Role of Antioxidant-1 in Extracellular Superoxide Dismutase Function and Expression

Viktoria Jeney, Shinichi Itoh, Maria Wendt, Quinton Gradek, Masuko Ushio-Fukai, David G. Harrison, Tohru Fukai

Abstract—The extracellular superoxide dismutase (ecSOD or SOD3) is a copper-containing enzyme which is highly expressed in the vasculature. Copper-containing enzymes require copper chaperones for their activity however the chaperone which delivers copper to SOD3 has not previously been defined. Atox1 is a copper chaperone proposed to deliver copper to the trans-Golgi network. Because SOD3 is secreted via the trans-Golgi network, we sought to determine whether Atox1 acts as a copper chaperone for SOD3. Using recombinant human SOD3, we found that the specific activity of SOD3 directly correlates with its copper content ($R^2=0.99$). SOD3 specific activity in the conditioned medium from cultured Atox1$^{-/}$ fibroblasts was markedly decreased, but could be recovered to that of wild-type cells by copper addition. These results indicated that Atox1 is required for delivering copper to SOD3 for its full activity. Unexpectedly, the protein and mRNA levels of SOD3 were dramatically decreased in cultured Atox1$^{-/}$ fibroblasts. This was associated with a marked decrease in SOD3 transcription rate but no change in SOD3 mRNA stability. Overexpression of Atox1 markedly increased SOD3 mRNA in both Atox1$^{-/}$ and Atox1$^{+/+}$ cells. These findings indicate that Atox1 positively regulates SOD3 transcription. Because SOD3 protein is upregulated in atherosclerotic vessels, we examined expression of Atox1 in vessels from ApoE$^{-/-}$ mice. Western and immunohistochemical analysis in ApoE$^{-/-}$ mice revealed that both Atox1 and SOD3 protein levels are markedly increased in atherosclerotic intimal lesions. In summary, Atox1 functions not only as a copper chaperone for SOD3 but also as a positive regulator for SOD3 transcription and may have an important role in modulating oxidative stress in the cardiovascular system. (Circ Res. 2005;96:723-729.)

Key Words: superoxide dismutase | antioxidant-1 | copper | atherosclerosis

The vascular production of superoxide (O$_2^-$) is increased in many common cardiovascular diseases including atherosclerosis, hypercholesterolemia, hypertension, ischemic heart disease, diabetic cardiomyopathy, and heart failure. The superoxide dismutases represent the major cellular defense against O$_2^-$. In mammalian tissue, three isoforms of SODs have been identified: the cytoplasmic CuZnSOD (SOD1), the mitochondrial MnSOD (SOD2), and the extracellular SOD (SOD3). In vascular tissue, up to one half of the total SOD is SOD3, depending on the species. It is secreted by vascular smooth muscle cells and fibroblasts and anchored to the extracellular matrix and endothelial cell surface through binding to the heparan sulfate proteoglycan and fibulin. Because of its location, the SOD3 plays an important role in regulating blood pressure and endothelial function by modulating the levels of O$_2^-$ in the vasculature.

We and others have demonstrated that SOD3 expression is influenced by multiple stimuli, including angiotensin II, nitric oxide, exercise training, and in pathological states, such as atherosclerosis and hypertension.

Because SOD3 is a copper-containing enzyme, its activity may be regulated by its copper content within the enzyme. Under physiological conditions, the level of intracellular free copper is extraordinarily restricted. Thus, soluble cytosolic copper carrier proteins termed “copper chaperones” are required for trafficking of copper to specific copper-containing enzymes through direct protein-protein interaction. Three copper chaperones have been characterized thus far: (1) Cox1, which delivers copper to cytochrome c oxidase in the mitochondria; (2) CCS (copper chaperone for SOD1), which delivers copper to SOD1 in the cytosol; and (3) Atox1, which delivers copper to some of the secretory copper enzymes via the copper transporter in the trans-Golgi network. Because SOD3 is a secretory protein, we hypothesized that SOD3 might obtain copper through Atox1.

Atox1 is a cytosolic protein that contains a single amino terminal copy of the MTCXXC metal-binding motif also found in other metal-binding proteins. Yeast genetic studies revealed that Atx1 (a yeast homologue of Atox1) delivers copper to Fet3P, another copper containing enzyme, which is...
the yeast homologue of human ceruloplasmin and is secreted via the yeast-trans-Golgi network. In addition, Atox1−/− mice have diminished activities of the secretory copper enzymes lysyl oxidase, dopamine monooxygenase, and tyrosinase leading, respectively, to impaired connective tissue integrity, impaired temperature regulation, and hypoglycemia. Interestingly, Atox1 was originally isolated as Antioxidant-1 from yeast, because it protects oxidative damage in yeast. The mechanisms underlying its antioxidant activity are unclear.

We performed the present study to determine whether Atox1 functions as a copper chaperone for SOD3. We demonstrated that Atox1, but not CCS, is required for full activity of SOD3 in a copper-dependent manner. We also found that Atox1 modulates SOD3 mRNA and protein levels, suggesting an additional role of Atox1 in modulating O2− scavenging. We demonstrated that SOD3 and Atox1 are concomitantly increased in atherosclerotic lesions of Apo(E)-deficient mice. These results indicate Atox1 has an important role in modulating SOD3 function and expression and thus has a critical antioxidant function in vivo.

Materials and Methods

Materials, Animals, and Cell Culture Used

Radiochemicals were purchased from DuPont Corp. All other reagents were purchased from Sigma Chemical Co, except when specified. CCS−/− mice (n = 15) and CCS+/+ mice (n = 15),14 Atox1−/− mice (n = 12) and Atox1+/+ mice (n = 12),12 Atox1−/− and Atox1+/+ immortalized mouse embryonic fibroblast cells, human Atox1 cDNA in pcDNA3.16 rabbit polyclonal antisera to Atox116 were a kind gift from Dr Jonathan Gitlin, Washington University, St. Louis, Mo. Mouse aortic endothelial cells were a kind gift from Dr Hanjoong Jo (Emory University, Atlanta, Ga). Other rabbit polyclonal antisera to Atox117 was a kind gift from Dr Diane Cox, University of Alberta, Edmonton, Canada. The polyclonal antibody for SOD3 detection has been described previously. Other antibodies were obtained commercially, including anti-SOD1 (BioDesign International), a biotin-conjugated goat anti-rabbit IgG (Bio-Rad Laboratories), and anti-rabbit IgG (Bio-Rad Laboratories Inc). ApoE−/− mice on the C57Bl/6 genetic background (n = 5) were obtained from Jackson Laboratories (Bar Harbor, Maine). C57Bl/6 mice were used as controls for ApoE−/− mice (n = 5). Atox1−/− and Atox1+/+ immortalized mouse embryonic fibroblast cells were cultured in DMEM with 10% fetal bovine serum supplemented with penicillin/streptomycin/glutamine and G418 (50 μg/mL). Experiments were performed with 1% serum with no additives. Transient transfection of human Atox1 cDNA in pcDNA3.1 into cells was performed using the calcium phosphate precipitation procedure according to the manufacturer’s recommendations.

Overexpression and Purification of Recombinant SOD3 and Copper Measurements

Human SOD3 was produced using Drosophila Schneider expression system (Invitrogen Corp). The human SOD3 coding region without the terminal codon was amplified by PCR using a forward primer with an EcoRI site incorporated at the 5′ end (underlined), (5′-CCGGATCTGCAGCTTCTTTCA-3′) and a reverse primer with an XbaI site inserted at the 3′ end (underlined), (5′-CTTCTTTCA-CCGGATCTGCAGCTTCTTTCA-3′). This product was subcloned into the EcoRI/XbaI sites of the pAc5.1/V7-His plasmid (Invitrogen Corp), which adds a C-terminal His, and V7 tag (pAc5.1/SOD3-V7-His). Schneider 2 cells stably expressing SOD3 with V7-His tag were obtained according to the manufacturer’s instructions. Successful protein production was confirmed by immunoblotting.

For amplification and purification of recombinant proteins, cells were grown in serum-free media (Invitrogen Corp) that contained 300 μg/mL hygromycin B with 0, 50, and 300 μmol/L of CuSO4. On harvesting, the cells were spun at 4000g for 10 minutes at 4°C, and the supernatant was collected. The recombinant SOD3 protein secreted into the media was purified by a nickel-chelating Ni-NTA chromatography (Qiagen Inc) and by chromatography on Con A-Sepharose (Pharmacia Biotech). Protein purity was confirmed by Western blot and by Coomassie Blue staining of SDS-polyacrylamide gel (SDS-PAGE).

The copper and zinc contents of recombinant SOD3 were analyzed in triplicate by inductively coupled plasma mass spectrometry (ICP-MS) using a PlasmaQuad3. Copper and zinc concentrations were calculated from calibration curves, and values for water blank were subtracted.

Western Blot Analysis

Western blot analysis was performed as previously described. The primary antibodies used included a rabbit polyclonal antibody against murine SOD3, a sheep antibody against human SOD1 (Biodesign International), and rabbit polyclonal antisera to Atox1. For Atox1 detection, samples were separated by 16.5% Tricine-SDS-PAGE (BioRad). Equal loading of proteins was confirmed by Ponceau or Coomassie blue staining.

Superoxide Dismutase Activity Assays

SOD activity was assayed by monitoring inhibition of the rate of xanthine/xanthine oxidase–mediated reduction of cytochrome c as previously described. Con A-Sepharose chromatography (Pharmacia Biotech) was used to isolate SOD3 from Atox1−/− and Atox1+/+ mouse vessels and conditioned media of Atox1−/− and Atox1+/+ cells as previously described.

Detection of SOD3 mRNA

In some experiments, we used real-time PCR to quantify SOD3 mRNA. Total RNA was extracted using TRI reagent (Molecular Research Center, Inc) and the RNeasy kit (Qiagen), including DNase treatment, according to the manufacturer’s instructions. Two micrograms of RNA were reverse transcribed using Superscript III reverse transcriptase (Invitrogen Corp). Real-time PCR was performed using the Light Cycler Thermocycler (Roche Diagnostics Corp). The 10-μL reaction mixtures contained 0.3 mM/L of forward and reverse primers for SOD3 (5′-TTTGTGTACGCGGCTTGCTAC-3′ and 5′-CTCCA-TCCAGATCTCCAGACT-3′) or those for GAPDH (5′-TTTGTGTACGCGGCTTGCTAC-3′ and 5′-CTCCA-TCCAGATCTCCAGACT-3′) or those for GAPDH (5′-TTTGTGTACGCGGCTTGCTAC-3′ and 5′-CTCCA-TCCAGATCTCCAGACT-3′). Atox1+/+ and Atox1−/− cells were cultured in DMEM with 10% fetal bovine serum supplemented with penicillin/streptomycin/glutamine and G418 (50 μg/mL). Experiments were performed with 1% serum with no additives. Transient transfection of human Atox1 cDNA in pcDNA3.1 into cells was performed using the calcium phosphate precipitation procedure according to the manufacturer’s recommendations.

Histochemical Study

Aortic segments were embedded in OCT (Miles Laboratories Inc) and frozen in liquid nitrogen. For immunohistochemical studies, cryosections were stained with a rabbit polyclonal antibody against human Atox1 (1:100), followed by a biotin-conjugated goat anti-rabbit IgG (1:200; Bio-Rad Laboratories). Staining was developed using the ABC-AP Kit (Vector Laboratories).

Statistical Analysis

All data are expressed as mean ± SEM. Comparisons were made by one-way ANOVA followed by the Tukey-Kramer post hoc test. Values of P < 0.05 were considered statistically significant.

Results

SOD3 Activity Is Copper Dependent

To determine whether copper regulates SOD3 activity, recombinant human SOD3 was expressed in Drosophila Schneider cells cultured in the presence of different copper.
SOD1 and SOD3 was determined as previously described. Activity was assayed by examining the inhibition of cytochrome c reduction by xanthine/xanthine oxidase at pH 7.4. Activity of SOD3 showed a linear relationship with its copper content (Figure 1A). The maximum specific activity of the recombinant human SOD3 was overexpressed in Drosophila Schneider cells with media containing different copper concentrations. SOD3 was purified from the media by concanavalin A sepharose chromatography and enzyme activity was determined by cytochrome c reduction assay. Copper content was measured by inductively coupled plasma mass spectrometry (ICP-MS). B and C, Activity and protein expression of SOD1 (B) and SOD3 (C) in aortas from CCS+/− and CCS−/− mice. Aortas were homogenized, and SOD activity was assayed by examining the inhibition of cytochrome c reduction by xanthine/xanthine oxidase at pH 7.4. Activity of SOD1 and SOD3 was determined as previously described. Aortas from four mice were combined for each experiment (n=3 for each data point). Results are presented as mean±SEM, and the values are expressed as units per milligram of total protein. †P<0.01 vs control. Protein levels of SOD1 and SOD3 were determined by Western analysis with antibodies specific to either the SOD1 or the murine SOD3. Representative blots are from four individual experiments.

**Figure 1.** Relation between copper content and specific activity of SOD3. Recombinant human SOD3 was overexpressed in Drosophila Schneider cells with media containing different copper concentrations. SOD3 was purified from the media by concanavalin A sepharose chromatography and enzyme activity was determined by cytochrome c reduction assay. Copper content was measured by inductively coupled plasma mass spectrometry (ICP-MS). B and C, Activity and protein expression of SOD1 (B) and SOD3 (C) in aortas from CCS+/− and CCS−/− mice. Aortas were homogenized, and SOD activity was assayed by examining the inhibition of cytochrome c reduction by xanthine/xanthine oxidase at pH 7.4. Activity of SOD1 and SOD3 was determined as previously described. Aortas from four mice were combined for each experiment (n=3 for each data point). Results are presented as mean±SEM, and the values are expressed as units per milligram of total protein. †P<0.01 vs control. Protein levels of SOD1 and SOD3 were determined by Western analysis with antibodies specific to either the SOD1 or the murine SOD3. Representative blots are from four individual experiments.

**Activation of SOD3 Does Not Require CCS in Vascular Tissue**

Because SOD3 activity was found to be copper dependent, we examined whether the known copper chaperone for SOD1 (CCS) is required for the full activity of SOD3 in vivo using CCS−/− and CCS+/+ mice. The activity of SOD1 was dramatically decreased in aortas from CCS−/− mice as compared with those from CCS+/+ mice (3.2±0.31 versus 8.8±0.5 U/mg protein, P<0.05; Figure 1B), whereas protein expression of SOD1 was not changed. In contrast, both the activity and protein levels of SOD3 were not changed in CCS−/− when compared with control aortas (1.6±0.1 versus 1.5±0.1 U/mg protein; P=NS) (Figure 1C). These results indicate that CCS is not required for activity of SOD3.

**Both Activity and Protein Expression of SOD3 Are Decreased in Vascular Tissue and the Conditioned Culture Medium From Fibroblasts in Atox1−/− mice**

The copper chaperone Atox1 has been shown to deliver copper to other copper-containing enzymes that are secreted via the trans-Golgi network. We thus hypothesized that Atox1 is required for the full activity of SOD3. To test this hypothesis, we first determined if Atox1 protein is expressed in cells of the cardiovascular system. Western analysis revealed that Atox1 is abundantly expressed in mouse aortic smooth muscle cells, aortic endothelial cells, fibroblasts, heart, and aorta as a 7.4-kDa protein (Figure 2). This molecular mass of Atox1 is consistent with that previously reported in other cells and tissues.

To determine whether Atox1 modulates SOD3 activity, SOD3 from the media of Atox1−/− and Atox1+/+ mouse fibroblasts was purified using concanavalin A sepharose chromatography. The activity of SOD3 in the culture medium was dramatically decreased in Atox1−/− fibroblasts compared with Atox1+/+ cells (0.06±0.01 versus 2.79±0.15 U/mg) (Figure 3A). Unexpectedly, SOD3 protein expression in Atox1−/− fibroblasts was also decreased compared with that in Atox1+/+ cells (Figure 3B), suggesting that Atox1 is involved in regulating SOD3 protein expression. In contrast, neither the activity nor protein expression of SOD1 was altered in Atox1−/− fibroblasts (Figure 3C and 3D). Consistent with this, both activity and protein expression of SOD3, not SOD1, were decreased in aortas from Atox1−/− mice compared with those from Atox1+/+ mice (supplemental Figure I, available online at http://circres.ahajournals.org). Furthermore, siRNA knockdown of Atox1 markedly decreased SOD3 protein expression in the culture medium of mouse fibroblasts (supplemental Figure II). Taken together, Atox1 deficiency markedly decreased both activity and pro-
tein of SOD3 in vascular tissue as well as in cultured fibroblasts.

**Full Activity of SOD3 Requires the Copper Chaperone Atox1**

We next examined whether SOD3-specific activity is altered in Atox1$^{-/-}$ cells. The relative amount of SOD3 protein secreted in the culture medium was determined by Western analysis. As shown in Figure 4A and 4B, the specific activity of SOD3 in the culture medium, as determined by ratio of SOD3 activity to SOD3 protein, was markedly decreased in Atox1$^{-/-}$ fibroblasts compared with Atox1$^{+/+}$ cells (5.19 ± 1.52 versus 17.59 ± 1.13 U/relative SOD3 protein amounts). The decrease in SOD3-specific activity in Atox1$^{-/-}$ fibroblasts was rescued by addition of copper in the culture medium. Although SOD3-specific activity in Atox1$^{+/+}$ cells was not significantly changed by copper addition (Figure 4B), it was decreased by the copper chelator BCS (bathocuproine disulfonate) (data not shown). These results suggest that most of the enzyme secreted from the Atox1$^{+/+}$ cells is fully metallated. In contrast, both activity and protein expression of SOD1 were not changed in Atox1$^{-/-}$ cells (Figure 3C and 3D), suggesting that Atox1 is not required for delivery of copper to SOD1. Taken together, these findings indicate that Atox1 is essential for the full activation of SOD3 but not SOD1.

**Figure 3.** Activity and protein expression of secreted SOD3 (A and B) and SOD1 (C and D) in Atox1$^{+/+}$ (WT) and Atox1$^{-/-}$ (Atox1-KO) fibroblast cells. Cells were cultured in 1% serum containing DMEM for 24 to 96 hours. SOD3 secreted in the culture medium was collected at the 72-hour time point or as indicated, and concentrated by Concanavalin-A sepharose chromatography. Activity and protein expression of SOD3 in concentrated culture medium (A and B) and SOD1 in cell lysates (C and D) were examined as described in Figure 1. Equal protein loading was confirmed by Ponceau staining or Coomassie blue staining. Top panels in B and D show representative Western blots, whereas the bottom panels in B and D depict mean values for four separate experiments.

**Figure 4.** A, SOD3-specific activity secreted from Atox1$^{+/+}$ (WT) and Atox1$^{-/-}$ (Atox1-KO) cells. Relative amount of SOD3 secreted in the culture medium was determined by Western analysis, and SOD3 activity was assessed as in Figure 1. B, Effect of in vitro copper treatment on SOD3 specific activity purified from Atox1$^{+/+}$ (WT) and Atox1$^{-/-}$ (Atox1-KO) cells. Both cells were cultured in 1% serum containing DMEM for 72 hours, and the cultured medium was harvested and concentrated as described in Figure 3. Relative SOD3 protein content was determined by identical methods as described for Figure 4A. After equal amounts of SOD3 protein were treated with or without CuSO$_4$ (10 μmol/L, 60 minutes, RT), SOD3 activity was measured. Graph shows mean data for three separate experiments. *$P<0.05$ vs vehicle alone. NS indicates not significant.
Atox1 Directly Regulates SOD3 mRNA Level

Because SOD3 protein level was decreased in Atox1−/− cells, we sought to determine whether Atox1 regulates SOD3 expression at the mRNA level. RNase protection assays showed that SOD3 mRNA level was decreased by 76±6% in Atox1−/− cells compared with Atox1+/+ cells (Figure 5A). Transfection of Atox1 cDNA increased SOD3 mRNA levels in both Atox1−/− and Atox1+/+ cells by 2.9±0.1- and 1.9±0.2-fold, respectively (Figure 5B). These findings suggest that Atox1 governs SOD3 mRNA levels in a positive fashion. The decrease in SOD3 mRNA in Atox1−/− cells could be due to either an increase in the rate of mRNA decay, a decrease in the rate of mRNA transcription, or both. The stability of SOD3 mRNA, as determined by RNase protection assays after blockade of transcription with actinomycin D (10 μg/mL), was identical between Atox1+/+ and Atox1−/− cells (Figure 6A). In contrast, the rate of SOD3 transcription, as determined using nuclear run-on assays, was decreased in Atox1−/− cells by 52±2%-fold compared with Atox1+/+ cells (Figure 6B). Taken together, these results suggest that Atox1 positively regulates SOD3 transcription without affecting mRNA stability.

Both SOD3 and Atox1 Are Increased in Atherosclerotic Vessels

We have previously shown that protein levels of SOD3 are markedly increased in atherosclerotic vessels.18 We therefore examined expression of Atox1 in aortas from ApoE−/− mice. Western analysis revealed that not only Atox1 but also Atox3 protein expression was significantly increased in ApoE−/− mouse aortas compared with those of wild-type mice (Figure 7A). Furthermore, immunohistochemical analysis of aortic sections of ApoE−/− mice showed that both Atox1 and SOD3 protein were increased in the intima of atherosclerotic lesions (Figure 7B). Note that Atox1 was identified in the nucleus of cells within the intimal region in ApoE−/− mice. These results suggest that Atox1 is increased and localized in the nucleus in atherosclerotic vessels.

Discussion

The present study demonstrates that the copper chaperone Atox1, but not CCS, is required for full activation of SOD3 in a copper-dependent manner. We also found that Atox1 positively regulates SOD3 mRNA levels at transcriptional level. Furthermore, Atox1 protein is markedly increased in the intimal lesion of atherosclerosis where SOD3 is increased. These findings suggest that Atox1 functions not only as a copper chaperone for SOD3 but also as a positive regulator for SOD3 transcription.

Using recombinant human SOD3, we demonstrated that the specific activity of SOD3 was linearly related to its copper content (Figure 1A), suggesting that SOD3-specific activity is copper dependent. This is consistent with SOD1, which also requires copper for its activity.23 Scavenging O2 by SOD1 requires copper for its activity, and Atox1 is increased and localized in the nucleus in atherosclerotic vessels.
SOD3 and Atox1 are prominent in the intima lesion in ApoE−/− mice. Immunoreactivity for Atox1 was performed using an anti-Atox1 antibody followed by a biotin-conjugated goat anti-rabbit IgG, and immunostaining for SOD3 was performed using an anti-SOD3 antibody followed by a biotin-conjugated goat anti-rabbit IgG. Immunoblotting showed that SOD1 (CCS) in modulating SOD3 activity. Our findings from CCS−/− and CCS+/+ mouse aortas suggest that CCS is not required for full activity of SOD3. We thus examined whether another copper chaperone, Atox1, which is essential for copper delivery to the secretory pathway, is involved in regulating SOD3 activity. We found that the specific activity of SOD3 secreted by Atox1−/− fibroblasts was markedly decreased compared with SOD3 secreted by Atox+/+ cells. Taken together, these results suggest that full enzymatic activity of SOD3 requires Atox1, but not CCS, although both Atox1 and CCS have the MXCXXC copper-binding motif in their active sites. Moreover, because SOD3 activity in Atox1−/− cells was normalized by copper addition to the culture medium, this further indicates that Atox1 plays a critical role in copper delivery to SOD3. The mechanisms of how SOD3 obtains copper through Atox1, however, remain unknown. Atox1 delivers copper to copper-containing enzymes via transporters such as Menkes or Wilson protein, which are important in the ultimate delivery of copper to the trans-Golgi network. Our present data indicate that SOD3 obtains copper via a similar pathway. Of note, the SOD3 expressed by Atox1−/− cells is not completely devoid of enzymatic activity, raising the possibility that an Atox1-independent copper delivery system for SOD3 exists in mammals.

We unexpectedly found that protein levels of SOD3 were dramatically decreased in aortas and in cultured fibroblasts from Atox1−/− mice as well as by siRNA knockdown of Atox1 in wild-type mouse fibroblasts. Furthermore, RNase protection assays and real-time PCR demonstrated that decrease in SOD3 protein in cells lacking Atox1 is associated with decrease in SOD3 mRNA. Actinomycin D-chase studies and nuclear run-on assays showed that this was due to diminished SOD3 gene transcription rather than reduced SOD3 mRNA stability. Importantly, overexpression of Atox1 increased SOD3 mRNA in both Atox1−/− and Atox1+/+ cells, indicating that Atox1 positively regulates SOD3 transcription. Although the mechanisms by which Atox1 regulates SOD3 mRNA transcription remain unknown, it is conceivable that Atox1 may act as a transcription factor. The highly conserved lysine residues at the carboxyl terminus of Atox1 are consistent with a classical nuclear location signal and may contribute to active import of this protein into nucleus. In preliminary studies, we have found that Atox1 localizes to the nucleus, and previously Atox1 has been identified in the nucleus of HeLa cells. It is also possible that Atox1 may deliver copper to copper-dependent transcription factors, thereby upregulating SOD3 mRNA. Indeed, several copper responsive transcription factors, including Ace1, Ame1, and Mac1 have been found in yeast, although homologs of these have not yet been reported in mammalian cells. Moreover, Sp1/Sp3 transcription factors have been shown to contribute to transcription of SOD3 gene, and activation of Sp1 can be inhibited by a copper chelator. Intriguingly, we found that copper chelator markedly decreased SOD3 mRNA levels in mouse fibroblasts (supplemental Figure III). Thus, Atox1 may increase SOD3 transcription directly or indirectly through regulation of Sp1/Sp3 in a copper-dependent manner. Further studies will be required to investigate the mechanisms.
of how Atox1 regulates SOD3 transcription and expression in various tissues and cell types.6

We have previously demonstrated that SOD3 is upregulated in atherosclerotic vessels.18 In the present study, we examined expression of Atox1 in aortas from ApoE−/− mice using Western and immunohistochemical analysis, and found that Atox1 expression was markedly increased in the nucleus of intimal lesions with a concomitant increase in SOD3 in the extracellular matrix. These in vivo findings support our observations in cultured cells that SOD3 and Atox1 are concomitantly regulated.

In summary, the present study demonstrates that Atox1 functions not only as a copper chaperone for SOD3 but also as a positive regulator for SOD3 transcription. In the cardiovascular system, SOD3 is highly expressed, particularly in vessels, and seems to play a major role in scavenging superoxide in the extracellular space. It is therefore likely that Atox1 plays a critical role in modulating extracellular superoxide in vascular cells and understanding how Atox1 functions will provide greater insight into how the oxidative state of cells is controlled.

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We also thank Drs Bibuendu Sarkar, Steven Moore, and Diane W. Cox of the University of Alberta for the gift of antibody to Atox1, and Suzanne Mertens for technical assistance.

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Supplemental methods:

RNA Interference and Cell Transfection. To knockdown mouse Atox1 mRNAs, the annealed siRNA duplexes for Atox1 (sense: 5’-GGAGUGGAGUCAACAUUGtt -3’, antisense: 5’-CAAUGUUGAACUCCACUCCtc-3’) and for scrambled siRNA (sense: 5’GGAUUGUAUGACGUAAGGtt, antisense: 5’-GCCUUACGUCAUACAAUCCtt-3’), respectively, were purchased from Ambion. We performed a Blast search and confirmed that the Atox1 and scrambled siRNA sequences we used in this study have no overlap with other proteins. Mouse fibroblasts were seeded into 100 mm dishes one day prior to transfection. At the time of transfection with siRNA, the cells were about 60% confluent in 6 ml of DMEM with 10% fetal bovine serum supplemented with penicillin/streptomycin/glutamine. Transfections of siRNA (at 25-100nM) were performed using Oligofectamine (Invitrogen) according to the manufacturer’s protocol.
Supplemental figure I: Activity and protein expression of SOD1 and SOD3 in aortas from Atox1<sup>+/+</sup> and Atox1<sup>−/−</sup> mice. The activity of SOD1 and SOD3 was determined by identical methods as described for Figure 1B and 1C. Aortas from four mice were combined for each experiment (n=3 for each data point). The results are presented as mean ± SEM, and the values are expressed as units per milligram of total protein. † p<0.01 vs. control. Protein levels of SOD1 and SOD3 were determined by Western analysis with antibodies specific to either the SOD1 or the murine SOD3. Representative blots are from 3 individual experiments.
**Supplemental figure II:** Effect of Atox1 siRNA on ecSOD protein in mouse fibroblasts. Forty eight hours after transfection with scrambled siRNA (control) or Atox1 siRNA, SOD3 and Atox1 protein levels were determined by identical methods as described for Figure 2 and 3B. Column graph shows mean data of three separate experiments.
Supplemental figure III: Effect of the copper chelator BCS on SOD3 mRNA in mouse fibroblasts. Mouse fibroblasts were exposed to the copper chelator BCS (bathocuproine disulfonate) (200µM) for 24 hours. SOD3 and GAPDH mRNA levels were assessed by quantitative real-time PCR, and GAPDH mRNA levels were used as internal control. Column graph shows mean data of three separate experiments.
Data Supplement

Supplemental methods:

RNA Interference and Cell Transfection. To knockdown mouse Atox1 mRNAs, the annealed siRNA duplexes for Atox1 (sense: 5’-GGAGUGGAGUCAACAUGGtt -3’, antisense: 5’-CAAUGUUGAACUCCACUCtt-3’) and for scrambled siRNA (sense: 5’GGAUUGUAUGACGUAAGGtt, antisense: 5’-GCCUUACGUCAUACAAUCCtt-3’), respectively, were purchased from Ambion. We performed a Blast search and confirmed that the Atox1 and scrambled siRNA sequences we used in this study have no overlap with other proteins. Mouse fibroblasts were seeded into 100 mm dishes one day prior to transfection. At the time of transfection with siRNA, the cells were about 60% confluent in 6 ml of DMEM with 10% fetal bovine serum supplemented with penicillin/streptomycin/glutamine. Transfections of siRNA (at 25-100nM) were performed using Oligofectamine (Invitrogen) according to the manufacturer’s protocol.
Supplemental figure I: Activity and protein expression of SOD1 and SOD3 in aortas from Atox1\textsuperscript{+/+} and Atox1\textsuperscript{-/-} mice. The activity of SOD1 and SOD3 was determined by identical methods as described for Figure 1B and 1C. Aortas from four mice were combined for each experiment (n=3 for each data point). The results are presented as mean ± SEM, and the values are expressed as units per milligram of total protein. † p<0.01 vs. control. Protein levels of SOD1 and SOD3 were determined by Western analysis with antibodies specific to either the SOD1 or the murine SOD3. Representative blots are from 3 individual experiments.
**Supplemental figure II:** Effect of Atox1 siRNA on ecSOD protein in mouse fibroblasts. Forty eight hours after transfection with scrambled siRNA (control) or Atox1 siRNA, SOD3 and Atox1 protein levels were determined by identical methods as described for Figure 2 and 3B. Column graph shows mean data of three separate experiments.
Supplemental figure III: Effect of the copper chelator BCS on SOD3 mRNA in mouse fibroblasts. Mouse fibroblasts were exposed to the copper chelator BCS (bathocuproine disulfonate) (200µM) for 24 hours. SOD3 and GAPDH mRNA levels were assessed by quantitative real-time PCR, and GAPDH mRNA levels were used as internal control. Column graph shows mean data of three separate experiments.