Copper Transporter ATP7A Protects Against Endothelial Dysfunction in Type 1 Diabetic Mice by Regulating Extracellular Superoxide Dismutase

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Oxidative stress and endothelial dysfunction contribute to vascular complication in diabetes. Extracellular superoxide dismutase (SOD3) is one of the key antioxidant enzymes that obtains copper via copper transporter ATP7A. SOD3 is secreted from vascular smooth muscles cells (VSMCs) and anchors at the endothelial surface. The role of SOD3 and ATP7A in endothelial dysfunction in type 1 diabetes mellitus (T1DM) is entirely unknown. Here we show that the specific activity of SOD3, but not SOD1, is decreased, which is associated with increased $O_2^{\bullet-}$ production in aortas of streptozotocin-induced and genetically induced $Ins2^{Akita}$ T1DM mice. Exogenous copper partially rescued SOD3 activity in isolated T1DM vessels. Functionally, acetylcholine-induced, endothelium-dependent relaxation is impaired in T1DM mesenteric arteries, which is rescued by SOD mimetic tempol or gene transfer of SOD3. Mechanistically, ATP7A expression in T1DM vessels is dramatically decreased whereas other copper transport proteins are not altered. T1DM-induced endothelial dysfunction and decrease of SOD3 activity are rescued in transgenic mice overexpressing ATP7A. Furthermore, SOD3-deficient T1DM mice or ATP7A mutant T1DM mice augment endothelial dysfunction and vascular $O_2^{\bullet-}$ production versus T1DM mice. These effects are in part due to hypoinsulinemia in T1DM mice, since insulin treatment, but not high glucose, increases ATP7A expression in VSMCs and restores SOD3 activity in the organoid culture of T1DM vessels. In summary, a decrease in ATP7A protein expression contributes to impaired SOD3 activity, resulting in $O_2^{\bullet-}$ overproduction and endothelial dysfunction in blood vessels of T1DM. Thus, restoring copper transporter function is an essential therapeutic approach for oxidant stress–dependent vascular and metabolic diseases. ***Diabetes*** **62:3839–3850, 2013**

Endothelial dysfunction plays important roles in the development of vascular complications in type 1 diabetes mellitus (T1DM), which is the most common cause of morbidity and mortality and is characterized by insulin deficiency or impaired insulin signaling (1–3). Although the role of oxidative stress in vascular dysfunction in T1DM has been extensively studied (4), the function of antioxidant enzymes in these pathological diseases remains unknown. One of the major antioxidant defense systems in the vasculature are the superoxide dismutases (SODs), which consist of the cytoplasmic Cu/Zn SOD (SOD1), the mitochondrial MnSOD (SOD2), and the extracellular SOD (SOD3) (5,6). SOD3 is a major extracellular antioxidant enzyme highly expressed in the vasculature and synthesized by vascular smooth muscle cells (VSMCs) and fibroblasts. It is secreted and anchored to the extracellular matrix and endothelial cell surface through the heparin-binding domain (HBD). Because of its extracellular location, SOD3 plays a major role in protecting against inactivation of nitric oxide (NO) by superoxide ($O_2^{\bullet-}$), thereby preventing endothelial dysfunction in oxidative stress–dependent cardiovascular diseases (7–10). Gene transfer of SOD3 decreases endothelial dysfunction and arterial pressure in hypertension (11) and aging (12) and restores erectile function in streptozotocin (STZ)-induced diabetes (13). Furthermore, diabetic patients showed elevated plasma SOD3 levels (14). Of note, the R213G polymorphism in the *SOD3* gene, which reduces binding to the endothelial surface and increases serum SOD3 levels, has been linked to an increase in cardiovascular risk (15). Little is known about the activities of SOD3 as well as the role of endogenous SOD3 in endothelial dysfunction in T1DM.

SOD3 is a secretory copper enzyme that requires copper as a catalytic cofactor for its full enzymatic activity in a fashion similar to SOD1 (5). Under physiological conditions, the intracellular level of free copper is extraordinarily restricted due to copper toxicity (16). Thus, soluble copper transport proteins are required to directly transfer copper to specific cellular target proteins. SOD1 obtains copper through interaction with the cytosolic copper chaperone CCS, whereas secretory copper enzyme SOD3 receives copper via the copper chaperone antioxidant-1 (Atox1)–copper transporter ATP7A (Menkes ATPase) pathway (5,17,18). Patients with Menkes disease show multiple abnormalities secondary to deficiencies in the activity of some secretory copper enzymes, such as dopamine β-mono-oxygenase, tyrosinase, and lysyl oxidase, leading to death in infancy (19). We previously reported that specific SOD3 activity is decreased in blood vessels of ATP7A dysfunctional mutant mice, which is rescued by copper addition (20). However, the role of copper transport proteins in vascular dysfunction in T1DM is entirely unknown.

We performed the current study to determine the role of SOD3 and copper transport proteins in modulating $O_2^{\bullet-}$-mediated endothelial dysfunction in T1DM animals. Here we show that specific activity of SOD3, but not SOD1, is decreased in diabetic vessels, thereby increasing $O_2^{\bullet-}$ production and impaired endothelium-dependent relaxation

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of resistant arteries, which is rescued by SOD mimetic tempol and gene transfer of SOD3. Mechanistically, copper transporter ATP7A protein expression is significantly reduced in blood vessels from T1DM mice in part due to the insulin deficiency but not high glucose. Transgenic mice overexpressing ATP7A restore T1DM-induced impaired SOD3 activity and endothelial function by reducing $O_2^{\bullet-}$ levels. The SOD3-deficient or ATP7A mutant T1DM mice further enhance endothelial dysfunction and vascular $O_2^{\bullet-}$ production versus T1DM mice. These findings provide new insights into the protective role of the endogenous ATP7A-SOD3 pathway in vascular dysfunction in oxidant stress–dependent metabolic and cardiovascular diseases.

RESEARCH DESIGN AND METHODS

Animals and experimental design. Heterozygous blotchy ATP7A mutant ($ATP7A^{mut}$) mice (21) back-crossed to the C57BL/6J background for 10 generations, $SOD3^{-/-}$ mice on C57BL/6J background (22), and heterozygous transgenic mice overexpressing ATP7A on C57BL/6J background were weaned at 4 weeks of age and maintained on regular chow for 3 months. C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). $ATP7A^{mut}$ mice carrying the X-linked blotchy ATP7A mutation have a splice site mutation introducing a new stop codon at amino acid residue 794 and show impaired copper transport function, but survive to more than 6 months of age (20). ATP7A transgenic mice that overexpress human ATP7A from the composite β-actin promoter (CAG) were generated as previously described (23). The University of Illinois at Chicago Animal Care and Use Committee approved the protocol for animal use.

Mice were studied between 8 and 12 weeks of age. Diabetes was induced by STZ (100 mg/kg body weight in 0.05 mol/L citrate buffer, pH.4.5, intraperitoneally) on two consecutive days after overnight fasting, and control mice were injected with citrate buffer, as previously described (24). Thirty days after STZ treatment, blood glucose was elevated significantly from 165 mg/dL in control mice to 552 mg/dL in diabetic mice. Regarding mouse body weight, it was not significantly changed at 30 days after STZ treatment as compared with those with vehicle treatment (28.7 ± 3.1 vs. 27.6 ± 3.2; NS), whereas it was significantly decreased at 60 days (24.5 ± 1.51 vs. 28.83 ± 0.75 in control; $P < 0.05$). The STZ mice were not treated with insulin in this study. Mice became hyperglycemic at 7–10 days after STZ injection for ~20 days and were killed for experiments at day 30. All reagents were purchased from Sigma-Aldrich (St. Louis, MO), except when specified. For a genetic model of T1DM, we used $Ins2^{Akita}$ diabetic mice on C57BL/6J background (Jackson Laboratory) which is a well-known animal model of T1DM with an autosomal dominant mutation in the *Ins2* gene (25).

Adenoviral vector and in vivo gene transfer. Adenovirus expressing human SOD3 (Ad.SOD3) and human SOD3 lacking HBD (Ad.SOD3-ΔHBD) were from the adenovirus core at University of Iowa (Iowa City, IA) (11). Ad.SOD3, Ad. SOD3-ΔHBD, and Lac Z (0.25 mL of 1×10^{12} particles/mL in 3% sucrose in PBS) were injected intravenously. Three days after viral injection, mice were killed and vascular tissue and plasma were collected for further experiments. Plasma SOD3 proteins were identified by zymography (11).

SOD activity assays. Tissue harvesting and SOD activity assay were performed as described previously (21). Con A–Sepharose chromatography was used to isolate SOD3 from vessels of diabetic and control mice.

Western blot analysis. Total tissue and cell lysate were used for Western blot analysis as previously described (26).

Cell culture. VSMCs were isolated from male Sprague-Dawley rat thoracic aortas by enzymatic digestion. Cells were grown in Dulbecco's modified Eagle's medium (DMEM), containing 10% bovine serum and 4.5 g/L glucose, as previously described (27). Before stimulation with insulin, cells were starved for 24 h with serum-free media.

Organoid cultures of diabetic vessels. To examine the effect of insulin on intact diabetic vessels in the absence of neurohormonal adaptations and changes in blood pressure, we used organoid cultures, as previously described (28). In brief, after dissection of adventitial tissue, aortic segments were immersed in six-well dishes that contained DMEM and antibiotics (penicillin 100 units/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.

Measurements of vascular superoxide production. Control littermate, $SOD3^{-/-}$, $ATP7A^{mut}$, and ATP7A transgenic mice were killed by CO_2 inhalation. Vascular $O_2^{\bullet-}$ production was determined using lucigenin-enhanced chemiluminescence as described previously (21).

Vascular reactivity studies. Isometric tension of mesenteric resistance arteries and mouse aorta were measured using wire myograph as described previously (29).

Statistical analysis. Data are presented as mean ± SE. Data were compared between groups of cells and animals by Student *t* test when one comparison was performed or by ANOVA for multiple comparisons. When significance was indicated by ANOVA, the Tukey-Kramer post hoc test was used to specify between group differences. Values of $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ were considered statistically significant.

RESULTS

Specific activity of SOD3, but not SOD1, is decreased, whereas $O_2^{\bullet-}$ production is enhanced in aorta from diabetic mice. T1DM mice were created by STZ administration, as described in RESEARCH DESIGN AND METHODS. Thirty days after the STZ injection, blood glucose was elevated to 552.5 ± 25.15 mg/dL in diabetic mice vs. 165.2 ± 11.71 mg/dL in the C57BL/6J controls, whereas body weight was not significantly changed without insulin (28.7 ± 3.1 vs. 27.6 ± 3.2; NS) (Supplementary Fig. 1). Plasma insulin levels were significantly lowered in STZ-induced diabetes compared with controls.

We examined SOD activity and expression in aortas of STZ-induced diabetic mice. Figure 1*A* shows that SOD3 activity was significantly ($P < 0.05$) decreased in diabetic mice, as compared with control mice, whereas protein levels of SOD3 were significantly increased (Fig. 1*B*). In contrast, SOD1 activity and protein levels were not altered in diabetic vessels. Thus, the specific activity of SOD3, as determined by the ratio of activity to protein, was markedly ($P < 0.001$) decreased in diabetic vessels (i.e., increased "inactive" SOD3 protein), whereas that of SOD1 was not changed (Fig. 1*C*). Consistent results were observed in genetic T1DM $Ins2^{Akita}$ mice (Supplementary Fig. 2*A*), indicating that the decrease in vascular SOD3 specific activity in STZ-induced diabetic mice is not due to a toxic effect of STZ but due to the diabetic condition. Of note, a decrease in SOD3 specific activity in aorta from T1DM mice was associated with a marked increase in $O_2^{\bullet-}$ production (Fig. 1*D*) and nitrotyrosine staining, an indicator of peroxynitrite ($ONOO^-$) (data not shown).

Since the activity of SOD1 and SOD3 is dependent on the catalytic copper cofactor (5,17), we examined whether decreased SOD3 specific activity in diabetic vessels is due to deficiency of copper. Figure 1*E* shows that copper addition restored the decreased specific activity of SOD3 purified from diabetic vessels. In contrast, the specific activities of SOD3 purified from control vessels or that of SOD1 from either diabetic or control vessels were not affected by copper addition. These results suggest that copper loading to SOD3 is selectively impaired in diabetic vessels, whereas SOD3 enzyme from control vessels or SOD1 enzyme from either diabetic or control vessels is fully metallated.

Endothelium-dependent relaxation is impaired in mesenteric resistance arteries from diabetic mice, which is rescued by SOD mimetic tempol or gene transfer of SOD3. Because increased $O_2^{\bullet-}$ production can alter endothelial function (5), we examined endothelium-dependent vasorelaxation in diabetic and control mice (Fig. 2). For this purpose, we used mesenteric arteries (~200 μm in diameter) with the wire myograph approach, which contributes to blood pressure and tissue perfusion (30) as well as aortic segments. Acetylcholine (ACh)-induced endothelium-dependent vasorelaxation was significantly impaired in resistance arteries of diabetic mice compared with control mice (maximum relaxation 68 ± 2 vs. 92 ± 2%, respectively,

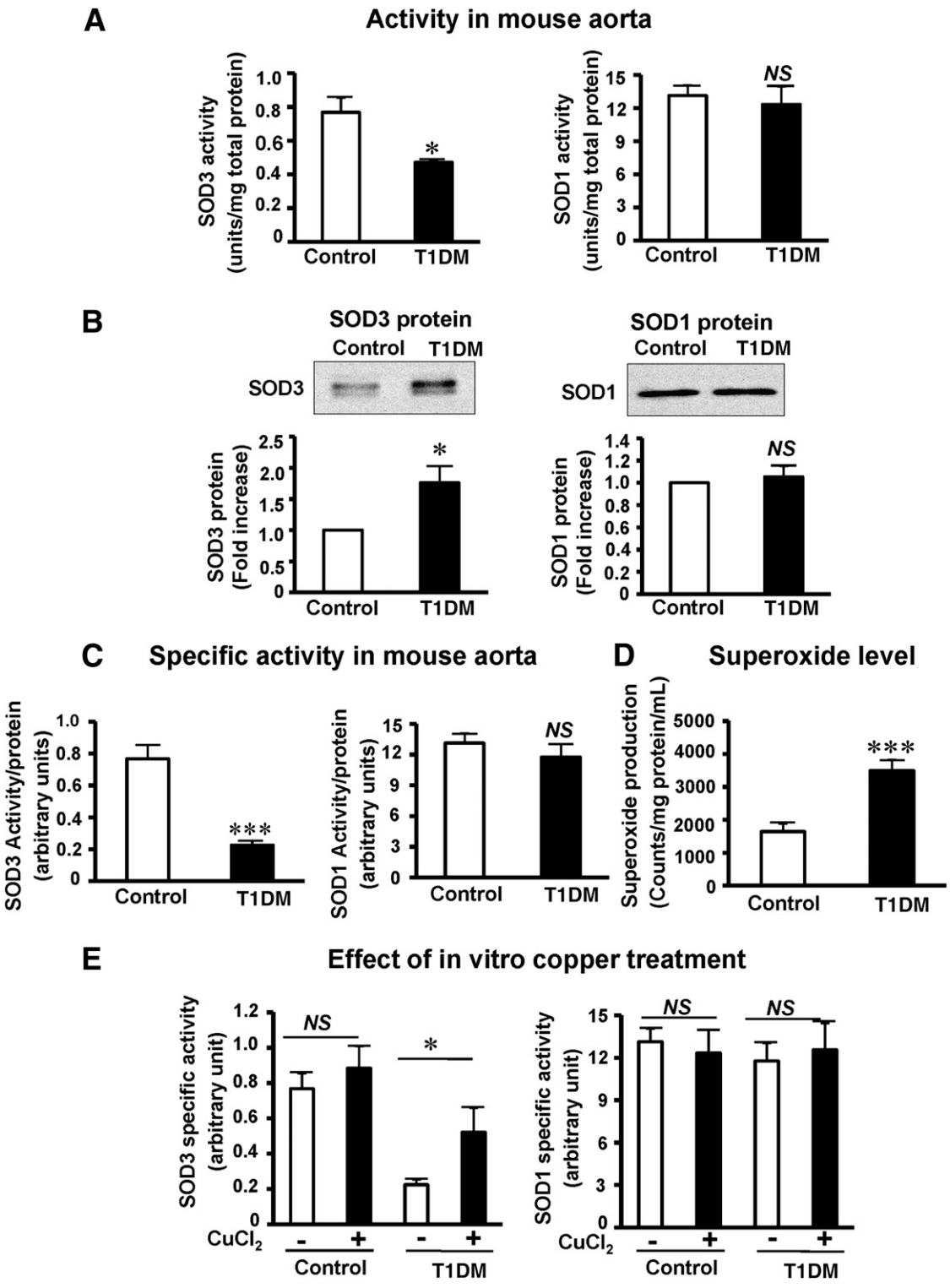

FIG. 1. Activity, protein level, and specific activity of SOD3 and SOD1 as well as vascular $O_2^{\bullet-}$ level in aortas of T1DM mice. *A*: Activities of SOD3 and SOD1 in homogenates from STZ-injected DM or control mice aorta were assayed by inhibition of cytochrome *c* reduction by xanthine/xanthine oxidase at pH 7.4. Con A–Sepharose chromatography was used to isolate SOD3 from tissue homogenates. *B*: Protein levels of SOD1 and SOD3 were determined by Western analysis with SOD1 or SOD3 antibody (*top*). Densitometric analysis was shown (*bottom*). *C*: Specific activity of SOD1 and SOD3 (*bottom*) was determined by the ratio of activity to relative amount of protein as previously described (20). *D*: Aortic $O_2^{\bullet-}$ production in control and diabetic mice was measured by a lucigenin-enhanced chemiluminescence (5 μmol/L) method. *E*: Con A-Sepharose–bound SOD3 or unbound SOD1 proteins were treated with or without $CuCl_2$ (10 μmol/L, 1 h at room temperature), and then specific activity of SOD3 and SOD1 was measured, as described above. Results are presented as mean ± SEM (n = 4). ***P < 0.001; *P < 0.05 vs. control. NS, not significant.

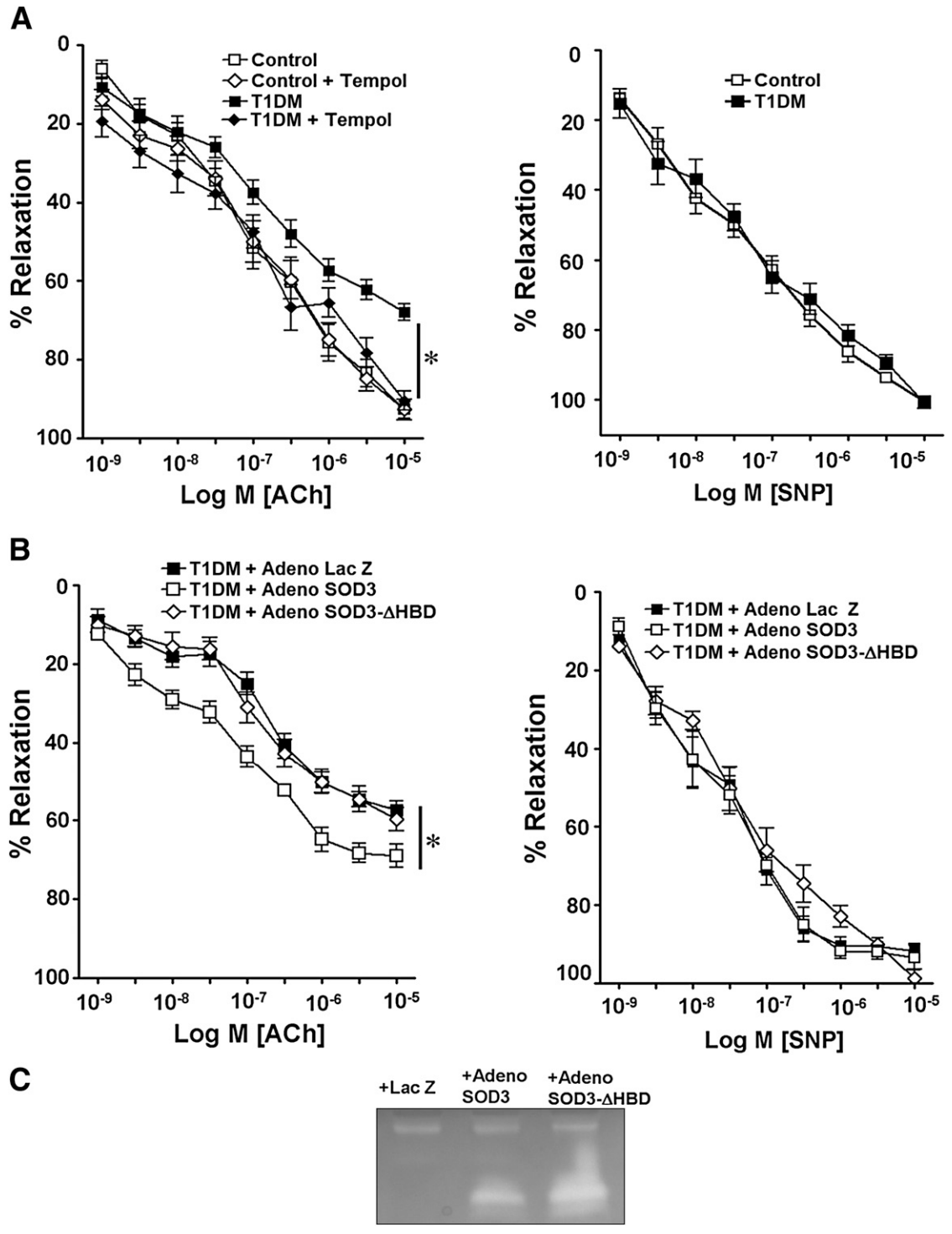

FIG. 2. Effect of SOD mimetic tempol or adenovirus-mediated gene transfer of SOD3 on relaxation to ACh and SNP in mesenteric resistance arteries from T1DM mice. *A*: Isometric tension of mesenteric resistance arteries (~180 μm) from STZ-injected DM or control mice was measured in isolated organ chambers using a wire myograph. Vasodilation was evoked by ACh and SNP after preconstriction with phenylephrine (1–5 μmol/L) in the presence and absence of cell-permeable SOD mimetic tempol (1 mmol/L) (*A*) or 3 days after injection of adeno-SOD3 or adeno-SOD3-ΔHBD (1×10^{12} particles per mice) to tail vein of mice (*B*). *C*: In-gel zymography for SOD activity of plasma from adenovirus-injected mice. Results are presented as mean ± SEM (n = 4–8). *$P < 0.05$ vs. control.

$P < 0.001$), which was rescued by the SOD mimetic tempol (Fig. 2*A*). Of note, sodium nitroprusside (SNP)–induced endothelium-independent vasorelaxation was not different between diabetic and control mice. A similar response was observed in mouse aorta from diabetic mice (Supplementary Fig. 3). These results suggest that impaired endothelium-dependent vasorelaxation in diabetic mice is largely due to increased $O_2^{\bullet-}$ levels.

Since specific activity of SOD3, which is anchored to endothelial surfaces through HBD, is significantly decreased in diabetic vessels, we next examined the effects of adenoviral-mediated gene transfer of SOD3 (Ad.SOD3)

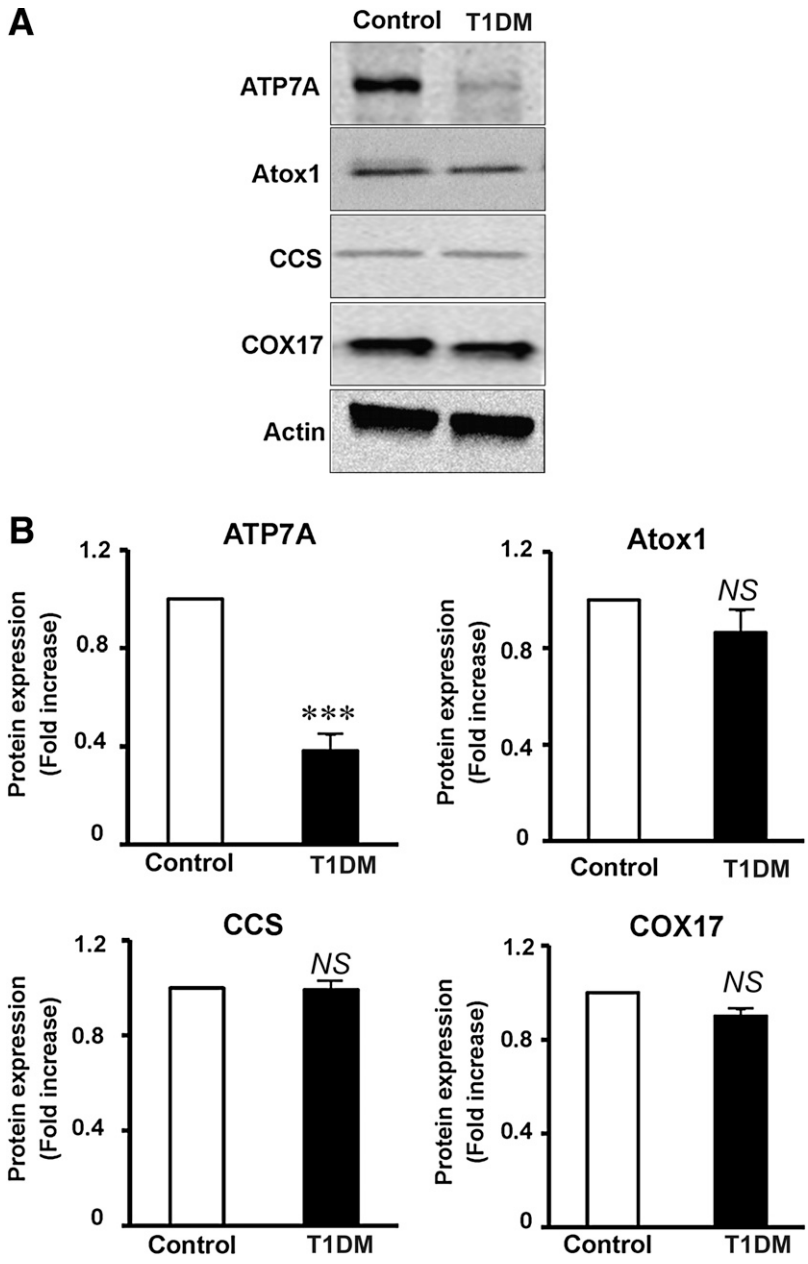

FIG. 3. Protein expression of ATP7A, but not other copper trafficking regulators, is decreased in aorta of T1DM mice. ***A*: Protein expression for ATP7A, Atox1, CCS, and COX17 in STZ-injected DM or control mice was determined by Western analysis with antibodies specific to their respective protein. *B*: Densitometric analysis was shown. Results are presented as mean ± SEM (n = 4). ***P < 0.001 vs. control. NS, not significant.**

and SOD3 lacking HBD (Ad.SOD3-ΔHBD) on endothelial dysfunction in diabetic mice. Ad.SOD3 or Ad.SOD3-ΔHBD were injected into diabetic mice intravenously, and the enzymatic activity of SOD3 or SOD3-ΔHBD in plasma was confirmed by in-gel zymography (Fig. 2*C*), as previously reported (11). Interestingly, a more intense band was detected for Ad.SOD3-ΔHBD than for Ad.SOD3, because SOD3 binds to vascular tissues, whereas SOD3-ΔHBD circulates without binding to vascular tissues. Figure 2*B* shows that ACh-induced endothelium-dependent vasorelaxation was significantly improved after gene transfer of SOD3, but not SOD3-ΔHBD, in diabetic mice (maximal relaxation 69 ± 3 vs. 57.4 ± 2.4%, respectively). Further, SNP-induced relaxation in diabetic mesenteric arteries was not altered by gene transfer of either SOD3 or SOD3-ΔHBD. These results suggest that increased $O_2^{\bullet-}$ production in diabetic vessels is in part due to decreased SOD3 activity.

Copper transporter ATP7A expression is decreased in diabetic vessels. Because impaired SOD3 specific activity in diabetic vessels is due to copper deficiency, we next examined the expression of copper transport proteins in diabetic and control mice. Figure 3 shows that protein expression of ATP7A, but not Atox1, was decreased (P < 0.001) in diabetic aorta compared with control aorta. In contrast, protein expression for CCS, a copper chaperone for SOD1 in the cytoplasm (17), and COX17, a copper chaperone for cytochrome *c* oxidase in the mitochondria (18), was not changed in diabetic vessels. A similar response was also observed in mesenteric arteries from diabetic mice (Supplementary Fig. 4). Of note, $Ins2^{Akita}$ mice,

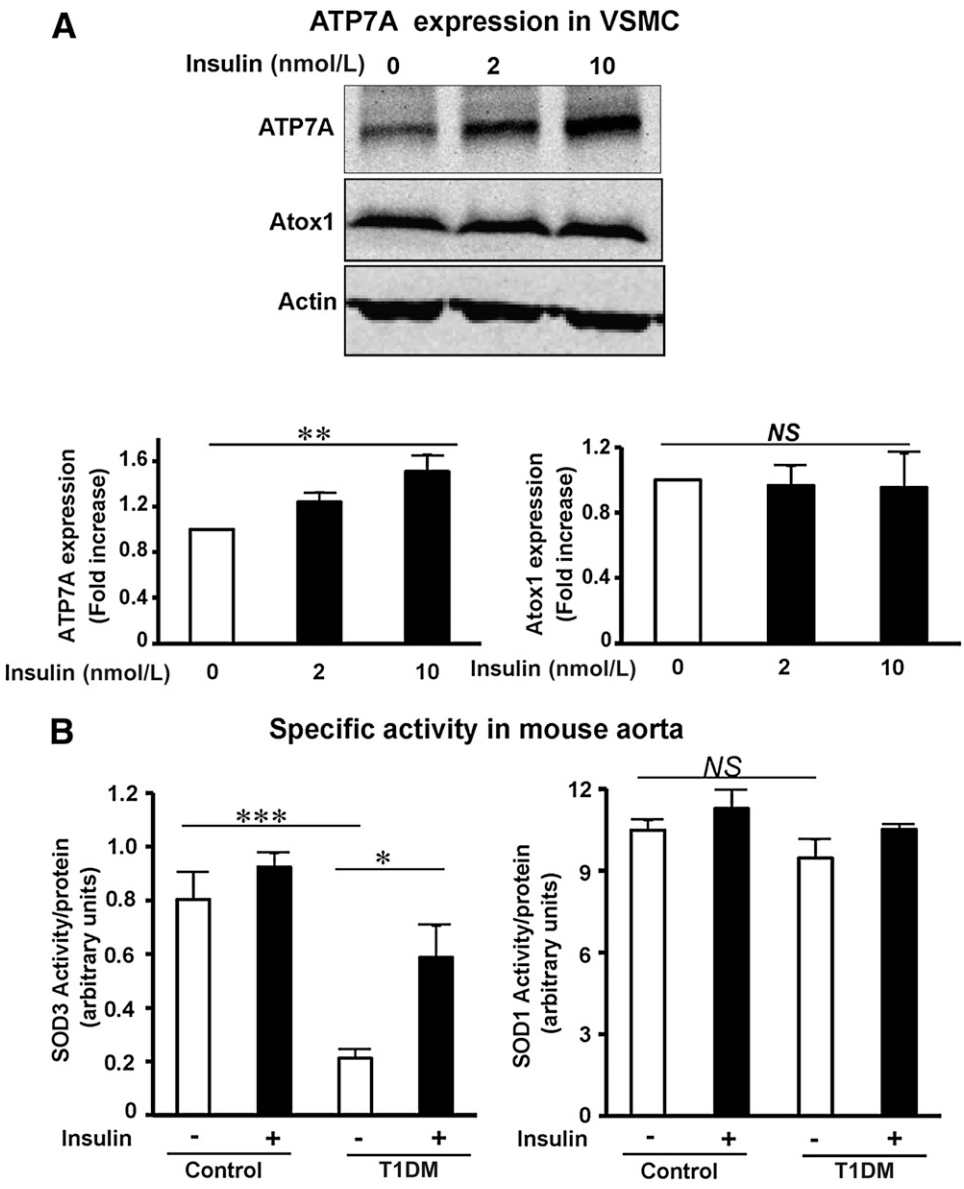

FIG. 4. Insulin restores reduced SOD3 specific activity in organoid culture from diabetic mouse aorta by increasing ATP7A protein expression in VSMCs. *A*: VSMCs were treated with insulin (10 nmol/L) for 12 h and used for Western analysis of ATP7A and Atox1 protein expression. *B*: Isolated STZ-injected DM or control mice aorta were exposed to insulin (10 nmol/L) for 24 h in organoid culture. Activity of SOD3 and SOD1 in homogenate was assayed as in Fig. 1. Results are presented as mean ± SEM (n = 3). ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$ vs. control. NS, not significant.

a genetic model of T1DM, exhibited results similar to STZ-induced diabetic mice (Supplementary Fig. 2*B*), indicating that the decrease in ATP7A expression in STZ-induced diabetic mice is not due to a toxic effect of STZ, but due to the diabetic condition. Thus, diabetic mice show a significant decrease in the expression of vascular copper transporter ATP7A.

Insulin increases ATP7A protein expression in cultured VSMCs and restores vascular SOD3 activity in organoid culture of diabetic vessels. To address the mechanism by which ATP7A protein expression is decreased in T1DM vessels, we next examined the role of hyperglycemia or hypoinsulinemia, which are characteristics of T1DM, using cultured VSMCs. In this cell type, ATP7A delivers copper to SOD3 at the secretory pathway, including the trans-Golgi network, and then SOD3 is secreted to the extracellular space (5). Figure 4*A* shows that insulin treatment (10 nmol/L) for 12 h significantly increased ATP7A protein expression ($P < 0.01$), but not another copper transport protein (Atox1), in VSMCs, whereas high glucose had no effects (data not shown). Insulin treatment also rescued the T1DM-induced decrease in SOD3 activity (Fig. 4*B*) and ATP7A protein expression (Supplementary Fig. 5) without affecting SOD1 activity in the organoid culture. These results suggest that hypoinsulinemia in T1DM may contribute to a decrease in ATP7A protein expression, resulting in decreased SOD3 specific activity.

Endothelial dysfunction, enhanced $O_2^{\bullet-}$ production, and decreased SOD3 activity are restored in diabetic transgenic mice overexpressing ATP7A. Given that ATP7A plays a critical role in delivering cofactor copper to SOD3 for its full activation, we hypothesized that impaired endothelium-dependent vasorelaxation and SOD3 activity in diabetic mice might be due to a reduction of ATP7A

expression. To address this question, we used ATP7A-overexpressing transgenic mice (23) and found that ATP7A protein expression was significantly increased by three- to fourfold in ATP7A transgenic compared with wild-type (WT) mice (Fig. 5*A*). Figure 5*C* and *D* shows that the decrease in SOD3 activity and specific activity in diabetic WT mice aorta was significantly improved in diabetic ATP7A transgenic mice. In parallel, diabetes-induced enhanced $O_2^{\bullet-}$ production was significantly decreased in diabetic ATP7A transgenic mice compared with WT mice (Fig. 5*B*). ACh-induced endothelium-dependent relaxation was significantly improved in resistance arteries of diabetic ATP7A transgenic mice compared with diabetic WT mice (maximal relaxation 74.8 ± 3.9 vs. 60.1 ± 3%, respectively) (Fig. 6*A*), whereas endothelium-independent relaxation to SNP was not changed between the two groups (Fig. 6*B*). These findings suggest that decreased ATP7A expression in diabetic vessels contributes to decreased SOD3 activity, thereby enhancing $O_2^{\bullet-}$ production and endothelial dysfunction.

Diabetic SOD3-deficient and ATP7A^{mut} mice enhance endothelial dysfunction and $O_2^{\bullet-}$ production. To examine the role of endogenous ATP7A and SOD3 in diabetic vessels, we used mice lacking SOD3 (SOD3$^{-/-}$ mice) or ATP7A dysfunctional mutant mice (ATP7A^{mut} mice) (20). Endothelium-dependent relaxation to ACh was markedly ($P < 0.001$) impaired in resistance arteries of diabetic SOD3$^{-/-}$ and ATP7A^{mut} mice compared with diabetic WT mice, which was rescued by addition of the SOD mimetic tempol (Fig. 7*A*). In contrast, endothelium-independent relaxation to SNP was not changed between the two groups (Fig. 7*A*). Vascular $O_2^{\bullet-}$ production in T1DM was significantly increased in SOD3$^{-/-}$ and ATP7A^{mut} mice to

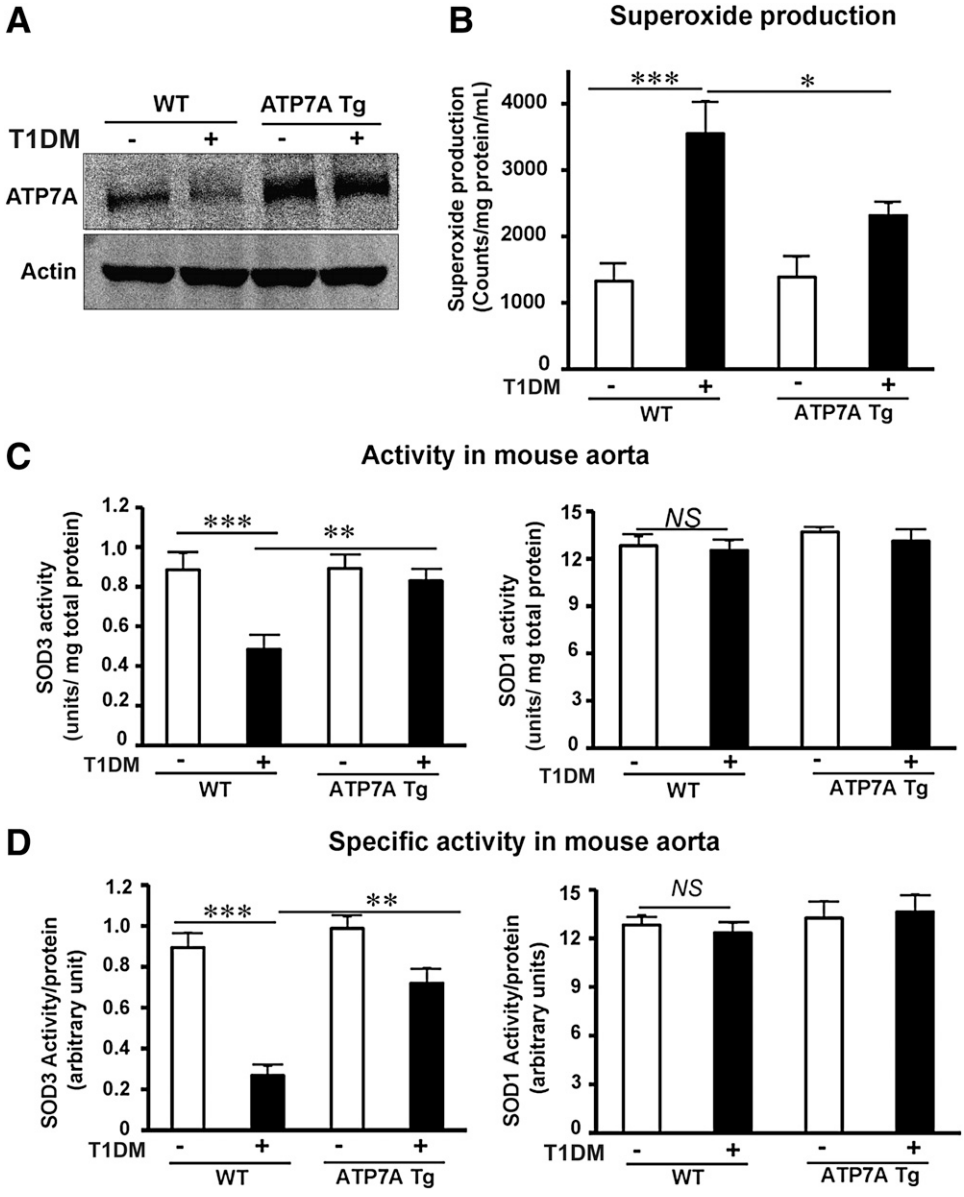

FIG. 5. Activity and specific activity of SOD3 and SOD1 as well as vascular $O_2^{\bullet-}$ level in aorta of diabetic transgenic mice overexpressing ATP7A (ATP7A Tg). Protein level of ATP7A (*A*) and $O_2^{\bullet-}$ production (*B*) in aortas from WT mice or transgenic mice overexpressing ATP7A with or without STZ injection (DM) were measured by Western analysis and lucigenin-enhanced chemiluminescence assay, respectively. Activity (*C*) and specific activity (*D*) of SOD3 and SOD1 in tissue homogenate were assayed as described in Fig. 1. Results are presented as mean ± SEM ($n = 4$). ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$ vs. control. NS, not significant.

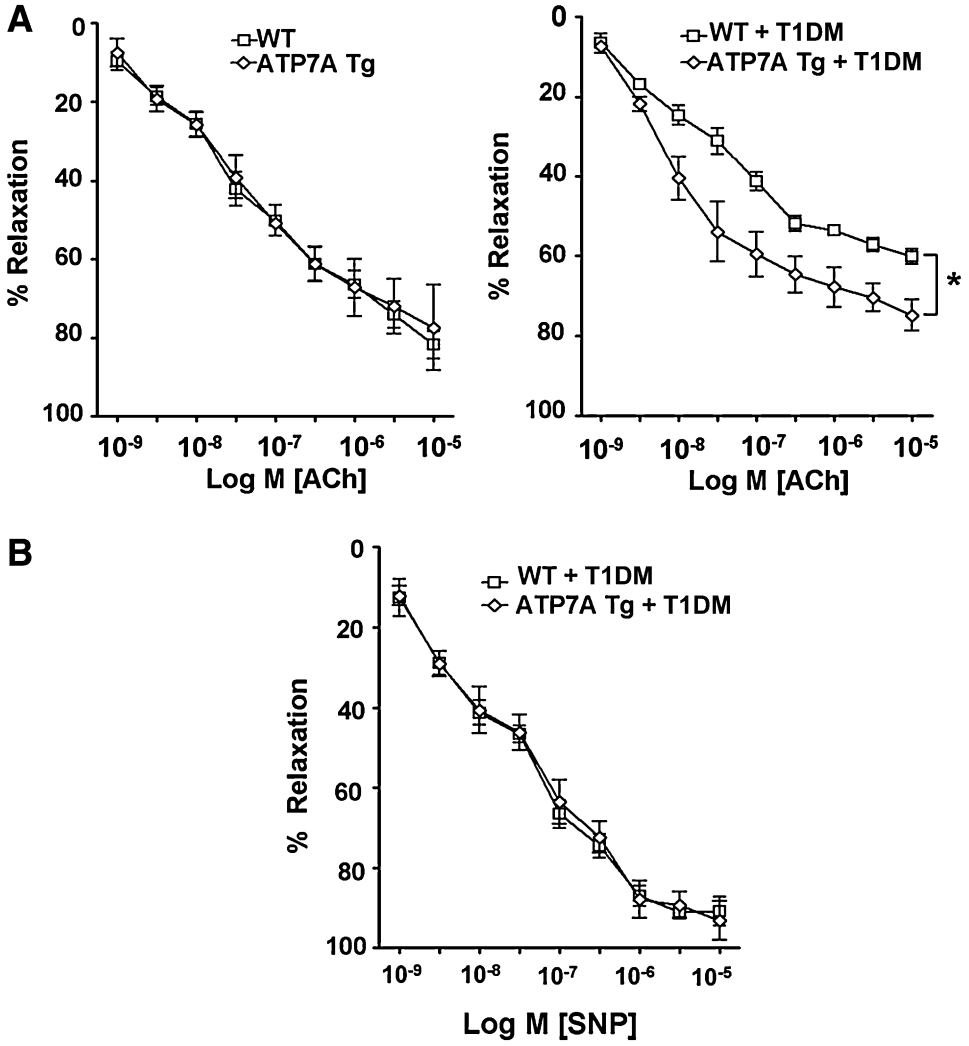

FIG. 6. Endothelium-dependent or -independent relaxation of mesenteric resistance arteries from diabetic transgenic mice overexpressing ATP7A (ATP7A Tg). Isometric tension of mouse mesenteric resistance arteries from WT mice or transgenic mice overexpressing ATP7A with or without STZ injection (DM) was measured as in Fig. 2. Vasodilation was evoked by ACh (*A*) and SNP (*B*) after preconstriction with phenylephrine (1–5 μmol/L). Results are presented as mean ± SEM (n = 6–8). *$P < 0.05$ vs. control.

a greater extent than in diabetic WT mice (Fig. 7*B*). These findings further suggest that endogenous ATP7A and SOD3 play an important role in protecting endothelial function by regulating vascular $O_2^{\bullet-}$.

DISCUSSION

The current study demonstrates a protective role for the Cu transporter ATP7A in T1DM-induced endothelial dysfunction by regulating SOD3 activity and vascular $O_2^{\bullet-}$ levels (Fig. 8). We found the following. *1*) Specific activity of SOD3, but not SOD1, is significantly decreased in diabetic vessels, which is associated with increased $O_2^{\bullet-}$ production compared with control vessels. *2*) In ex vivo experiments, addition of copper partially rescues decreased SOD3 specific activity in diabetic vessels. *3*) Functionally, endothelium-dependent relaxation is impaired in mesenteric arteries of T1DM, which is rescued by SOD mimetic tempol or gene transfer of SOD3. *4*) Mechanistically, ATP7A expression in T1DM aorta is dramatically decreased whereas other copper transport proteins (Atox1, CCS, and COX17) are not changed. *5*) These effects may be due to hypoinsulinemia in T1DM mice, since insulin treatment, but not high glucose, increases ATP7A expression in VSMCs and restores SOD3 activity in organoid culture of T1DM vessels. *6*) Transgenic mice overexpressing ATP7A exhibit restored T1DM-induced impaired endothelial function and SOD3 activity and increased vascular $O_2^{\bullet-}$ production. *7*) The $SOD3^{-/-}$ T1DM or $ATP7A^{mut}$ T1DM mice show augmented endothelial dysfunction and increased $O_2^{\bullet-}$ production versus T1DM mice. Thus, restoring ATP7A-SOD3 function is an important therapeutic strategy for oxidant stress–dependent cardiovascular and metabolic diseases.

Previous studies have reported either increased or decreased total SOD activity (31–33) as well as either unaltered or decreased SOD1 expression in vascular tissues from diabetic animals (34–36) or in vascular progenitor cells from diabetic patients (37). However, the role of endogenous SOD3 in endothelial dysfunction in T1DM has not been investigated. In the current study, we provide compelling evidence that the specific activity of SOD3 is markedly decreased, and SOD3 protein expression is increased in aortas from STZ-induced diabetic mice, whereas activity or expression of SOD1 is not altered. This result is

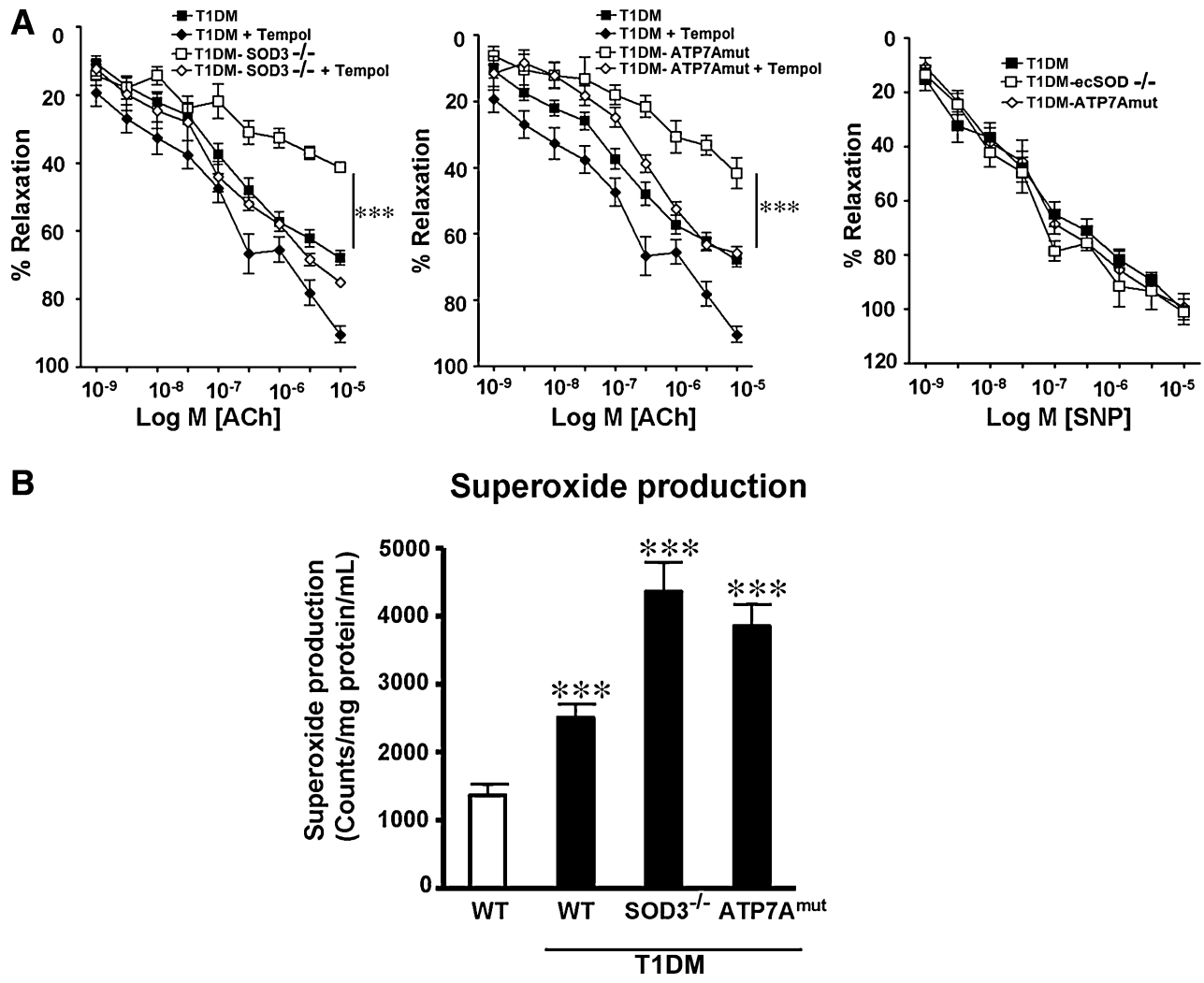

FIG. 7. Vascular function and $O_2^{\bullet-}$ level in diabetic ATP7A^{mut} and SOD3$^{-/-}$ mice. *A*: Isometric tension of mesenteric resistance arteries in STZ-injected diabetic (DM) WT, ATP7A^{mut}, and SOD3$^{-/-}$ mice was measured in isolated organ chambers as in Fig. 2. *A*: Vasodilation was evoked by ACh in the presence and absence of SOD mimetic tempol (1 mmol/L) or SNP after preconstriction with phenylephrine (1–5 μmol/L). *B*: Vascular $O_2^{\bullet-}$ production in WT, diabetic WT, ATP7A^{mut}, and SOD3$^{-/-}$ mice was measured by lucigenin-enhanced chemiluminescence assay. Results are presented as mean ± SEM (n = 6–8). ***P < 0.001 vs. control. ecSOD, extracellular SOD.

consistently observed in the genetic model of Ins2$^{Akita+/-}$ T1DM mice. Our findings are divergent with a previous report showing that glycated SOD3 is increased in the serum (14), and tissue-bound SOD3 is decreased in diabetic patients or animals compared with nondiabetic controls (9,35), which is likely due to the reduction of heparin affinity of SOD3 via nonenzymatic glycation without changing the enzymatic activity (14,38). Given that pediatric patients show a significant decrease in plasma SOD3 compared with control subjects (39), it is conceivable that tissue-bound SOD3 levels in diabetes may be regulated by aging. Indeed, the current study has used young mice (3–4 months of age) to demonstrate that the tissue SOD3 level is increased in T1DM mice aorta. Alternatively, since copper loading to SOD3 in diabetic vessels is impaired, as discussed below, increased SOD3 protein levels in diabetic vessels (aortas and mesenteric arteries) may be caused by the accumulation of immature SOD3, as copper deficiency increases the ceruloplasmin accumulation in a pre-Golgi compartment in hepatocytes (40). Taken together, these results suggest that changes of SOD3 protein levels do not reflect its activity and that T1DM mice exhibit decreased SOD3 activity without altering SOD1 activity, which may contribute to increased vascular $O_2^{\bullet-}$ production.

The functional significance of decreased SOD3 activity in diabetic vessels is demonstrated by the finding that endothelium-dependent relaxation in mesenteric arteries is impaired in T1DM mice, which is rescued by gene transfer of SOD3 as well as the SOD mimetic tempol. By contrast, endothelium-independent vasorelaxation is not affected in diabetic mice, suggesting that inhibition of ACh-induced vasodilation in T1DM is likely attributed to a decrease in endothelial NO bioavailability, which is supported by increased $ONOO^-$ formation, assessed by nitrotyrosine staining. Consistent with our results, previous studies reported that gene transfer of SOD3 rescues the endothelial function in other pathological conditions, such as pulmonary hypoxia, hypertension, and aging (5), and in different vascular beds (13). In the current study, we also found that SOD3-deficient T1DM mice show augmented impaired endothelium-dependent vasodilation and $O_2^{\bullet-}$ production versus T1DM mice, supporting the protective role of endogenous SOD3 in endothelial function. Our current study also shows that gene transfer of SOD3

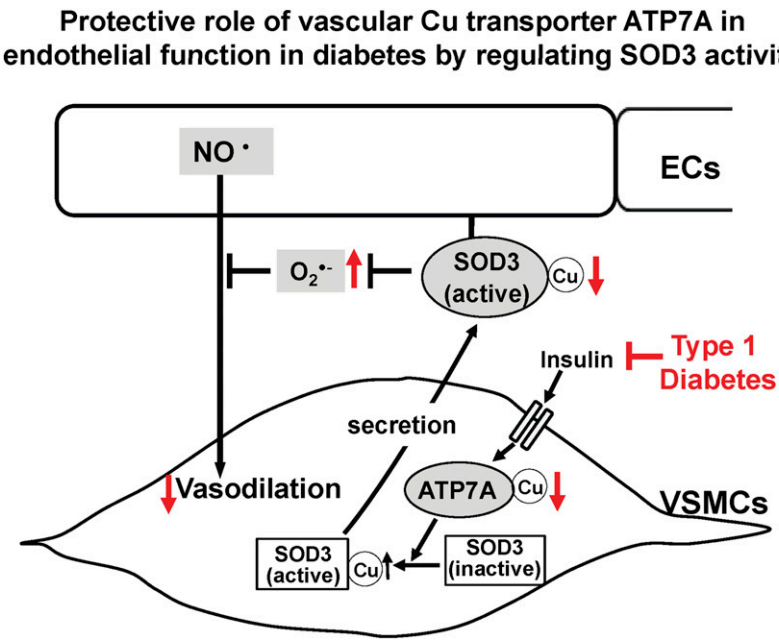

FIG. 8. Proposed model for the protective role of the ATP7A-SOD3 pathway in T1DM-induced endothelial dysfunction. ATP7A delivers copper and activates SOD3 protein, which scavenges $O_2^{\bullet-}$ in the extracellular space, thereby increasing bioavailability of NO and preserving endothelial function. STZ-induced diabetes decreases ATP7A protein levels primarily due to the hypoinsulinemia, thereby decreasing SOD3 activity, leading to overproduction of $O_2^{\bullet-}$ and endothelial dysfunction. EC, endothelial cell.

lacking HBD does not rescue impaired endothelium-dependent relaxation in diabetic vessels. This result strongly supports the notion that binding of SOD3 to the endothelial surface and extracellular matrix of vascular tissue via HBD is essential for protecting against inactivation of endothelium-derived NO by $O_2^{\bullet-}$ during diffusion to vascular smooth muscle to induce vasorelaxation (5,8,11). Taken together, SOD3 plays an important role in protecting endothelial function by scavenging extracellular $O_2^{\bullet-}$ in the diabetic vessel wall.

Mechanistically, we found that the decreased specific activity of SOD3 purified from T1DM aorta is partially restored by Cu addition, suggesting that Cu loading to SOD3 is impaired in diabetic vessels. It has been shown that full activation of SOD3 requires copper transporter ATP7A and copper chaperone Atox1, which are involved in copper delivery to SOD3 (20,21,29,41), whereas SOD1 gets copper through copper chaperone CCS (17). In the current study, we provide the first evidence that ATP7A protein expression, but not Atox1, CCS, or COX17 (copper chaperone for cytochrome *c* oxidase), is selectively and significantly decreased in STZ-induced T1DM vessels. Consistent results are observed in genetically induced (i.e., $Ins2^{Akita}$) T1DM mice. Thus, these findings may explain why the activity of SOD3, but not SOD1, is decreased in T1DM vessels. Furthermore, our data imply that copper transport systems coupled to distinct copper enzymes can be differently regulated in response to T1DM, as reported for hypoxia (42). To address the mechanism by which ATP7A protein expression is decreased in T1DM vessels, we next examined the role of hyperglycemia or hypoinsulinemia, which are characteristics of T1DM, in VSMCs. Here we show that insulin treatment of VSMCs increases ATP7A expression without affecting the Atox1 level, whereas high glucose alone has no effects. Consistent with this, Hardman et al. (43) reported that insulin regulates ATP7A expression in human placental Jeg-3 cells. Furthermore, we found that insulin treatment directly restores the T1DM-induced decrease in SOD3 activity and ATP7A protein expression without affecting SOD1 activity in the organoid culture of T1DM vessels, which can exclude the possibility of a neurohormonal effect of insulin. Thus, these results indicate that hypoinsulinemia in T1DM may contribute to the decrease in ATP7A protein expression in VSMCs, thus reducing SOD3 activity in the blood vessels. Our preliminary study found that insulin increases ATP7A protein stability in a phosphatidylinositol (PI) 3-kinase/Akt-dependent manner. Given that the insulin–PI 3-kinase/Akt pathway has been shown to be impaired in vascular tissue from T2DM mice (44,45), it is tempting to speculate that a decrease in the insulin–PI 3-kinase/Akt pathway in T1DM and T2DM may contribute to the downregulation of ATP7A expression in VSMCs. This may result in a decrease in SOD3 activity and subsequent overproduction of $O_2^{\bullet-}$, thereby inducing endothelial dysfunction. This issue should be investigated more in detail in future studies.

In this study, we have demonstrated the functional role of ATP7A in regulating SOD3 activity and endothelial function in diabetic vessels using transgenic mice overexpressing ATP7A or $ATP7A^{mut}$ mice. Here we show that T1DM-induced endothelial dysfunction and decrease of SOD3 activity are rescued in transgenic mice overexpressing ATP7A, whereas SOD3-deficient T1DM mice or $ATP7A^{mut}$ T1DM mice accelerate endothelial dysfunction and vascular $O_2^{\bullet-}$ production compared with T1DM mice. Consistent with this, we previously reported that angiotensin II–induced hypertension and endothelial dysfunction are further augmented in $ATP7A^{mut}$ mice (21). Importantly, ATP7A plays a role not only in providing copper to some secretory cuproenzymes but also in regulating intracellular levels of copper by exporting copper (46). It has been shown that ATP7A-overexpressing

transgenic mice alter tissue copper homeostasis (23), and that tissue copper levels are increased in STZ-induced diabetes, which is rescued by insulin treatment (47). Thus, it is tempting to speculate that increased copper levels in diabetes may be caused by decreased ATP7A expression and subsequent inhibition of copper export. Furthermore, due to its toxicity, increased copper levels may contribute to the pathogenesis of diabetic vascular complications. Indeed, copper chelation therapy has been shown to mitigate various pathogenic states of diabetes, such as left ventricular hypertrophy in diabetic patients (48), diabetic neuropathy (49), and diabetic nephropathy (50). Taken together, it is likely that the copper transporter protein ATP7A plays an important role in preventing diabetes-induced endothelial dysfunction by regulating SOD3 activity as well as intracellular copper homeostasis.

In summary, the current study provides direct evidence for the protective role of the ATP7A-SOD3 pathway in endothelial function by reducing extracellular $O_2^{\bullet -}$ levels and increasing bioavailability of NO in T1DM (Fig. 8). These findings provide novel insight into ATP7A as a potential therapeutic target for the treatment of oxidative stress–dependent cardiovascular and metabolic diseases such as T1DM.

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V.S. designed the study, performed experiments, analyzed data, and wrote the manuscript. N.U. and J.O. helped to make diabetic mice. R.D.M. conducted mouse husbandry and genotyping. R.M.L. and J.F.B.M. developed the ATP7A transgenic mice. M.U.-F. and T.F. designed the overall study, analyzed data, and wrote, reviewed, and edited the manuscript. T.F. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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REFERENCES

1. Nathan DM, Cleary PA, Backlund JY, et al.; Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) Study Research Group. Intensive diabetes treatment and cardiovascular disease in patients with type 1 diabetes. N Engl J Med 2005; 353:2643–2653
2. Rask-Madsen C, King GL. Mechanisms of disease: endothelial dysfunction in insulin resistance and diabetes. Nat Clin Pract Endocrinol Metab 2007;3: 46–56
3. Calles-Escandon J, Cipolla M. Diabetes and endothelial dysfunction: a clinical perspective. Endocr Rev 2001;22:36–52
4. Jay D, Hitomi H, Griendling KK. Oxidative stress and diabetic cardiovascular complications. Free Radic Biol Med 2006;40:183–192
5. Fukai T, Ushio-Fukai M. Superoxide dismutases: role in redox signaling, vascular function, and diseases. Antioxid Redox Signal 2011;15:1583–1606
6. Zelko IN, Mariani TJ, Folz RJ. Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. Free Radic Biol Med 2002;33:337–349
7. Fukai T, Folz RJ, Landmesser U, Harrison DG. Extracellular superoxide dismutase and cardiovascular disease. Cardiovasc Res 2002;55:239–249
8. Oury TD, Day BJ, Crapo JD. Extracellular superoxide dismutase: a regulator of nitric oxide bioavailability. Lab Invest 1996;75:617–636
9. Fattman CL, Schaefer LM, Oury TD. Extracellular superoxide dismutase in biology and medicine. Free Radic Biol Med 2003;35:236–256
10. Jung O, Marklund SL, Geiger H, Pedrazzini T, Busse R, Brandes RP. Extracellular superoxide dismutase is a major determinant of nitric oxide bioavailability: in vivo and ex vivo evidence from ecSOD-deficient mice. Circ Res 2003;93:622–629
11. Chu Y, Iida S, Lund DD, et al. Gene transfer of extracellular superoxide dismutase reduces arterial pressure in spontaneously hypertensive rats: role of heparin-binding domain. Circ Res 2003;92:461–468
12. Lund DD, Chu Y, Miller JD, Heistad DD. Protective effect of extracellular superoxide dismutase on endothelial function during aging. Am J Physiol Heart Circ Physiol 2009;296:H1920–H1925
13. Bivalacqua TJ, Usta MF, Kendirci M, et al. Superoxide anion production in the rat penis impairs erectile function in diabetes: influence of in vivo extracellular superoxide dismutase gene therapy. J Sex Med 2005;2:187–197; discussion 197–198
14. Adachi T, Ohta H, Hirano K, Hayashi K, Marklund SL. Non-enzymic glycation of human extracellular superoxide dismutase. Biochem J 1991;279: 263–267
15. Juul K, Tybjaerg-Hansen A, Marklund S, et al. Genetically reduced antioxidative protection and increased ischemic heart disease risk: the Copenhagen City Heart Study. Circulation 2004;109:59–65
16. Rae TD, Schmidt PJ, Pufahl RA, Culotta VC, O'Halloran TV. Undetectable intracellular free copper: the requirement of a copper chaperone for superoxide dismutase. Science 1999;284:805–808
17. Culotta VC, Yang M, O'Halloran TV. Activation of superoxide dismutases: putting the metal to the pedal. Biochim Biophys Acta 2006;1763:747–758
18. Kim BE, Nevitt T, Thiele DJ. Mechanisms for copper acquisition, distribution and regulation. Nat Chem Biol 2008;4:176–185
19. Mercer JF. The molecular basis of copper-transport diseases. Trends Mol Med 2001;7:64–69
20. Qin Z, Itoh S, Jeney V, Ushio-Fukai M, Fukai T. Essential role for the Menkes ATPase in activation of extracellular superoxide dismutase: implication for vascular oxidative stress. FASEB J 2006;20:334–336
21. Qin Z, Gongora MC, Ozumi K, et al. Role of Menkes ATPase in angiotensin II-induced hypertension: a key modulator for extracellular superoxide dismutase function. Hypertension 2008;52:945–951
22. Gongora MC, Qin Z, Laude K, et al. Role of extracellular superoxide dismutase in hypertension. Hypertension 2006;48:473–481
23. Ke BX, Llanos RM, Wright M, Deal Y, Mercer JF. Alteration of copper physiology in mice overexpressing the human Menkes protein ATP7A. Am J Physiol Regul Integr Comp Physiol 2006;290:R1460–R1467
24. Geraldes P, Hiraoka-Yamamoto J, Matsumoto M, et al. Activation of PKC-delta and SHP-1 by hyperglycemia causes vascular cell apoptosis and diabetic retinopathy. Nat Med 2009;15:1298–1306
25. Wang J, Takeuchi T, Tanaka S, et al. A mutation in the insulin 2 gene induces diabetes with severe pancreatic beta-cell dysfunction in the Mody mouse. J Clin Invest 1999;103:27–37
26. Ashino T, Sudhahar V, Urao N, et al. Unexpected role of the copper transporter ATP7A in PDGF-induced vascular smooth muscle cell migration. Circ Res 2010;107:787–799
27. Taniyama Y, Ushio-Fukai M, Hitomi H, et al. Role of p38 MAPK and MAPKAPK-2 in angiotensin II-induced Akt activation in vascular smooth muscle cells. Am J Physiol Cell Physiol 2004;287:C494–C499
28. Fukai T, Siegfried MR, Ushio-Fukai M, Griendling KK, Harrison DG. Modulation of extracellular superoxide dismutase expression by angiotensin II and hypertension. Circ Res 1999;85:23–28
29. Ozumi K, Sudhahar V, Kim HW, et al. Role of copper transport protein antioxidant 1 in angiotensin II-induced hypertension: a key regulator of extracellular superoxide dismutase. Hypertension 2012;60:476–486
30. Intengan HD, Schiffrin EL. Structure and mechanical properties of resistance arteries in hypertension: role of adhesion molecules and extracellular matrix determinants. Hypertension 2000;36:312–318
31. Pieper GM, Jordan M, Dondlinger LA, Adams MB, Roza AM. Peroxidative stress in diabetic blood vessels. Reversal by pancreatic islet transplantation. Diabetes 1995;44:884–889
32. Kakkar R, Mantha SV, Kalra J, Prasad K. Time course study of oxidative stress in aorta and heart of diabetic rat. Clin Sci (Lond) 1996;91: 441–448

33. Baluchnejadmojarad T, Roghani M. Chronic administration of genistein improves aortic reactivity of streptozotocin-diabetic rats: mode of action. Vascul Pharmacol 2008;49:1–5
34. Tagami S, Kondo T, Yoshida K, Hirokawa J, Ohtsuka Y, Kawakami Y. Effect of insulin on impaired antioxidant activities in aortic endothelial cells from diabetic rabbits. Metabolism 1992;41:1053–1058
35. Turkseven S, Kruger A, Mingone CJ, et al. Antioxidant mechanism of heme oxygenase-1 involves an increase in superoxide dismutase and catalase in experimental diabetes. Am J Physiol Heart Circ Physiol 2005;289:H701–H707
36. Kamata K, Kobayashi T. Changes in superoxide dismutase mRNA expression by streptozotocin-induced diabetes. Br J Pharmacol 1996;119:583–589
37. Hamed S, Brenner B, Aharon A, Daoud D, Roguin A. Nitric oxide and superoxide dismutase modulate endothelial progenitor cell function in type 2 diabetes mellitus. Cardiovasc Diabetol 2009;8:56
38. Adachi T, Ohta H, Hayashi K, Hirano K, Marklund SL. The site of non-enzymic glycation of human extracellular-superoxide dismutase in vitro. Free Radic Biol Med 1992;13:205–210
39. Ookawara T, Matsuura N, Oh-ishi T, et al. Serum extracellular superoxide dismutase in pediatric patients with various diseases as judged by an ELISA. Res Commun Mol Pathol Pharmacol 2000;107:291–296
40. Pisu P, Bellovino D, Gaetani S. Copper regulated synthesis, secretion and degradation of ceruloplasmin in a mouse immortalized hepatocytic cell line. Cell Mol Biol (Noisy-le-grand) 2005;(Suppl. 51):OL859–OL867
41. Jeney V, Itoh S, Wendt M, et al. Role of antioxidant-1 in extracellular superoxide dismutase function and expression. Circ Res 2005;96:723–729
42. White C, Kambe T, Fulcher YG, et al. Copper transport into the secretory pathway is regulated by oxygen in macrophages. J Cell Sci 2009;122:1315–1321
43. Hardman B, Michalczyk A, Greenough M, Camakaris J, Mercer JF, Ackland ML. Hormonal regulation of the Menkes and Wilson copper-transporting ATPases in human placental Jeg-3 cells. Biochem J 2007;402:241–250
44. Groop PH, Forsblom C, Thomas MC. Mechanisms of disease: pathway-selective insulin resistance and microvascular complications of diabetes. Nat Clin Pract Endocrinol Metab 2005;1:100–110
45. Kim JA, Montagnani M, Koh KK, Quon MJ. Reciprocal relationships between insulin resistance and endothelial dysfunction: molecular and pathophysiological mechanisms. Circulation 2006;113:1888–1904
46. La Fontaine S, Mercer JF. Trafficking of the copper-ATPases, ATP7A and ATP7B: role in copper homeostasis. Arch Biochem Biophys 2007;463:149–167
47. Failla ML, Kiser RA. Altered tissue content and cytosol distribution of trace metals in experimental diabetes. J Nutr 1981;111:1900–1909
48. Cooper GJ, Young AA, Gamble GD, et al. A copper(II)-selective chelator ameliorates left-ventricular hypertrophy in type 2 diabetic patients: a randomised placebo-controlled study. Diabetologia 2009;52:715–722
49. Cameron NE, Cotter MA. Neurovascular dysfunction in diabetic rats. Potential contribution of autoxidation and free radicals examined using transition metal chelating agents. J Clin Invest 1995;96:1159–1163
50. Gong D, Lu J, Chen X, et al. A copper(II)-selective chelator ameliorates diabetes-evoked renal fibrosis and albuminuria, and suppresses pathogenic TGF-beta activation in the kidneys of rats used as a model of diabetes. Diabetologia 2008;51:1741–1751