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Role of copper transport protein Antioxidant-1 in Angiotensin II-induced hypertension: A key regulator of Extracellular SOD

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Abstract

Extracellular superoxide dismutase (SOD3) is a secretory copper enzyme involved in protecting angiotensin II (Ang II)-induced hypertension. We previously found that Ang II upregulates SOD3 expression and activity as a counter-regulatory mechanism; however, underlying mechanisms are unclear. Antioxidant-1 (Atox1) is shown to act as a copper-dependent transcription factor as well as copper chaperone for SOD3 *in vitro*, but its role in Ang II-induced hypertension *in vivo* is unknown. Here we show that Ang II infusion increases Atox1 expression as well as SOD3 expression and activity in aortas of wild-type mice, which are inhibited in mice lacking Atox1. Accordingly, Ang II increases vascular $O_2^{\bullet-}$ production, reduces endothelium-dependent vasodilation and increases vasoconstriction in mesenteric arterioles to a greater extent in Atox1$^{-/-}$ than in wild-type mice. This contributes to augmented hypertensive response to Ang II in Atox1$^{-/-}$ mice. In cultured vascular smooth muscle cells, Ang II promotes translocation of Atox1 to the nucleus, thereby increasing SOD3 transcription by binding to Atox1 responsive element in the SOD3 promoter. Furthermore, Ang II increases Atox1 binding to the copper exporter ATP7A which obtains copper from Atox1 as well as translocation of ATP7A to plasma membranes where it colocalizes with SOD3. As its consequence, Ang II decreases vascular copper levels, which is inhibited in Atox1$^{-/-}$ mice. In summary, Atox1 functions to prevent Ang II-induced endothelial dysfunction and hyper-contraction in resistant vessels as well as hypertension in vivo by reducing extracellular $O_2^{\bullet-}$ levels via increasing vascular SOD3 expression and activity.

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Disclosures
None

Keywords

Angiotensin II; Hypertension; Oxidative stress; Antioxidant 1; SOD3; SOD1; Copper

Introduction

Angiotensin II (Ang II), the principal effector peptide of the renin-angiotensin system, plays a major role in the initiation and progression of vascular diseases such as hypertension in part through reactive oxygen species (ROS).[1] Ang II-induced increase in ROS in particular, superoxide ($O_2^{\bullet-}$), leads to decreased bioavailability of nitric oxide (NO), which impairs endothelium-dependent vasodilatation and promotes vasoconstriction. A major antioxidant defense system against $O_2^{\bullet-}$ is the superoxide dismutases (SODs): the cytoplasmic Cu/ZnSOD (SOD1), the mitochondrial MnSOD (SOD2), and the extracellular Cu/ZnSOD (SOD3, ecSOD).[2] SOD3 is a major extracellular antioxidant enzyme highly expressed in the vasculature and it plays an important role in regulating blood pressure and endothelial function by reducing extracellular $O_2^{\bullet-}$ level, thereby preventing oxidative inactivation of NO released from endothelium.[3-5] We and others reported that Ang II-induced increase in blood pressure, vascular $O_2^{\bullet-}$ levels, and endothelial dysfunction is augmented in SOD3$^{-/-}$ mice,[4, 5] which are ameliorated by treatment with recombinant SOD3.[4] We have also shown that Ang II increases expression and activity of SOD3 *in vitro* and *in vivo*.[6] These findings suggest that Ang II-induced modulation of SOD3 is important protective mechanism that prevents overproduction of $O_2^{\bullet-}$; however, underlying regulatory mechanisms remain unknown.

Copper is an essential micronutrient by serving as a cofactor for key metabolic and redox related proteins, and SOD1 and SOD3 require catalytic copper for their full enzymatic activity[2] . Under physiological conditions, the intracellular level of free copper is extraordinarily restricted.[7] Thus, soluble copper carrier proteins termed “copper chaperones” are required to directly transfer copper to specific cellular target proteins. SOD1 obtains copper through interaction with the cytosolic copper chaperone for SOD1 (CCS).[8] Using cultured fibroblasts, we demonstrated that exogenous copper-induced full activation of SOD3 requires copper chaperone Antioxidant-1 (Atox1), which delivers copper to the secretory copper enzymes, likely via interaction with the copper exporter ATP7A (Menkes ATPase).[9-11] Of note, Atox1-ATP7A pathway is involved in not only to activate secretory copper enzyme but also to control intracellular copper levels by regulating secretion of excess copper. Recently, we have shown that Atox1 also serves as a transcription factor for copper-induced increase in SOD3 and cyclin D1 expression *in vitro*.[9, 12] The association of copper homeostasis with hypertension has been reported.[13] Tissue levels of copper are either decreased or increased in hypertensive rats, which have been proposed to be affected by severity of hypertension.[14, 15] Serum copper concentration is increased in humans characterized by either essential or pulmonary hypertension,[16, 17] as well as various rodent hypertension model.[14] Copper deficient diet alters blood pressure in an age-dependent manner.[18, 19] However, the role of Atox1 in hypertension *in vivo* has not been investigated.

We performed the present study to test the hypothesis that Atox1 may play a critical role in modulating blood pressure and vascular function in resistance vessels via regulating vascular SOD3 during Ang II-induced hypertension. Here we demonstrate that Ang II infusion markedly increases Atox1 protein expression as well as SOD3 expression and specific activity in aortas from wild type mice, which are inhibited in Atox1$^{-/-}$ mice. Ang II increases vascular $O_2^{\bullet-}$ production and promotes endothelial dysfunction in mesenteric arterioles to a greater extent in Atox1$^{-/-}$ than in wild-type mice. This contributes to the exaggerated hypertensive response to Ang II in Atox1$^{-/-}$ mice. Mechanistically, Ang II

increases Atox1 binding to the SOD3 promoter to increase SOD3 transcription as well as Atox1 binding to copper exporter ATP7A to increase specific activity of SOD3 in cultured vascular smooth muscle cells (VSMCs). Thus, Ang II-induced upregulation of Atox1 may represent an important counter-regulatory mechanism that blunts overproduction of $O_2^{\bullet-}$ or elevating blood pressure through regulating SOD3 expression and activity in the vasculature.

Materials and Methods

Animal and Cell Culture Studies

Atox1$^{-/-}$ mice (backcrossed eight times to C57Bl/6) were obtained from Mutant Mouse Regional Resource Centers. Atox1$^{-/-}$ mice were originally reported to have phenotypes which show perinatal death (45% of pups) or survive more than one month.[20] Our laboratory further backcrossed Atox1$^{-/-}$ mice to C57Bl/6 mice more than ten times, and thus used C57Bl/6 mice as control. “Survivor” mice were intercrossed with more than 10 times and Atox1$^{-/-}$ mice used in the present study survived more than six months (90%). For in vivo experiments, we performed Ang II infusion by osmotic minipumps, blood pressure measurement by the tail cuff method, immunohistochemical and western analysis, real-time PCR, SOD activity assay, vascular $O_2^{\bullet-}$ production, vascular reactivity studies by wire myograph, copper measurements by inductively coupled plasma mass spectrometry (ICP-MS), and synchrotron X-ray Fluorescence Microscopy analysis. All studies were approved by the Animal Care and Use Committee of the University of Illinois-Chicago. For in vitro experiments, rat vascular smooth muscle cells (VSMCs) (between passage 7 and 15) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% bovine serum. Using them, we performed nuclear/cytoplasmic fractionation, immunofluorescence, plasmid constructs, transient transfection and reporter assay, and DNA pull down assay.

An expanded Materials and Methods section is available in the online Data Supplement (please see http;//hyper.ahajournals.org).

Results

Atox1 expression is increased in aortas of Ang II-infused mice

We first examined the Atox1 expression in aorta from mice with Ang II-induced hypertension. Western (Figure 1A) and immunohistochemical (Figure 1B) analysis showed that Ang II infusion (0.7 mg/kg/day) for 7 days significantly increased Atox1 protein expression in the medial layer of aorta. Importantly, some of Atox1-positive cells were found in the nucleus (Figure 1B).

Effect of Ang II infusion on SOD3 expression and activity in aortas of wild-type and Atox1$^{-/-}$ mice

Atox1 has been shown to function as a copper chaperone for secretory copper-containing enzymes[20, 21] and a copper-dependent transcription factor in vitro.[9, 10, 12] Thus, we examined the SOD3 expression and activity in aorta of Ang II-infused Atox1$^{-/-}$ mice. The body weights and various organ weights (heart, kidney, lung and brain) were not different between C57Bl/6 wild-type (WT) and Atox1$^{-/-}$ mice (Figure S1). Ang II infusion for 7 days significantly increased mRNA and protein levels (Figure 2A and 2B) as well as activity of SOD3 (Figure 2C), but not those of SOD1, in aortas from WT mice. These Ang II-induced effects were almost completely inhibited in Atox1$^{-/-}$ mice (Figure 2). At baseline, SOD3 protein and mRNA levels as well as activity were decreased in Atox1$^{-/-}$ compared with WT aortas. In contrast, total SOD activity, protein expression of other major Cu enzymes

including ceruloplasmin or lysyl oxidase in aortas were similar between WT and Atox1$^{-/-}$ mice at baseline and after Ang II infusion (Figures S2 and S3, and Figure 2). These findings suggest that Atox1 plays an important role in regulating expression and activity of SOD3 in vessels of Ang II-infused mice.

Effect of Ang II on blood pressure and vascular $O_2^{\bullet-}$ production in WT mice and Atox1$^{-/-}$ mice

We next examined the functional role of Atox1 in Ang II-induced hypertension and $O_2^{\bullet-}$ levels using Atox1$^{-/-}$ mice. The basal blood pressure and the initial hypertensive response to Ang II by day 4 were similar in the Atox1$^{-/-}$ and WT mice. However, Ang II infusion for 7 days increased systolic blood pressure to greater extent in Atox1$^{-/-}$ mice than in wild-type mice (Figure 3A). Furthermore, lucigenin chemiluminescence analysis showed that Ang II-induced increase in $O_2^{\bullet-}$ production was significantly enhanced in Atox1$^{-/-}$ aorta as compared with WT mice (Figure 3B), while basal $O_2^{\bullet-}$ production was modestly, but not significantly, increased in Atox1$^{-/-}$ aorta. Ang II-induced alteration of blood pressure and vascular $O_2^{\bullet-}$ production in Atox1$^{-/-}$ mice and WT mice were rescued by co-infusion of the SOD mimetic, tempol (Figures. 3A and 3B). These results suggest that augmentation of Ang II-induced increase in blood pressure in Atox1$^{-/-}$ mice is due to increased $O_2^{\bullet-}$ levels.

Effect of Ang II on mesenteric vascular function in WT mice and Atox1$^{-/-}$ mice

To determine the role of Atox1 in endothelium-dependent vasodilation, we used mesenteric arteries (200 μm in diameter) with the wire myograph approach. Under basal conditions, endothelium-dependent relaxations to acetylcholine (ACh) were impaired in Atox1$^{-/-}$ mice compared with those of WT mice (Figure 4A, left panel). Ang II caused a marked impairment of ACh-evoked relaxation to a greater extent in arteries of Atox1$^{-/-}$ than WT mice (max relaxation 44.8±1.72 vs. 61.3±3.02%, $p<0.01$). Relaxations to endothelium-independent vasodilator sodium nitroprusside (SNP) were similar in WT and Atox1$^{-/-}$ mice at baseline and were not altered by Ang II (Figure 4B). The impairment of ACh-induced vasorelaxation in Ang II-infused Atox1$^{-/-}$ mice and WT mice was rescued by co-infusion of the SOD mimetic tempol (Figure 4A, right panel). In mice without Ang II infusion, direct contractile responses to Ang II were markedly increased in mesenteric arteries of Atox1$^{-/-}$ mice compared with WT mice, which was rescued by the SOD mimetic tempol (Figure S4). By contrast, contractions induced by phenylephrine, which did not produce $O_2^{\bullet-}$, or KCl were not different in WT and Atox1$^{-/-}$ mice. Thus, non-KCl normalized data for Ang II-induced vasoconstriction in WT and Atox1$^{-/-}$ mice was similar to the one after normalization (not shown). Thus, these results suggest that Atox1 is required for full activity of SOD3 and thus decreasing $O_2^{\bullet-}$ level, thereby inhibiting Ang II-induced endothelial dysfunction and vasoconstriction.

Ang II promotes translocation of Atox1 into the nucleus in VSMCs

To examine the molecular mechanisms by which Atox1 regulates SOD3 expression and activity in the vasculature, we next examined the subcellular localization of Atox1. Immunofluorescence analysis in VSMCs shows that Ang II stimulation promoted translocation of Atox1 from the cytosol to the nucleus in a time-dependent manner with peak at 30 min (Figure 5A). Consistently, subcellular fractionation shows that Atox1 protein level is increased in nuclear fraction with a peak at 30 min after Ang II stimulation, and then decreased after 120 min (Figure 5B). We confirmed the purity of nuclear and cytosolic fractions by detecting the markers, laminin B and tubulin, respectively.

Ang II stimulates Atox1 binding to the SOD3 promoter and its promoter activity in VSMCs

To determine if Atox1 functions as a transcription factor for SOD3 in Ang II-stimulated VSMCs, we performed luciferase reporter gene assays. Ang II stimulation increased SOD3 promoter activity, which was blocked by the mutation of Atox1 responsive elements (Atox1-RE)[12] in the SOD3 promoter (Figure 6A). DNA pull-down assays show that Atox1 directly bound to the Atox1 responsive element including DNA segment (Figure 6B). These results suggest that Ang II increases SOD3 transcription by promoting Atox1 binding to the SOD3 promoter.

Ang II stimulates Atox1 binding to ATP7A as well as translocation of ATP7A to plasma membrane where it colocalizes with SOD3 in VSMCs

Atox1 also functions to deliver copper to secretory copper enzymes via interaction with copper exporter ATP7A in yeast system.[21, 22] Co-immunoprecipitation assay reveals that Ang II increased Atox1 binding to ATP7A (Figure 7A) as well as ATP7A binding to the secretory copper enzyme SOD3 (Figure 7B) in a time-dependent manner. This Ang II-induced formation of Atox1/ATP7A/SOD3 complex may be required for increasing SOD3 specific activity. To gain further insights the mechanism by which Atox1 increases SOD3 specific activity, we examined the subcellular localization of ATP7A in response to Ang II in VSMCs. Immunofluorescence analysis shows that Ang II stimulation promoted the translocation of ATP7A from TGN to plasma membrane and post-Golgi vesicles where it colocalized with SOD3. This Ang II-induced alternation of ATP7A localization was inhibited by Atox1 siRNA (Figures 7C and 7D).

Effect of Ang II on vascular copper levels in WT mice and Atox1$^{-/-}$ mice

Because copper exporter ATP7A is involved in delivering copper to the SOD3 which is secreted to the extracellular space, we examined the copper levels in aorta from WT and Atox1$^{-/-}$ mice with or without Ang II infusion. Inductively coupled plasma mass spectrometry (ICP-MS) (Figure 8A) and synchrotron-based X-ray fluorescence microscopy (SXFM) analysis (Figure 8B) show that Ang II infusion significantly decreased copper levels in the medial layers of aorta. Specific decrease in copper levels was normalized by sulfur levels, which was used as a surrogate for total cellular protein and to visualize the morphology of tissue sections[23]. By contrast, Atox1$^{-/-}$ mice showed increased copper levels in aorta at basal state, which was not affected by Ang II infusion (Figures 8A and 8B).

Discussion

Previous studies have implicated a protective role for intracellular SOD1 as well as extracellular SOD3 in limiting endothelial dysfunction produced by AngII.[4, 5, 24] In the present study, we demonstrate that Ang II infusion increases Atox1 expression in aortas of wild-type mice. Activity and expression of SOD3, but not those of SOD1, are decreased in Atox1$^{-/-}$ mice treated with Ang II. This leads to exaggerated Ang II-induced vascular $O_2^{\bullet-}$ production, thereby promoting Ang II-induced endothelial dysfunction and vasoconstriction, which in turn augments systolic blood pressure in Atox1$^{-/-}$ mice. These changes induced by Atox1 deficiency are rescued by adding SOD mimetic tempol. Thus, Atox1 functions to prevent Ang II-induced endothelial dysfunction and hypertension by reducing $O_2^{\bullet-}$ levels via increasing vascular SOD3 expression and activity. Moreover, Atox1 regulates copper levels in the vasculature during Ang II-induced hypertension.

Impairment of endothelium-dependent ACh-induced relaxation in mesenteric arteries from Ang II-infused Atox1$^{-/-}$ mice is likely due to a decrease in endothelial NO· bioavailability, because endothelium-independent vasodilation is not affected by Ang II in Atox1$^{-/-}$ mice. There is significant reduction in vascular reactivity in vehicle-infused Atox1$^{-/-}$ mice, which

may be in part due to the slight, but not significant, increase in $O_2^{\bullet-}$ levels in these mice at baseline. However, Ang II-induced hypertension, but not basal blood pressure, is significantly altered in Atox1$^{-/-}$ mice. In addition, it has been reported that Ang II increases vasoconstriction partially through $O_2^{\bullet-}$-induced inactivation of vasorelaxant NO[1], although this response depends on vascular-bed and genetic background.[25, 26] In the present study, we found that contractions to the Ang II, but not KCl, are significantly increased in mesenteric arteries of the Atox1$^{-/-}$ mice compared with WT mice. This is inhibited by SOD mimetic tempol. Thus, the loss of NO bioavailability induced by decrease in SOD3 activity and subsequent increase in $O_2^{\bullet-}$ levels in Atox1 deficient arteries may contribute not only to impaired endothelium-dependent vasodilation but also to enhanced Ang II-induced vasoconstriction. This in turn likely increases systemic vascular resistance in Atox1$^{-/-}$ mice during Ang II infusion and, thus, augments the hypertensive response observed in these animals. Of note, it has been shown that endothelium-dependent vasorelaxation in mesenteric arteries is dependent on not only NO but also epoxyeicosatrienoic acids (EETs) and H_2O_2, which have been proposed as endothelium-dependent hyperpolarizing factor (EDHF)[27]. Thus, Atox-1$^{-/-}$ mice have increased vascular $O_2^{\bullet-}$ which would scavenge NO, but also may have reduced vascular H_2O_2 to inhibit action of EDHF. This point requires further investigation in future study.

We found that the levels of total SOD activity were similar between WT and Atox1$^{-/-}$ mice and were not changed by Ang II infusion. This result may reflect the fact that SOD1 activity, which consists of more than half of total SOD activity, is not dependent on Atox1. Nevertheless, the dramatic decrease in SOD3 activity by Atox1 deficiency likely has a major impact on increasing $O_2^{\bullet-}$ levels in the interstitial space, where this enzyme is localized. This will efficiently promote oxidative inactivation of NO which traverses from the endothelial cells to VSMCs, thereby preventing endothelium-dependent vasorelaxation, which in turn increases blood pressure in Atox1$^{-/-}$ mice, as reported for SOD3$^{-/-}$ mice.[2] Of note, endothelial dysfunction in the coronary circulation of humans has profound prognostic implications in that it predicts adverse cardiovascular events and long-term outcome.[28] The R213G polymorphism in the SOD3 gene, which reduces binding to endothelial surface and increases serum SOD3 levels,[3] has been linked to an increase in cardiovascular risk.[29] Thus, protective role of Atox1 in endothelial function through SOD3 would have potential clinical impact.

In this study, SOD3 protein and mRNA expression as well as nuclear Atox1 expression in blood vessels are increased by Ang II infusion, which are abolished in Atox1$^{-/-}$ mice. Consistent with our results, Hamza and Gitlin originally showed that Atox1 is localized in the nucleus in cultured cells[30]; however, functional significance of nuclear Atox1 was not addressed. Using promoter analysis and DNA pull-down assay in cultured VSMCs, we demonstrate that Ang II stimulation promotes translocation of Atox1 from the cytosol to the nucleus as well as Atox1 binding to the Atox1 response element in SOD3 promoter. This observation is consistent with our earlier study that Atox1 serves as a transcription factor for copper-induced increase in SOD3 and cyclin D1 expression in fibroblasts.[9, 12] The present study also found that expression of other copper containing enzymes including lysyl oxidase, ceruloplasmin or SOD1 was not changed in Atox1$^{-/-}$ arteries as compared to WT with or without Ang II infusion. This is consistent with the fact that SOD3, but not lysyl oxidase, ceruloplasmin or SOD1, has Atox1-responsive elements in its promoters. Thus, current study provides the first evidence that “agonist-induced” transcription factor function of Atox1 for SOD3 plays a potentially important role in Ang II-induced upregulation of SOD3 expression in blood vessels, and thereby modulating $O_2^{\bullet-}$ levels and hypertensive responses induced by Ang II (Figure 9).

Copper chaperone function of Atox1 to deliver copper to SOD3 in Ang II-treated VSMCs is also demonstrated in this study. We found that Ang II promotes Atox1 binding to the copper exporter ATP7A which obtains copper from Atox1. It also stimulates ATP7A translocation from TGN to plasma membrane where it colocalizes with SOD3. Consistent with our results, Atox1 is shown to deliver copper to secretory copper enzymes via interaction with ATP7A in yeast.[21, 22] Furthermore, ATP7A is reported to deliver copper to the secretory copper enzymes in the postGolgi vesicles rather than in TGN where copper loading normally takes place.[31, 32] We previously reported that Ang II infusion-induced increase in blood pressure and vascular $O_2^{\bullet-}$ production are augmented in ATP7A mutant mice due to decrease in SOD3 activity.[33] In line with this, the present study provides the additional new evidence that Ang II promotes copper chaperone function of Atox1 by facilitating formation of Atox1/ATP7A/SOD3 complex, thereby increasing specific SOD3 activity (Figure 9).

The association of copper metabolism with hypertension has been implicated.[13] However, there is no information regarding vascular copper levels in hypertension, or involvement of Atox1 in this response. The present study shows that Ang II treatment significantly decreases vascular copper levels as assessed by ICP-MS and SXFM analysis, which is inhibited in Atox1$^{-/-}$ mice. This might in part reflect the Ang II-stimulated secretion of copper-loaded SOD3 to the circulation via the Atox1-ATP7A pathway, despite the majority of SOD3 will bind to the extracellular matrix. In addition, Ang II-induced decrease in vascular copper levels may be also due to secretion of secretory copper containing proteins, or excess copper to protect cells from its toxicity. This is consistent with previous study that levels of copper in some tissues such as liver and kidney are significantly lower in hypertensive rodents.[14] The physiological consequence of copper export via ATP7A is placental copper transport to the developing fetus during pregnancy or to provide copper as part of a neuronal protective mechanism.[34] Thus, the present study provide the first evidence that vascular copper levels are altered during Ang II-induced hypertension, which is at least in part regulated by Atox1. Detailed analysis of molecular mechanism and functional significance of altered copper levels in hypertension requires future investigation.

Previous studies have shown that SOD3$^{-/-}$ mice have normal blood pressure at baseline but Ang II-induced increase in blood pressure is augmented in these mice in a time dependent manner with a peak at day 7, which remained elevated at least until day 14.[5] Consistent with this, the present study found that Atox1$^{-/-}$ mice showed similar time course of exaggerated hypertensive response to Ang II, as reported for SOD3$^{-/-}$ mice. It has been reported that deletion of SOD3 in the circumventricular organ (CVO) in the brain increases blood pressure in the basal state and after Ang II infusion, in part by modulating sympathetic outflow.[35] Intriguingly, Atox1 expression is observed in several regions of brain including choroid plexus which belongs to CVO, where ATP7A is also expressed.[36] Thus, it is conceivable that Atox1 may be involved in regulation of blood pressure, either by regulation of SOD3 in CVO or by other secretory copper enzymes in the brain. Atox1 is also expressed in the kidney, including glomeruli in both the juxtramedullary and cortical nephrons and medulla associated with the loops of Henle,[37] which regulates oxidative stress-dependent hypertension.[38] In particular, $O_2^{\bullet-}$ production in the brain is required for the genesis of hypertension.[35, 39] In current study, we used non-invasive tail cuff method to measure blood pressure. However, it does not give the information, such as 24-hour blood pressure and blood pressure variability. Thus, Atox1-SOD3-mediated regulation of Ang II-induced hypertension should be further confirmed using telemetry system. Furthermore, investigating role of Atox1 in other oxidative stress-dependent pathophysiologies such as diabetes mellitus, obesity, and atherosclerosis in which blood pressure is not consistently increased[38] is the subject of future studies.

Perspectives

The present study provides compelling evidence that Atox1 plays an important role in regulating vascular function and hypertension induced by Ang II in vivo by decreasing extracellular $O_2^{\bullet-}$ and increasing bioavailability of NO through copper chaperone and transcription factor function for SOD3 in blood vessels (Figure 9). Atox1 is also involved in regulating copper homeostasis during Ang II induced hypertension. Our findings provide novel insight into Atox1 as a potential therapeutic target for treatment of hypertension and various other oxidative stress-dependent cardiovascular diseases.

Supplementary Material

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Novelty and Significance

What Is New?

- Copper transport protein Atox1 plays an important role in preventing endothelial dysfunction, augmented vasoconstriction, and hypertension induced by Ang II in vivo.
- Vascular Atox1 modulates NO-mediated vascular function via copper chaperone and transcription factor for SOD3, which scavenges extracellular $O_2^{\bullet -}$.
- Vascular Atox1 regulates copper homeostasis in Ang II induced hypertension.

What is Relevant?

- Atox1 regulates blood pressure and vascular function in Ang II-induced hypertension by regulating vascular SOD3 expression and activity.

Summary

- Our findings provide novel insight into Atox1 as a potential therapeutic target for various oxidative stress-dependent cardiovascular diseases, such as hypertension.

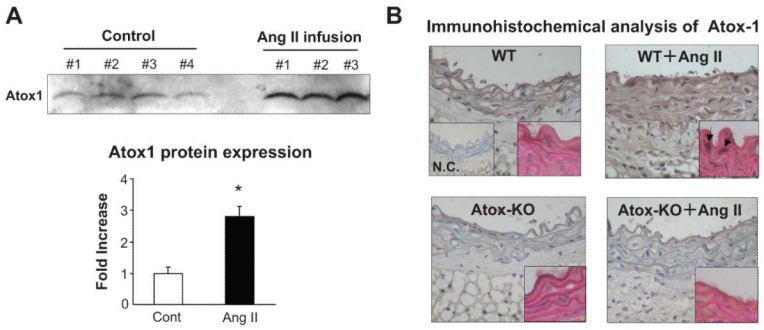

Figure 1. Effect of angiotensin II (Ang II) infusion on Atox1 expression in aortas of WT and Atox1$^{-/-}$ mice *in vivo* by immunoblotting (A) and immunohistochemistry (B)

Aortas of C57Bl/6 mice were harvested after a 7-day infusion of Ang II (0.7 mg/ kg/ day) or vehicle subcutaneously with osmotic minipumps, and subjected to Western analysis with antibodies specific to Atox1. Representative blots were from 3 individual experiments. *P<0.001 vs. WT mice. B, Immunohistochemical analysis of Atox1 expression in aortas from Ang II-treated C57Bl/6 (WT) and Atox1$^{-/-}$ (Atox1-KO) mice. Arrows in inset shows nuclear staining of Atox1. All images were taken at 5 different fields and the cell images are representative of >3 different experiments.

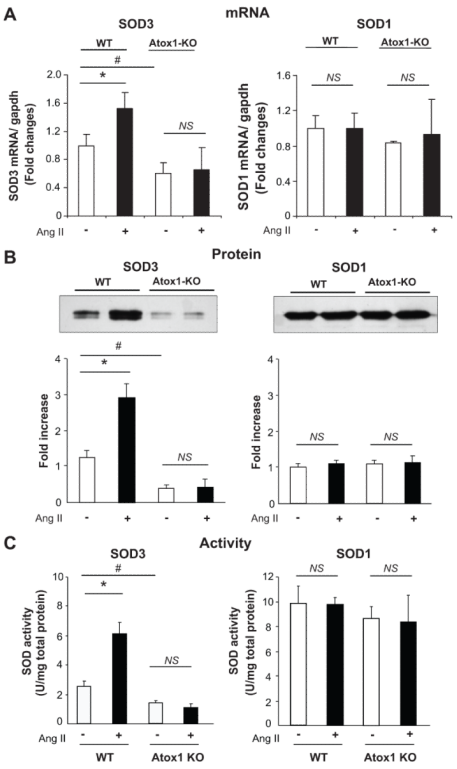

Figure 2. Effect of Ang II infusion on mRNA expression (A), protein level (B), and activity (C) of SOD3 and SOD1 in aortas from Atox1$^{-/-}$ mice

Mice were infused with Ang II as described in Figure 1. A and B, mRNA expression and protein levels of SOD1 and SOD3 in aortas of C57Bl/6 and Atox1$^{-/-}$ mice were measured by real-time PCR and by western analysis with antibodies specific to either SOD1 or SOD3. C, Activity of SOD3 and SOD1 in aortas were analyzed by the inhibition of cytochrome c reduction by xanthine/xanthine oxidase. SOD3 activity was determined after separation with Con A-Sepharose. The results are presented as mean ± SE from four separate experiments. Representative blots are from 3 individual experiments. *P<0.001; $^{\#}$P<0.01 vs. either vehicle-infused WT or Atox1$^{-/-}$ mice; *NS*, not significant

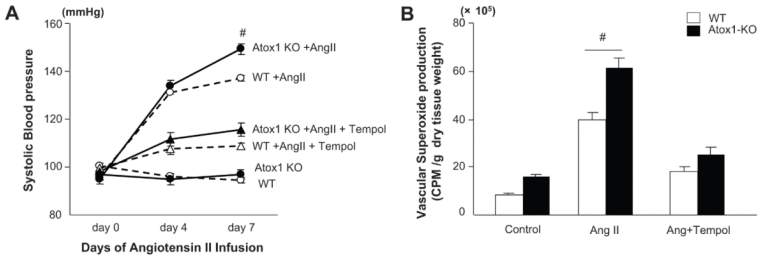

Figure 3. Effect of Ang II on blood pressure and vascular $O_2^{\bullet-}$ production in Atox1$^{-/-}$ mice

A, Either Ang II (0.7 mg/kg/day) or Ang II and the SOD mimetic Tempol (50 mg/kg/day) or vehicles were infused, and blood pressure was measured before, 4 and 7 days after minipumps implantation (n=5 per group). $^{\#}P<0.01$ vs. WT or Atox1$^{-/-}$ mice with Tempol treatment. B, $O_2^{\bullet-}$ production in aorta from C57Bl/6 (WT) and Atox1$^{-/-}$ mice as determined with lucigenin-enhanced chemiluminescence (5 μmol/L). $^{\#}P < 0.01$ vs. WT mice (n=6).

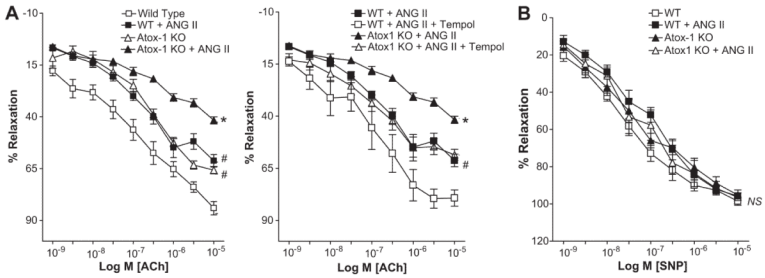

Figure 4. Effect of Ang II infusion on endothelium-dependent vascular relaxation in mesenteric arteries from Atox1$^{-/-}$ and WT mice

A and B, First or second-order mesenteric resistance arteries was studied using a wire myograph and precontracted with phenylephrine. Vasodilation was evoked by acetylcholine (Ach) in absence or presence of the SOD mimetic tempol (A) and sodium nitroprusside (SNP) (B). Data are expressed as mean ± SE (n=6-8 per group). * P<0.001 vs. remaining three groups, $^{\#}$P<0.01 vs. either untreated WT mice or Ang II-treated WT mice with Tempol treatment. *NS*, not significant.

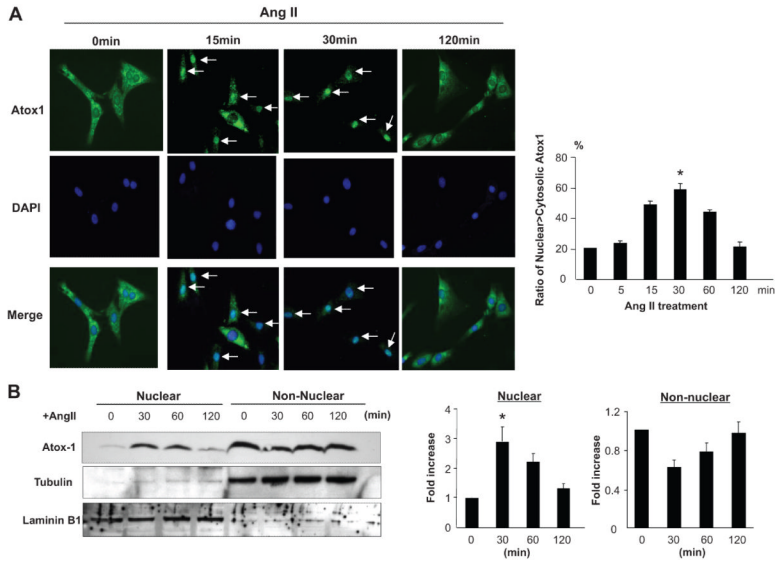

Figure 5. Effect of Ang II on nuclear translocation of Atox1 in vascular smooth muscle cells (VSMCs)

A, VSMCs were stimulated with Ang II (100 nM) for indicated time and cells were stained with anti-Atox 1 antibody and the nuclear marker, DAPI. In each image, ratio of Nuclear >Cytosolic Atox1 (immunofluorescence of nuclear Atox1 is higher than that of cytosolic Atox1) was calculated from 5 randomized view and the cell images are representative of >3 different experiments. *P<0.001vs. no treated cells. White arrows indicate nuclear Atox1 predominant cells. B, Nuclear and cytoplasmic fractions were immunoblotted with anti-Atox 1 antibody, cytoplasmic marker, tubulin and nuclear marker, laminin B1. Right panel shows averaged data for nuclear and non-nuclear expression of Atox1 protein, expressed as fold increased against control VSMCs (Mean ± SE, n=3). *P<0.001vs. untreated cells.

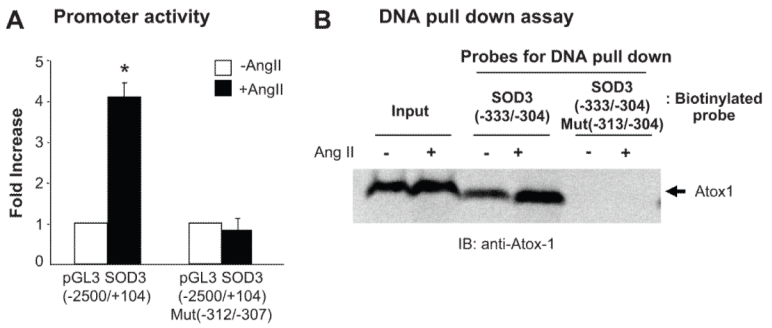

Figure 6. Role of Atox1 in Ang II-induced SOD3 promoter activity in VSMCs

A, Role of Atox1 on Ang II-induced transactivation of the SOD3 gene promoter in VSMCs. Cells were transiently transfected with SOD3 promoter luciferase reporter constructs (pGL3 SOD3 (−2500/+104) or mutated SOD3 promoter luciferase reporter constructs (pGL3 SOD3, Mut(−312/−307) along with Ang II (Mean ± SE, n=3). *P<0.001 vs untreated cells. B, DNA pull-down assay, showing Ang II-induced binding of Atox1 to the GAAAGA sequence (Atox1-reponsive element (Atox1-RE)) in the SOD3 promoter. Nuclear extract from VSMCs treated with or without Ang II was incubated with biotinylated oligonuclotide probes and streptavidin-Sepharose. The Protein-DNA complexes were subjected to SDS-PAGE, followed by immunoblotting with anti-Atox1 antibody. The experiments was performed using two different oligonucleotide, SOD3 (−333/−304) and SOD3 (−333/−304) Mut (−313/−304). Representative figure was from three independent experiments.

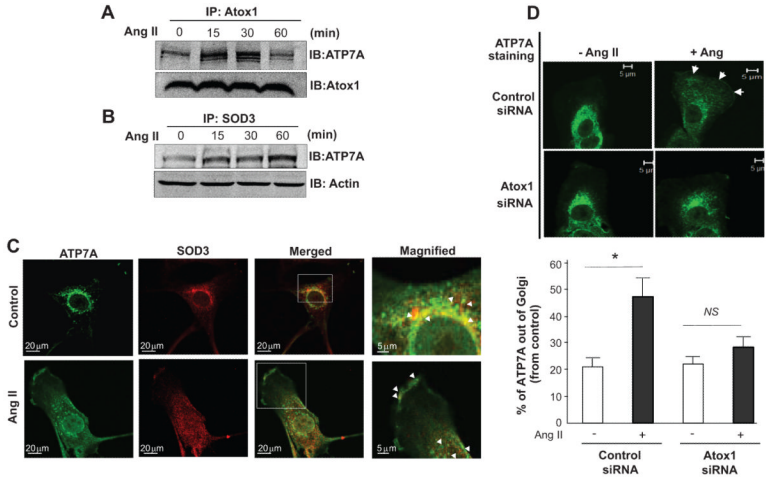

Figure 7. Effect of Ang II on interaction among Atox1, ATP7A, and ecSOD in VSMCs

A and B, VSMCs were stimulated with Ang II (100 nM), and lysates were immunoprecipitated (IP) with either anti-Atox1 or anti-ecSOD antibody, followed by immunoblotting (IB) with anti-ATP7A antibody. C, Effect of Ang II on subcellular localization of ATP7A (green) and SOD3 (red) in human aortic smooth muscle cells (HASMs). D, HASMs transfected with Atox1 or control siRNAs were stained with anti-ATP7A antibody. Images were representative of 3 different experiments taken at 5 different fields/well.

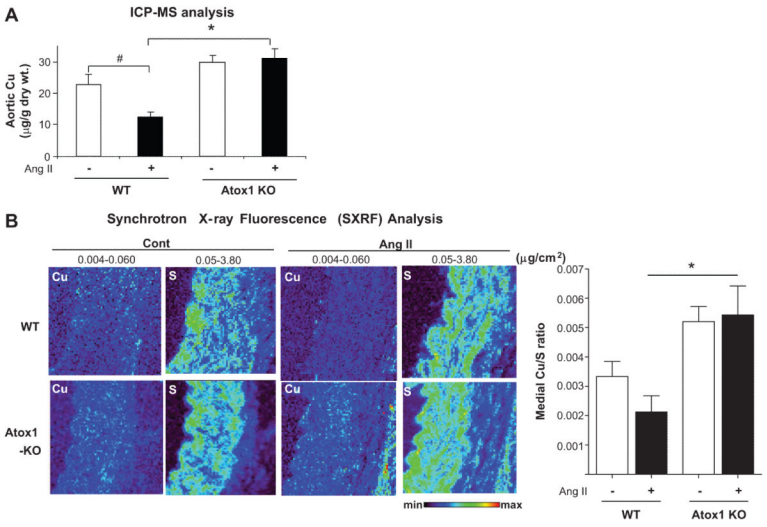

Figure 8. Effect of Ang II-induced hypertension on abundance and spatial distribution of copper in the vascular tissue from Atox1$^{-/-}$ and WT mice

A and B,, Aortic copper content in Atox1$^{-/-}$ and WT mice infused with Ang II or vehicle for 7 days, as described in Figure 1 was measured by inductively coupled plasma mass spectrometry (ICP-MS) or Synchrotron-based X-ray Fluorescence (SXRF). SXRF scans (1-2 sec per pixel) were performed in paraffin-embedded tissue (left). The maximum and minimum threshold values in microgram per square centimeter are given above each frame. Map of copper shows areas of the lowest to the highest content scaled to a rainbow color (bottom). Total sulfur is used as a surrogate for total cellular protein and to visualize the morphology of tissue sections. Data are quantified using three samples for each group, two to three scans/sample (right).

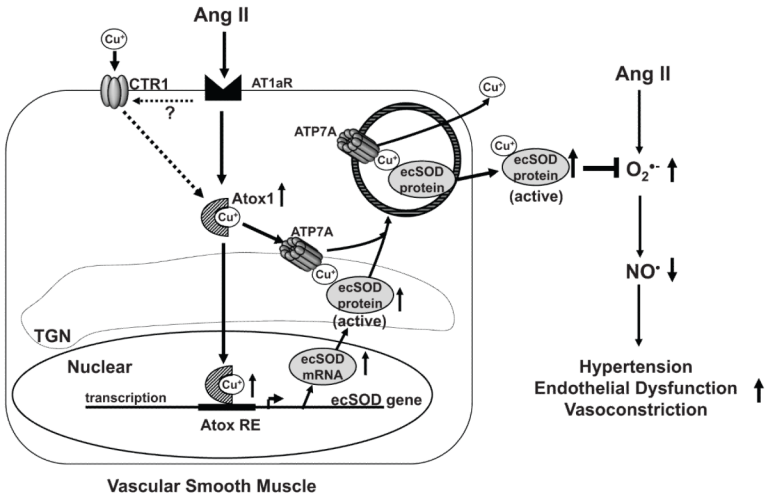

Figure 9. Proposed model for protective role of Atox1 in Ang II-induced hypertension through regulating SOD3 expression and activity

Atox1 plays an important role in Ang II-induced transcription and activity of SOD3 by increasing Atox1 binding to the Atox1 response element in SOD3 promoter as well as to copper transporter ATP7A protein required for copper delivery to SOD3 for enhancing its specific activity. As its consequence, Atox1 contributes to the decrease in extracellular $O_2^{\bullet -}$ levels in the vessel wall, thereby increasing available NO to preserve endothelial function, and reducing blood pressure.