOD and ecSOD and the cyanide-resistant MnSOD. For specific analysis of ecSOD in vessel extracts, chromatography on concanavalan A Sepharose (Pharmacia Biotech) was used. Unlike Cu/ZnSOD and MnSOD, the glycoprotein in ecSOD binds to the lectin concanavalin A.

Isolation of RNA and Ribonuclease Protection Assay
The above assays indicated that angiotensin II increased both ecSOD enzyme activity and protein expression. To determine whether this was associated with an increase in mRNA expression, RNase assays were performed as previously described. A 923-riboprobe nucleotide was used, which protected 828 nucleotides of ecSOD. The murine GAPDH riboprobe (Ambion, Inc) was used as a control.

Organoid Cultures of Mouse Aorta
To examine the effect of angiotensin II on intact aortas in the absence of neurohumoral adaptations and changes in blood pressure, we used organoid cultures. This also permitted the analysis of potential signaling mechanisms responsible for angiotensin II–modulation of ecSOD expression. After dissection of adventitial tissue, aortic segments were immersed in 6-well dishes that contained DMEM (GIBCO BRL) and antibiotics (penicillin 100 U/mL, streptomycin 100 mg/L) and was supplemented with 0.1% calf serum. The vessel segments were then maintained in a tissue culture incubator at 37°C and exposed to various experimental conditions for the times indicated.

Studies of Human Aortic Smooth Muscle Cells
To determine if angiotensin II might modulate ecSOD expression in human cells and to determine if this was mediated by either transcriptional or posttranscriptional mechanisms, human aortic smooth muscle cells (HASMs, Clonetics Corp) were studied. HASMs were cultured in smooth muscle basal medium (Clonetics) and 5% FBS. Experiments were performed with 0.5% serum and no additives at passages 4 to 8.

The human ecSOD cDNA was cloned with reverse transcription–polymerase chain reaction from total RNA obtained from HASMs. This approach used a forward primer from the 5' end of coding region with a SalI site at the 5' end (underlined), (5'-CACCCTGCAAGCAGTG CCCGACTCCC AGCCA TGCG-3', nt +52) and a reverse primer complimentary to 83 bp outside of the end of the coding region (with a EcoR1 site incorporated at the 5' end) (5'-CCGGGAATTCTGGTGTCGGGAGCAGAG C3', nt +875). The resultant polymerase chain reaction product was gel-purified, digested, and subcloned into the EcoRI/Sall sites of the pBluescript SK-vector (Stratagene Inc) and sequenced with the Sequenase 2.0 kit (U.S. Biochemical Corp). RNase protection assays were performed as described above except that 40 U of RNase T1 was used.

Nuclear run-on assays were performed as previously described. Identical numbers of nuclei from control and angiotensin II–treated HASMs were used for preparation of nascent transcripts. After linearization of the ecSOD DNA with BgIII, antisense ecSOD RNA was transcribed from the T3 promoter with T3 polymerase and sense ecSOD RNA transcribed from T7 polymerase. Equal amounts of these RNA probes (5 μg) and a human antisense GAPDH RNA were slot-blotted onto a Zeta-Probe GT membrane (Bio-Rad Laboratories). Hybridization was performed for 36 hours at 65°C. Signals were visualized by autoradiography and densitometric values for the nascent ecSOD transcripts were normalized to the GAPDH signal.

To examine ecSOD mRNA stability, HASMs were either untreated or pretreated with angiotensin II for 10 hours and then exposed to 10 μg/mL actinomycin D for 6 to 24 hours. Total RNA was collected at the times indicated, and RNase protection assays were performed as described above.

Materials
Radiochemicals were purchased from DuPont Corp. All other reagents were purchased from Sigma Chemical Co, except when specified.

Data Analysis and Statistical Evaluation
RNase protection assays and Western blots were analyzed by densitometry by use of National Institutes of Health Image software. For RNase protection assays, values were normalized to the GAPDH densitometry. In each of these cases, data for angiotensin II and other experimental conditions are expressed as a percentage of control vessels studied in parallel. Values are expressed as mean±SEM. Comparisons were performed with t tests and a Bonferroni correction for multiple comparisons when indicated. Values of P<0.05 were considered significant.

Results
Effect of Angiotensin II and Norepinephrine on Systolic Blood Pressure in Mice
Angiotensin II infusion (1.1 mg · kg⁻¹ · d⁻¹) caused a progressive increase in systolic blood pressure from 107±3 to 152±9 mm Hg. Blood pressure peaked by the third to fourth day of angiotensin II infusion and remained elevated for the duration of the infusion period. Norepinephrine infusion (5.6 mg · kg⁻¹ · d⁻¹) produced a similar increase in blood pressure from 109±6 to 141±7 mm Hg (Figure 1). Losartan prevented the effect of angiotensin II on blood pressure.
Effect of Angiotensin II on ecSOD Expression

Western blots consistently revealed 2 bands in mouse aortas that corresponded to ecSOD type C and A (proteolized form). Protein levels of ecSOD were increased by angiotensin II infusion in a dose-dependent manner (2.6±0.3-fold increase at 7 days after angiotensin II infusion, Figure 2A). In contrast, angiotensin II infusion did not change cytosolic Cu/ZnSOD protein (Figure 2A). Aortic ecSOD protein levels were not changed by norepinephrine infusion (Figure 2B). Thus, although both angiotensin II and norepinephrine increased blood pressure to a similar extent, only angiotensin II increases ecSOD expression. Losartan completely abrogated the effect of angiotensin II on ecSOD expression (Figure 2B).

Total SOD activity was similar in the aortas of control mice and mice made hypertensive by angiotensin II (Figure 3). However, when specific assays for each SOD isozyme were performed, ecSOD activity was increased significantly in aortas of mice with angiotensin II–induced hypertension (1.63±0.12 versus 0.73±0.17 U/mg protein; P<0.05; Figure 3).

Infusion of angiotensin II increased ecSOD mRNA in a time- and dose-dependent manner, as assessed by RNase protection assays (maximum 2.7±0.7-fold at 7 days of angiotensin II infusion, Figure 4).

Effect of Angiotensin II on ecSOD Expression in Organoid Culture of Mouse Aortas

As observed in vivo, angiotensin II also increased aortic ecSOD protein expression in organoid cultures (Figure 5A). This effect was time dependent, and by 12 hours of exposure to angiotensin II, ecSOD protein increased by 3.1±0.2-fold. Similar to the results in vivo, losartan (10 μmol/L) completely inhibited this effect (Figure 5B). Because angiotensin II is known to increase production of reactive oxygen species by the activation of an NADH/NADPH oxidase,2,4 we performed additional studies to determine whether angiotensin II induction of ecSOD was mediated via oxidative stress. Neither diphenyleneiodonium chloride (DPI, 10 μmol/L)2 an inhibitor of the NADH/NADPH oxidase, nor the superoxide scavenger Tiron (10 mmol/L)3 affected ecSOD upregulation induced by angiotensin II (Figure 5B). Thus, reactive oxygen
species seem unlikely to be crucial in the effect of angiotensin II on ecSOD expression.

A major signaling cascade initiated by angiotensin II in vascular tissue is the p42/44 MAP kinase pathway. We have previously found that angiotensin II activates the p42/44 MAP kinase in rat aortic smooth muscle cells in a redox-insensitive fashion. In preliminary studies with the use of antibodies specific for phosphorylated p42/p44 MAP kinase, we confirmed this finding in mice aortic organoid cultures (data not shown). Therefore, we examined a possible role of p42/44 MAP kinase in angiotensin II stimulation of ecSOD expression. As shown in Figure 6, the specific p42/44 MAP kinase kinase inhibitor PD98059 completely abolished the effect of angiotensin II on ecSOD protein expression.

Figure 6. Effect of MEK inhibition on ecSOD expression in response to Ang II. Mouse aortas were exposed to Ang II (100 nmol/L) for 12 hours in organoid culture in the absence and presence of PD98059 (30 μmol/L) for 12 hours. The top inset shows a representative Western blot analysis, and the bottom inset shows mean data for 3 separate experiments. *P<0.05 vs control.

Figure 7. Effect of Ang II on expression of ecSOD mRNA in HASMs. HASMs (passage 4 to 6) were exposed to Ang II (100 nmol/L) for the times indicated and mRNA levels were determined by use of RNase protection assays as in Figure 4, with human ecSOD and GAPDH riboprobes. Left, A representative RNase protection assay. Right, Mean data for 3 separate experiments. *P<0.05, †P<0.01 vs control cells.

Effect of Angiotensin II on ecSOD Expression in HASMs

To determine whether our findings in mouse vessels extend to humans, we performed additional studies with HASMs. As shown in Figure 7, exposure of HASMs to angiotensin II for 12 hours increased ecSOD mRNA expression by 3.8±0.2-fold. Nuclear run-on assays revealed that angiotensin II increased the transcription rate by 1.7±0.3-fold (Figure 8A). In addition, angiotensin II enhanced the half-life of ecSOD mRNA (Figure 8B). In control HASMs, the half-life of
ecSOD mRNA, which was assessed after exposure to actinomycin D, was \( \approx 16 \) hours. In contrast, angiotensin II increased the half-life of the ecSOD mRNA to \( \approx 24 \) hours.

**Discussion**

The present studies show that hypertension caused by angiotensin II increases ecSOD enzyme activity, protein levels, and mRNA expression by 2- to 3-fold. Induction of ecSOD during angiotensin II infusion is not the result of hypertension alone because norepinephrine infusion, which produces a similar degree of hypertension, does not affect ecSOD expression. In organoid culture, angiotensin II (100 nmol/L) also increases ecSOD mRNA and protein expression by \( \approx 2 \)-fold. This effect in organoid culture seems to be dependent on activation of the p42/44 MAP kinase pathway but independent of superoxide production by the vascular NADH/NADPH oxidase. This effect of angiotensin II also occurs in HASMs and is due to increases in both ecSOD transcriptional rate and mRNA stability.

Angiotensin II–induced hypertension is associated with increased vascular superoxide production.\(^4\) Interestingly, in rats (a species that lacks vascular ecSOD activity),\(^19\) angiotensin II infusion produces hypertension that is substantially more severe than that observed in the present study in mice.\(^5,6\)

In addition, angiotensin II–induced hypertension in rats can be prevented by either liposome-encapsulated SOD or heparin-binding SOD, which is similar to ecSOD.\(^5,6\) Thus, in species that express vascular ecSOD, such as the mouse, upregulation of endogenous vascular ecSOD may represent an important compensatory mechanism that blunts the blood pressure response during conditions when angiotensin II is chronically elevated.

It has become apparent that increases in cyclic strain, such as those that may be encountered in hypertension, can influence transcription of a variety of genes in vascular cells.\(^20,21\) Several lines of evidence from the present data suggest that the direct effect of hypertension was not the cause of increased ecSOD expression in response to angiotensin II. Norepinephrine, which also produced hypertension, had no effect on ecSOD expression. In addition, exposure of mouse aortas or HASMs to angiotensin II in culture also increased ecSOD expression. Thus, although increased pressure may contribute to this phenomenon, angiotensin II can directly increase expression of ecSOD in mouse aortas and HASMs independent of pressure.

Although the present studies focused on the effect of angiotensin II, they do not exclude the possibility that other neurohumoral mediators may also modulate ecSOD expression. The effect of angiotensin II is of particular interest however, because this octapeptide has been implicated in the pathogenesis of several vascular diseases, including atherosclerosis, hypertension, diabetes, and heart failure.

Both in vivo experiments and organoid cultures, the effect of angiotensin II on ecSOD expression was completely prevented by losartan, which indicated that the result is due to activation of the AT\(_1\) receptor. Angiotensin II directly activates an NADH/NADPH oxidase in cultured vascular smooth muscle cells\(^2\) and in vivo.\(^4\) We therefore determined whether upregulation of ecSOD by angiotensin II was mediated by reactive oxygen species. Angiotensin II–induced expression of ecSOD was not prevented by either DPI, an inhibitor of flavoproteins such as the NADH/NADPH oxidase, or by Tiron, a superoxide scavenger. These results suggest that angiotensin II directly upregulates ecSOD independently of superoxide anion production or activation of the NADH/NADPH oxidase.

In cultured smooth muscle cells, the p42/44 MAP kinase pathway is activated by angiotensin II.\(^16\) Interestingly, the selective MEK inhibitor PD98059\(^18\) completely prevented upregulation of ecSOD induced by angiotensin II. In keeping with these observations, we have recently demonstrated that angiotensin II activates p42/44 MAP kinase through DPI-insensitive mechanisms in cultured rat vascular smooth muscle cells.\(^17\) In combination, these data suggest that angiotensin II upregulates ecSOD expression via a p42/44 MAP kinase pathway independently of superoxide production. To our knowledge, this is the first evidence that the p42/44 MAP kinase pathway is involved in regulation of antioxidant enzymes in vascular tissue.

Nuclear run-on assays in HASMs indicated that a portion of the effect of angiotensin II on ecSOD expression was due to enhanced transcription of ecSOD. Angiotensin II is known to promote AP-1 complex formation,\(^22,23\) which is then probably signaled via p42/44 MAP kinase activation.\(^24\) Of note, the 5′ promoter region of the ecSOD gene contains an AP-1 consensus sequence that may be involved in its activation by angiotensin II.\(^25\)

It has become clear that modulation of mRNA half-life often plays a critical role in regulation of gene expression. In the present study, we found that angiotensin II increased ecSOD mRNA stability. There is ample precedent for angiotensin II affecting the stability of other mRNAs. In rat aortic smooth muscle cells, angiotensin II has been shown to increase expression of the parathyroid-related hormone by increasing gene transcription and by mRNA stabilization.\(^26\) In hepatocytes, angiotensin II increases stability of c-fos mRNA.\(^27\) Similarly, angiotensin II has been shown to increase angiotensinogen mRNA half-life, probably through the binding of stabilizing proteins to UCCUU sequences in the 3′ UTR.\(^28\) Interestingly, both the human and murine ecSOD contain similar UCCUU sequences in their respective 3′ UTR, which may be involved in the response to angiotensin II.

Of note, the total vascular SOD was not increased by angiotensin II infusion in vivo. Nevertheless, the increase in ecSOD caused by angiotensin II probably has a major effect on superoxide scavenging in the interstitial space, where this enzyme is localized. It has recently been shown that endothelial cells release superoxide extracellularly,\(^29\) and this may influence the biological half-life of nitric oxide when it traverses from the endothelium to the media. Enhanced superoxide scavenging by the ecSOD would increase nitric oxide bioactivity. Additionally, copper/zinc-containing SOD possesses peroxidative activity.\(^30,31\) Thus, increased ecSOD expression could increase peroxidative reactions in the interstitial space, thus influencing radical chain reactions and ultimately LDL oxidation.

Recently, we found that atherosclerosis is also associated with an increase in vascular ecSOD, seemingly from lipid-
laden macrophages. Given our present findings, it appears that both atherosclerosis and angiotensin II–induced hypertension increase vascular ecSOD levels, albeit from different cell types. The simplest interpretation of these findings is that increased ecSOD expression represents an important adaptive mechanism that allows increased scavenging of $O_2^-$ in these two conditions in which rates of vascular $O_2^-$ production are elevated. Because $O_2^-$ can increase blood pressure and increases in vascular levels of SOD can lower blood pressure, this response to angiotensin II may serve as a critical adaptation that blunts the effect of angiotensin II on blood pressure and vascular redox state. More importantly, there are several known polymorphisms of ecSOD in humans. Although none of these are associated with an obvious phenotype, it is unclear how they may respond to hypertension and activation of the renin-angiotensin system. It is also possible that increased ecSOD expression in these conditions may contribute to vascular pathology. The dismutation of $O_2^-$ by ecSOD would increase extracellular levels of $H_2O_2$, which may have important proatherogenic properties. The superoxide anion is more likely a reducing agent than an oxidizing agent, and $H_2O_2$ is a modestly potent oxidizing agent. $H_2O_2$ is substantially more stable than $O_2^-$, and because it is uncharged, it can freely diffuse into adjacent cells, in which it may serve to promote vascular smooth muscle hypertrophy, alter gene expression, and activate matrix metalloproteinases. Importantly, transgenic mice that overexpress Cu/ZnSOD, develop more extensive fatty-streak deposition than control mice when fed a high cholesterol diet. Thus, it is conceivable that increased vascular levels of ecSOD might have untoward effects, thus linking angiotensin II–induced hypertension and atherosclerosis.

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References

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