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

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ABSTRACT

Oxidative stress and endothelial dysfunction contribute to vascular complication in diabetes mellitus (DM). Extracellular superoxide dismutase (ecSOD, SOD3) is one of the key antioxidant enzymes which obtains copper via copper transporter ATP7A. SOD3 is secreted from vascular smooth muscles cells (VSMCs) and anchors at endothelial surface. Role of SOD3 and ATP7A in endothelial dysfunction in type 1 DM is entirely unknown. Here we show that the specific activity of SOD3, but not SOD1, is decreased, which is associated with increased O$_2^\cdot$ production in aortas of streptozotocin-induced and genetically-induced Ins2$^{Akita}$ type 1 DM mice. Exogenous copper partially rescued SOD3 activity in isolated DM vessels. Functionally, acetylcholine-induced endothelium-dependent relaxation is impaired in DM mesenteric arteries, which is rescued by SOD mimetic tempol or gene transfer of SOD3. Mechanistically, ATP7A expression in DM vessels is dramatically decreased while other copper transport proteins are not altered. DM-induced endothelial dysfunction and decrease of SOD3 activity are rescued in transgenic mice overexpressing ATP7A. Furthermore, SOD3 deficient DM mice or ATP7A mutant DM mice augment endothelial dysfunction and vascular O$_2^\cdot$ production vs. DM mice. These effects are in part due to hypoinsulinemia in type1-DM mice, since insulin treatment, but not high glucose, increases ATP7A expression in VSMCs and restores SOD3 activity in the organoid culture of DM vessels. In summary, decrease in ATP7A protein expression contributes to impaired SOD3 activity, resulting in O$_2^\cdot$ overproduction and endothelial dysfunction in blood vessels of type1 DM. Thus, restoring copper transporter function is an essential therapeutic approach for oxidant stress-dependent vascular and metabolic diseases.
INTRODUCTION

Endothelial dysfunction plays important roles in the development of vascular complications in diabetes mellitus (DM), which is the most common cause of morbidity and mortality and characterized by insulin deficiency or impaired insulin signaling (1-3). Although a role of oxidative stress in vascular dysfunction in DM 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 extracellular SOD (ecSOD, 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$_2^-$), and 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), aging (12), and restores erectile function in streptozotocin-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 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 role of endogenous SOD3 in endothelial dysfunction in DM.

SOD3 is a secretory copper enzyme, which 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,
while 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, role of copper transport proteins in vascular dysfunction in DM is entirely unknown.

We performed the present study to determine the role of SOD3 and copper transport proteins in modulating O$_2^\cdot$-mediated endothelial dysfunction in type I diabetic animals. Here we show that specific activity of SOD3, but not SOD1, is decreased in diabetic vessels, thereby increasing O$_2^\cdot$- production and impaired endothelium-dependent relaxation 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 type I DM (T1DM) mice in part due to the insulin deficiency, but not high glucose. Transgenic mice overexpressing ATP7A restore DM-induced impaired SOD3 activity and endothelial function by reducing O$_2^\cdot$- levels. The SOD3 deficient or ATP7A mutant DM mice further enhance endothelial dysfunction and vascular O$_2^\cdot$- production vs. DM mice. These findings provide new insights into the protective role of 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\textsuperscript{mut}) mice (21) backcrossed to the C57BL/6J background for 10 generations, SOD3\textsuperscript{t/t} 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, Maine). ATP7A\textsuperscript{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 the human ATP7A from the composite beta 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 streptozotocin (STZ, 100mg/kg body weight in 0.05M citrate buffer, pH 4.5, intraperitoneally) on two consecutive days after overnight fasting and injected control mice 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 to that with vehicle treatment (28.7±3.1 vs. 27.6±3.2; NS), while 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 about 20 days, and were sacrificed for experiments at day 30. All reagents were purchased from Sigma (St. Louis, MO), except when specified. For a genetic model of T1DM, we used Ins2\textsuperscript{Akita} diabetic mouse on C57BL/6J background (Jackson Laboratory) which is well-known animal model of T1DM with an autosomal dominant mutation in Ins2 gene (25).
Adenoviral vector and in vivo gene transfer. Adenovirus expressing human SOD3 (Ad.SOD3) and human SOD3 lacking heparin binding domain (Ad.SOD3-ΔHBD) were from adenovirus core at University of Iowa (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 euthanized and vascular tissue and plasma were collected for further experiments. Plasma SOD3 proteins were identified by zymography (11).

Superoxide dismutase 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 4.5g/l glucose as previously described (27). Before stimulation with insulin, cells were starved for 24 hours with serum free media.

Organoid Cultures of Diabetic Vessels: To examine 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). Briefly, after dissection of adventitial tissue, aortic segments were immersed in 6-well dishes that contained DMEM and antibiotics (penicillin 100U/mL, streptomycin 100mg/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 euthanized by CO₂ inhalation. Vascular O₂⁻ production was determined using lucigenin-enhanced chemiluminescence as described before (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 *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 while $\text{O}_2^{\bullet-}$ production is enhanced in aorta from diabetic mice. T1DM mice were created by STZ administration, as described in research design and methods. 30 days after the STZ injection, blood glucose was elevated to 552.5±25.15 mg/dl in diabetic mice versus 165.2±11.71 mg/dl in the C57BL/6J controls, while body weight was not significantly changed without insulin (28.7±3.1 vs. 27.6±3.2; NS) (Supplemental Fig. 1). Plasma insulin levels were significantly lowered in STZ-induced diabetes compared to controls.

We examined SOD activity and expression in aortas of STZ-induced diabetic mice. Figure 1A shows that SOD3 activity was significantly (P<0.05) decreased in diabetic mice, as compared to control mice, while protein levels of SOD3 were significantly increased (Fig. 1B). 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 vessel (i.e. increased “inactive” SOD3 protein), whereas that of SOD1 was not changed (Fig. 1C). Consistent results were observed in genetic type1 diabetic Ins2Akita mice (Supplemental Fig. 2A), indicating that decrease in vascular SOD3 specific activity in STZ-induced diabetic mice is not due to toxic effect of STZ, but due to diabetic condition. Of note, decrease in SOD3 specific activity in aorta from DM mice was associated with a marked increase in $\text{O}_2^{\bullet-}$ production (Fig. 1D) and nitrotyrosine staining, an indicator of peroxynitrite ($\text{ONOO}^-$) (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 vessel is due to deficiency of copper. Figure 1E shows that copper addition restored the decreased specific activity of SOD3 purified from diabetic vessel. 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, while SOD3 enzyme from control vessels or SOD1 enzyme from either diabetic or control vessels are fully metallated.

**Endothelium-dependent relaxation is impaired in mesenteric resistant arteries from diabetic mice, which is rescued by SOD mimetic tempol or gene transfer of SOD3.** Because increased $O_2^{-}$ 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-induced endothelium-dependent vasorelaxation was significantly impaired in resistance arteries of diabetic mice compared to control mice (Maximum relaxation 68±2% versus 92±2%, respectively, P<0.001), which was rescued by SOD mimetic, tempol (Fig. 2A). Of note, SNP-induced endothelium-independent vasorelaxation was not different between diabetic and control mice. Similar response was observed in mouse aorta from diabetic mice (Supplemental Fig.3). These results suggest that impaired endothelium-dependent vasorelaxation in diabetic mice is largely due to an increased $O_2^{-}$ 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) and SOD3 lacking HBD (Ad-SOD3∆HBD) on endothelial dysfunction in diabetic mice. Ad-SOD3 or Ad-SOD3∆HBD were injected to diabetic mice intravenously, and enzymatic activity of SOD3 or SOD3∆HBD in plasma was confirmed by in-gel zymography (Fig. 2C), as reported (11). Interestingly, a more intense band was detected for Ad-SOD3∆HBD than for Ad-SOD3, because SOD3 binds to vascular tissues, while SOD3-∆HBD circulates without binding to vascular tissues. Figure 2B shows that ACh-induced endothelium-dependent vasorelaxation was significantly improved after gene transfer of SOD3, but not SOD3∆HBD, in diabetic mice (Maximal relaxation 69±3% versus 57.4±2.4%,
respectively). Further, SNP-induced relaxation in diabetic mesenteric arteries was not altered by gene transfer of either SOD3 or SOD3ΔHBD. These results suggest that increased O$_2^-$ 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 to 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), were not changed in diabetic vessels. Similar response was also observed in mesenteric arteries from diabetic mice (Supplemental Fig. 4). Of note, Ins2$^{Akita}$ mice, a genetic model of T1DM, exhibited similar results to STZ-induced diabetic mice (Supplemental Fig. 2B), indicating that decrease in ATP7A expression in STZ-induced diabetic mice is not due to toxic effect of STZ, but due to diabetic condition. Thus, diabetic mice show a significant decrease in 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 vessel, 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 transGolgi network, and then SOD3 is secreted to the extracellular space (5). Figure 4A shows that insulin treatment (10 nM) for 12 hrs significantly increased ATP7A protein expression (P<0.01), but not another copper transport protein Atox1 in VSMCs, while high glucose had no effects (data not shown). Insulin treatment also rescued the DM-induced decrease in SOD activity (Fig. 4B) and ATP7A protein expression (Supplemental 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$_2^-$ 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 four fold in ATP7A transgenic mice compared to wild type (WT) mice (Fig. 5A). Figure 5C and 5D show that decrease in SOD3 activity and specific activity in diabetic WT mice aorta were significantly improved in diabetic ATP7A transgenic mice. In parallel, diabetes-induced enhanced O$_2^-$ production was significantly decreased in diabetic ATP7A transgenic mice than WT mice (Fig. 5B). ACh-induced endothelium-dependent relaxation was significantly improved in resistance arteries of diabetic ATP7A transgenic mice compared to diabetic WT mice (Maximal relaxation 74.8±3.9% versus 60.1±3% respectively, Fig. 6A), while endothelium-independent relaxation to SNP was not changed between two groups (Fig. 6B). These findings suggest that decreased ATP7A expression in diabetic vessels contributes to decreased SOD3 activity, thereby enhancing O$_2^-$ production and endothelial dysfunction.

**Diabetic SOD3 deficient and ATP7A$^{mut}$ mice enhance endothelial dysfunction and O$_2^-$ 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 to diabetic WT mice, which was rescued by addition of SOD mimetic tempol (Fig. 7A). In contrast, endothelium-independent relaxation to SNP was not changed between the two groups (Fig. 7A). Vascular O$_2^-$ production in DM was significantly increased in SOD3$^{-/-}$ and ATP7A$^{mut}$ mice to a greater extent than in
diabetic WT mice (Fig. 7B). These findings further suggest that endogenous ATP7A and SOD3 plays an important role in protecting endothelial function by regulating vascular $O_2^{*}$.

**DISCUSSION**

The present study demonstrates a protective role for the Cu transporter ATP7A in DM induced endothelial dysfunction by regulating SOD3 activity and vascular $O_2^{*}$ levels (Fig. 8). We found that: (1) Specific activity of SOD3, but not SOD1, is significantly decreased in diabetic vessels which is associated with increased $O_2^{*}$ production compared to 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 DM, which is rescued by SOD mimetic tempol or gene transfer of SOD3; (4) Mechanistically, ATP7A expression in DM aorta is dramatically decreased while other copper transport proteins (Atox1, CCS, 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 DM vessels; (6) Transgenic mice overexpressing ATP7A exhibits restored DM-induced impaired endothelial function, SOD3 activity and increased vascular $O_2^{*}$ production; (7) The SOD3$^{-/-}$ DM mice or ATP7A$^{mut}$ DM mice show augmented endothelial dysfunction and increased $O_2^{*}$ production vs. DM mice. Thus, restoring ATP7A-SOD3 function is 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 DM has not been investigated. In the present study, we provide compelling evidence that the specific activity of SOD3 is markedly decreased, whilst SOD3 protein expression is increased in aortas from STZ-induced diabetic mice, whereas
activity or expression of SOD1 is not altered. This result is consistently observed in the genetic model of Ins2Akita+/− T1DM mice. Our findings are divergent to 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 to non-diabetic controls (9; 35), which is likely due to the reduction of heparin affinity of SOD3 via non-enzymatic glycation without changing the enzymatic activity (14; 38). Given that pediatric patients show a significant decrease in plasma SOD3 compared to controls (39), it is conceivable that tissue-bound SOD3 levels in diabetes may be regulated by aging. Indeed, the present study has used young mice (3-4 month of age) to demonstrate that tissue SOD3 level is increased in DM 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 level do not reflect its activity and that T1DM mice exhibit decreased SOD3 activity without altering SOD1 activity, which may contribute to increased vascular O$_2^{-}$ 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 DM mice, which is rescued by gene transfer of SOD3 as well as SOD mimetic tempol. By contrast, endothelium-independent vasorelaxation is not affected in diabetic mice, suggesting that inhibition of ACh-induced vasodilation in DM 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 other different vascular beds (13). In the present study, we also found that SOD3 deficient DM mice show augmented impaired endothelium-dependent vasodilation and O$_2^{-}$ production vs. DM mice, supporting the protective role of
endogenous SOD3 in endothelial function. Our current study also shows that gene transfer of SOD3 lacking HBD does not rescue impaired endothelium-dependent relaxation in diabetic vessels. This result strongly supports the notion that binding of SOD3 to endothelial surface and extracellular matrix of vascular tissue via HBD is essential for protecting against inactivation of endothelium-derived NO by $\text{O}_2^{-}$ 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 $\text{O}_2^{-}$ in the diabetic vessel wall.

Mechanistically, we found that the decreased specific activity of SOD3 purified from DM 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), while SOD1 gets copper through copper chaperone CCS (17). In the present study, we provide the first evidence that ATP7A protein expression, but not Atox1 or 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. $\text{Ins}^2\text{Akita}$) T1DM mouse. Thus, these findings may explain why the activity of SOD3, but not SOD1, is decreased in DM 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 vessel, 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 Atox1 level, while high glucose alone has no effects. Consistent with this, Hardman et al. reported that insulin regulates ATP7A expression in human placental Jeg-3 cells (43). Furthermore, we found that insulin treatment directly restores the DM-induced decrease in SOD3 activity and ATP7A protein expression without affecting SOD1 activity in the organoid culture of DM vessels, which can exclude the possibility of
neurohormonal effect of insulin. Thus, these results indicate that hypoinsulinemia in T1DM may contribute to the decrease in ATP7A protein expression in VSMCs, and thus reducing SOD3 activity in the blood vessels. Our preliminary study found that insulin increases ATP7A protein stability in a PI3kinase/Akt-dependent manner. Given that insulin-PI3kinase/Akt pathway has been shown to be impaired in vascular tissue from type2 DM mice (44; 45), it is tempting to speculate that decrease in insulin-PI3kinase/Akt pathway in type1 and type2 DM may contributes to downregulation of ATP7A expression in VSMCs. This may result in decrease in SOD3 activity and subsequent overproduction of O$_2^{-}$, thereby inducing endothelial dysfunction. This issue should be investigated more in detail in future study.

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 DM-induced endothelial dysfunction and decrease of SOD3 activity are rescued in transgenic mice overexpressing ATP7A, while SOD3 deficient DM mice or ATP7A$^{mut}$ DM mice accelerate endothelial dysfunction and vascular O$_2^{-}$ production compared to DM 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 in not only providing copper to the 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 speculation 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 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 present study provides direct evidence for the protective role of the ATP7A-SOD3 pathway in endothelial function by reducing extracellular O$_2^-$ levels and increasing bioavailability of NO in diabetes mellitus (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 diabetes mellitus.

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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.M. developed the ATP7A- transgenic mice, MU-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 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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FIGURE LEGENDS

Figure 1. Activity, protein level, and specific activity of SOD3 and SOD1 as well as vascular O$_2^*$ level in aortas of type 1 diabetic 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 (lower) was determined by the ratio of activity to relative amount of protein as previously described (20). (D) Aortic O$_2^*$ production in control and diabetic mice were measured by lucigenin-enhanced chemiluminescence (5 μM) method. (E) Con A-Sepharose-bound SOD3 or -unbound SOD1 proteins were treated with or without CuCl$_2$ (10 μM, 1 hr at room temperature), and then specific activity of SOD3 and SOD1 were measured, as described above. Results are presented as mean ± SEM (n=4). ***P<0.001, *P<0.05 vs control. NS, not significant.

Figure 2. Effect of SOD mimetic Tempol or adenovirus-mediated gene transfer of SOD3 on relaxation to acetylcholine (ACh) and sodium nitroprusside (SNP) in mesenteric resistance arteries from type 1 diabetic 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 μM) in the presence and absence of cell permeable SOD mimetic, tempol (1mM)(A) or three days after injection of adeno-SOD3 or adeno-SOD3ΔHBD (1 x 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.

**Figure 3.** Protein expression of ATP7A, but not other copper trafficking regulators, is decreased in aorta of type 1 diabetic 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 respective protein. (B) Densitometric analysis was shown. Results are presented as mean±SEM (n=4). ***P<0.001 vs control.

**Figure 4.** Insulin restores reduced SOD3 specific activity in organoid culture from diabetic mouse aorta by increasing ATP7A protein expression in VSMCs. (A) Vascular smooth muscle cells (VSMCs) were treated with insulin (10 nM) for 12 hours 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 nM) for 24 hrs in organoid culture. Activity of SOD3 and SOD1 in homogenate was assayed as Figure 1. Results are presented as mean±SEM (n=3). ***P<0.001, *P<0.05 vs control.

**Figure 5.** Activity and specific activity of SOD3 and SOD1 as well as vascular O$_2^•$ level in aorta of diabetic transgenic mice overexpressing ATP7A. Protein level of ATP7A (A) and O$_2^•$-production (B) in aortas from wild type (WT) mice or transgenic mice overexpressing ATP7A with or without STZ injection (DM) were measured by western analysis and lucigenin-enhanced chemiluminescence assay, respectively. (C and D) Activity (C) and specific activity (D) of SOD3 and SOD1 in tissue homogenate were assayed as described in Figure 1. Results are presented as mean ± SEM (n=4). ***P<0.001, *P<0.05 vs control.
**Figure 6.** Endothelium-dependent or –independent relaxation of mesenteric resistance arteries from diabetic transgenic mice overexpressing ATP7A. Isometric tension of mouse mesenteric resistance arteries from WT mice or transgenic mice overexpressing ATP7A with or without STZ injection (DM) was measured as Figure 2. Vasodilation was evoked by (A) acetylcholine (ACh) and (B) sodium nitroprusside (SNP) after preconstriction with phenylephrine (1- 5 µM). Results are presented as mean ± SEM (n=6-8). *P<0.05 vs control.

**Figure 7.** Vascular function and O$_2$•- 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 were measured in isolated organ chambers as Figure 2. (A) Vasodilation was evoked by acetylcholine (ACh) in the presence and absence of SOD mimetic, tempol (1mM) or sodium nitroprusside (SNP) after preconstriction with phenylephrine (1- 5 µM). (B) Vascular O$_2$•- 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<0.05 vs control.

**Figure 8.** Proposed model for the protective role of ATP7A-SOD3 pathway in type 1 diabetes-induced endothelial dysfunction. ATP7A delivers copper and activates SOD3 protein which scavenges O$_2$•- in the extracellular space, thereby increasing bioavailability of nitric oxide (NO) and preserve endothelial function. STZ-induced diabetes decreases ATP7A protein levels primarily due to the hypoinsulinemia, thereby decreasing SOD3 activity leading to overproduction of O$_2$•- and endothelial dysfunction.
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Online Supplemental Materials

**Supplemental Figure 1.** Effect of Streptozotocin (STZ) on body weight, blood glucose and plasma insulin levels in mice. Blood glucose was measured using One Touch Ultra meter (B). Insulin (C) was measured according to manufacturer instructions using Ultra-Sensitive Mouse Insulin ELISA kit (Crystal Chem Inc, Downers Grove, IL). Results are presented as mean ± SEM (n=6). ***P<0.001 vs control.

**Supplemental Figure 2.** Activity of SOD3 and SOD1 (A) and protein levels of SODs and copper transport proteins (B) in vascular tissue of Akita mice. (A) Activity (top) and specific activity (bottom) of SOD3 and SOD1 in tissue homogenate were assayed as Figure 1. (B) Protein levels of SOD and copper transport proteins ATP7A and Atox1 were determined by western analysis. Results are presented as mean ± SEM (n=3). ***P<0.001, *P<0.05 vs control.

**Supplemental Figure 3.** Acetylcholine (ACh) and sodium nitroprusside (SNP) induced vasorelaxation in aortic tissue from type 1 diabetic mice. Isometric tension of aortic ring 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 µM). Results are presented as mean ± SEM (n=4-6). *P<0.05 vs control.

**Supplemental Figure 4.** Protein levels of SOD3, SOD1, and copper transport proteins ATP7A and Atox1 in mesenteric resistance arteries determined by western analysis. Representative blot from n=3.

**Supplemental Figure 5.** Insulin restores reduced ATP7A expression in organoid culture from diabetic mouse aorta. Isolated STZ-injected DM or control mice aorta was exposed to insulin
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