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Original Contribution

Novel mechanism for regulation of extracellular SOD transcription and activity by copper: Role of antioxidant-1

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ABSTRACT

Extracellular superoxide dismutase (SOD3), a secretory copper-containing antioxidant enzyme, plays an important role in various oxidative stress-dependent cardiovascular diseases. Although cofactor copper is required for SOD3 activity, it remains unknown whether it can regulate SOD3 transcription. We previously demonstrated that SOD3 activity requires the copper chaperone antioxidant-1 (Atox1), involved in copper delivery to SOD3 at the *trans*-Golgi network (TGN). Here we show that copper treatment in mouse fibroblasts significantly increases mRNA and protein levels of SOD3, but not SOD1, which is abolished in Atox1-deficient cells. Copper promotes Atox1 translocation to the nucleus. Promoter deletion analysis identifies copper- and Atox1-response elements (REs) at the SOD3 promoter. Gel-shift and ChIP assays reveal that Atox1 directly binds to the Atox1 RE in a copper-dependent manner in vitro and in vivo. Adenovirus-mediated reexpression in Atox1$^{-/-}$ cells of nucleus-targeted Atox1 (Atox1–NLS), but not TGN-targeted Atox1 (Atox1–TGN), increases SOD3 transcription without affecting SOD3 activity. Importantly, reexpression of both Atox1–NLS and Atox1–TGN together, but not either alone, in Atox1$^{-/-}$ cells increases SOD3 activity. SOD3 transcription is positively regulated by copper through the transcription factor function of Atox1, whereas the full activity of SOD3 requires both the copper chaperone and the transcription factor functions of Atox1. Thus, Atox1 is a potential therapeutic target for oxidant stress-dependent cardiovascular disease.

SOD3 is the major extracellular antioxidant enzyme against $O_2^{\cdot-}$. It is synthesized and secreted by vascular smooth muscle cells and fibroblasts, thereby preventing oxidative inactivation of nitric oxide released from endothelial cells and superoxide- or peroxynitrite-mediated degradation of extracellular matrix proteins [1–3]. Thus, SOD3 plays an important role in various oxidative stress-dependent pathophysiologies, including hypertension, ischemia–reperfusion injury, and lung injury [1–3]. Indeed, the R213G polymorphism in the SOD3 gene has been linked to an increase in cardiovascular risk [4]. Furthermore, mice deficient in SOD3 reveal an essential role for SOD3 in angiotensin II-induced hypertension, ischemia-induced cardiac remodeling, and angiogenesis [5–8]. Accumulating evidence reveals that SOD3 activity and expression are altered by various stimulants and pathological states at multiple levels such as transcriptional and posttranslational levels [9]. Little is known about the mechanism by which SOD3 transcription is regulated.

The activity of both SOD3 and SOD1 requires a catalytic copper to scavenge $O_2^{\cdot-}$ by reduction and reoxidation of the copper ion at the active site of the enzyme [10,11]. Under physiological conditions, the level of intracellular free copper is extraordinarily restricted [12]. Thus, soluble copper carrier proteins termed "*copper chaperones*" are required to directly transfer *copper* to specific cellular targets. SOD1 has been shown to obtain catalytic copper ion through interaction with the cytosolic copper carrier protein CCS, a copper chaperone for SOD1, thereby increasing its activity [13]. In contrast, we have previously demonstrated that SOD3 activity requires the copper chaperone antioxidant-1 (Atox1), which delivers copper to SOD3 by interacting with the copper transporter Menkes ATPase at the *trans*-Golgi network (TGN) [11,14]. Unexpectedly, our preliminary study showed that the cofactor copper upregulates SOD3 mRNA expression without affecting SOD1 mRNA. However, the underlying mechanisms are unknown.

Atox1 was originally isolated from yeast and has been reported to protect SOD1-deficient yeast from oxidative damage [15]. This oxidative protection is dependent on both conserved copper binding residues in the N-terminal domain and conserved lysine residues in the C-terminal domain, whereas the copper chaperone function of Atox1 is dependent on copper-binding residues, but not on its lysine

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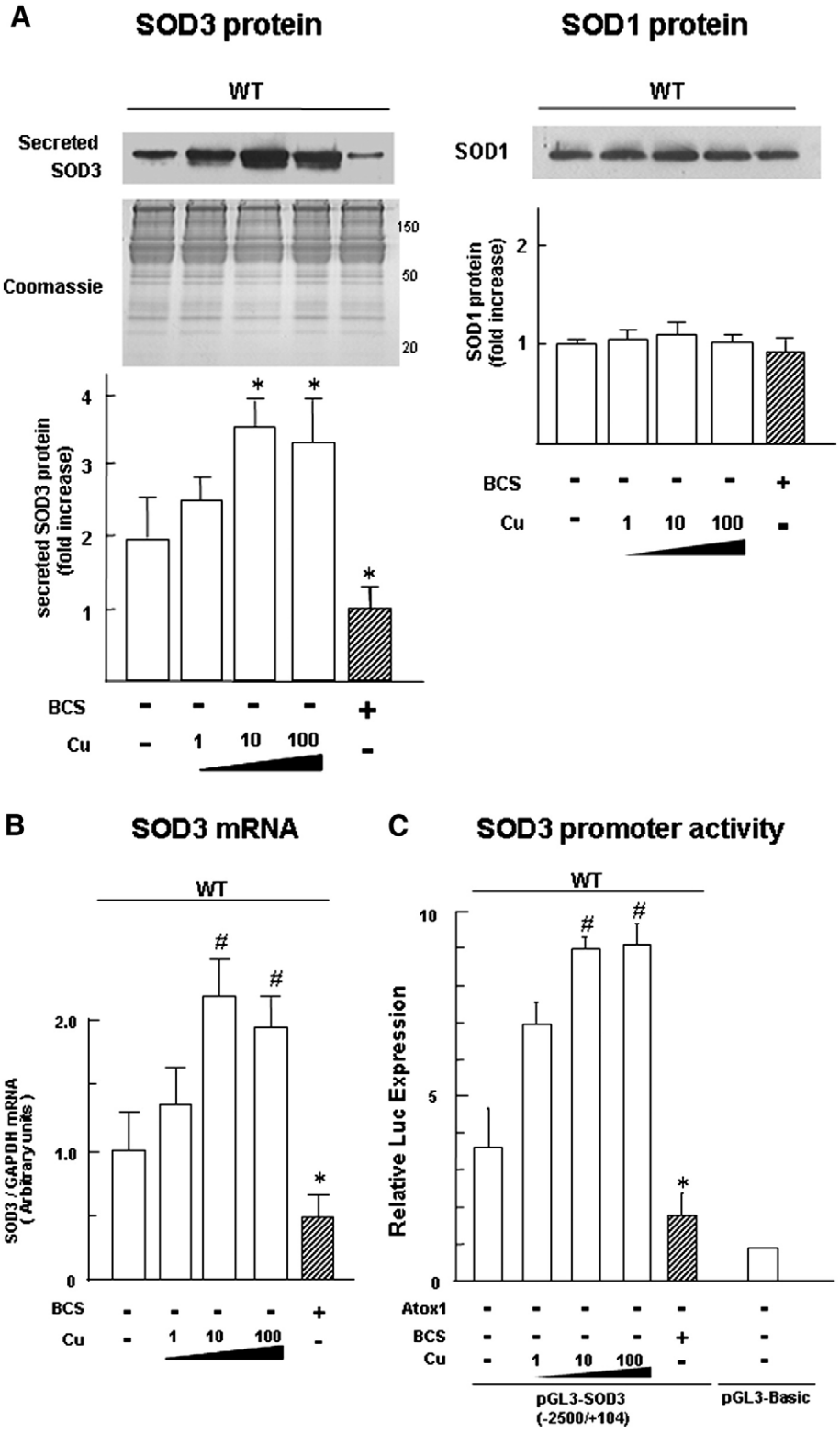

Fig. 1. Copper stimulates expression of SOD3, but not SOD1, at the transcription level in mouse fibroblasts. (A) Effect of copper on protein expression of secreted SOD3 (left) and SOD1 (right) in MEFs treated with either the copper chelator BCS (200 μM) or $CuCl_2$ at the dose indicated (in μM) for 72 h ($n=4$, *$p<0.01$ vs untreated cells). The SOD3 secreted into the culture medium was collected and concentrated by concanavalin A–Sepharose chromatography as previously described [11]. Protein expression of SOD3 in concentrated culture medium and of SOD1 in cell lysates was examined as previously described [11]. Equal protein loading was confirmed by Ponceau staining or Coomassie blue staining. (B) Quantitative real-time PCR analysis showing SOD3 mRNA levels in MEFs treated with either the copper chelator BCS (200 μM) or $CuCl_2$ at the dose indicated for 12 h ($n=4$, *$p<0.01$; #$p<0.001$ vs untreated cells). (C) Effect of copper on transactivation of the SOD3 promoter in MEFs ($n=3$, each in quadruplicate, *$p<0.01$; #$p<0.001$ vs untreated WT cells). Cells were transiently transfected with SOD3 promoter–luciferase reporter constructs and treated with either the copper chelator BCS (200 μM) or $CuCl_2$ at the dose indicated [11]. Forty-eight hours after transfection, the luciferase activity was assayed and normalized to the *Renilla* luciferase activity produced by the cotransfected control plasmid pRL-CMV.