HHS Public Access

Author manuscript

Arterioscler Thromb Vasc Biol. Author manuscript; available in PMC 2019 March 01.

Published in final edited form as:

Arterioscler Thromb Vasc Biol. 2018 March ; 38(3): 529–541. doi:10.1161/ATVBAHA.117.309819.

Akt2 Stabilizes ATP7A, a Cu transporter for SOD3, in Vascular Smooth Muscles: Novel Mechanism to limit Endothelial Dysfunction in Type2 diabetes

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Abstract

Objective—Copper (Cu) transporter ATP7A is required for full activation of extracellular SOD (SOD3), which is secreted from vascular smooth muscle cells (VSMCs) and anchors to endothelial cell surface to preserve endothelial function by scavenging extracellular superoxide. We reported

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Authors' contributions

V.S. designed the study, performed experiments, analyzed data, and wrote the manuscript. N.O. and J.P.O. performed ubiquitination assay. A.M. provided some T2DM sample. S.A.P., Z.B. and V.P. provided human biopsy sample. N.H. provided Akt1 and Akt2 KO mice. D.S. provided db/db/PTP1B−/− 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.

Competing financial interests

The authors declare no competing financial interests.

that ATP7A protein expression and SOD3 activity are decreased in insulin-deficient type1 diabetes mellitus (T1DM) vessels, thereby inducing superoxide-mediated endothelial dysfunction, which are rescued by insulin treatment. However, it is unknown regarding the mechanism by which insulin increases ATP7A expression in VSMCs and whether ATP7A downregulation is observed in T2DM mice and human in which insulin-Akt pathway is selectively impaired.

Approach and Results—Here we show that ATP7A protein is markedly downregulated in vessels isolated from T2DM patients as well as those from high fat diet-induced or db/db T2DM mice. Akt2 activated by insulin promotes ATP7A stabilization via preventing ubiquitination/degradation as well as translocation to plasma membrane in VSMCs, which contributes to activation of SOD3 that protects against T2DM-inudced endothelial dysfunction. Downregulation of ATP7A in T2DM vessels is restored by constitutive active Akt or PTP1B$^{-/-}$ T2DM mice which enhance insulin-Akt signaling. Immunoprecipitation, in vitro kinase assay and mass spectrometry analysis reveal that insulin stimulates Akt2 binding to ATP7A to induce phosphorylation at Ser1424/1463/1466. Furthermore, SOD3 activity is reduced in Akt2$^{-/-}$ vessels or VSMCs, which is rescued by ATP7A overexpression.

Conclusion—Akt2 plays a critical role in ATP7A protein stabilization and translocation to plasma membrane in VSMCs, which contributes to full activation of vascular SOD3 that protects against endothelial dysfunction in T2DM.

Keywords

Type 2 DM; endothelial dysfunction; ATP7A; Akt2; vascular smooth muscle cells

Introduction

Oxidative stress contributes to diabetes mellitus (DM)-induced endothelial dysfunction, which is one of the most common causes of cardiovascular morbidity and mortality[1, 2]. The major cellular defense against superoxide ($O_2^{\bullet-}$) is superoxide dismutases (SODs) which consists of the cytoplasmic Cu (copper)/Zn SOD (SOD1), the mitochondrial MnSOD (SOD2), and the extracellular SOD (SOD3). SOD3 is highly expressed in the vasculature, which is mainly secreted from vascular smooth muscle cells (VSMCs)[3], and is anchored to endothelial cell surface through binding to the heparin sulfate proteoglycan, collagen and fibrin-5[4]. By contrast, SOD3 in endothelial cells is epigenetically silenced[3–6]. Of note, the R213G polymorphism in the SOD3 gene, which reduces binding to the endothelial surface and increases serum SOD3 levels, is correlated with cardiovascular risk[7], while plasma SOD3 levels are altered in diabetic patients[8, 9]. Studies using SOD3$^{-/-}$ mice showed that vascular SOD3 plays an essential role in preserving endothelial function by scavenging extracellular $O_2^{\bullet-}$ and increasing NO bioavailability in hypertension[10, 11], and aging[12].

Cu-transporting/exporting ATPase (ATP7A) is essential not only for activation of secretory Cu enzymes such as SOD3, but also for exporting excess Cu to regulate intracellular bioavailable Cu[4, 13]. To achieve this, ATP7A translocates from the trans-Golgi network (TGN) to the plasma membrane or cytoplasmic vesicle, thereby exporting Cu or delivering Cu to the secretory Cu enzymes[13]. ATP7A trafficking is regulated by post-translational modification processes such as glycosylation[14] and phosphorylation[15]. We demonstrated

that ATP7A is involved in platelet-derived growth factor-stimulated VSMC migration via regulating lysyl oxidase (LOX) activity and Rac1[16]. We also reported that ATP7A protein expression is decreased in vessels of insulin-deficient type1 diabetes mellitus (T1DM) mice, thereby reducing SOD3 activity and inducing excess $O_2^{\bullet-}$-mediated endothelial dysfunction, which are rescued by insulin treatment. However, it is unknown regarding the molecular mechanism by which insulin increases ATP7A expression in VSMCs as well as whether ATP7A protein is downregulated in insulin-resistant T2DM mice and patients.

Insulin resistance in T2DM has been characterized by selective impairment of Akt dependent pathways without altering ERK pathways[17–19]. Vascular tissues express two Akt isoforms (Akt1 and Akt2) with below limit of detection of Akt3 as shown in previous reports[20]. Importantly, role of Akt1 and Akt2 in insulin resistance and blood vessel function seems to be different. Akt2$^{-/-}$ mice (in particular, male) have phenotypes such as insulin resistance and a DM-like syndrome[21], while insulin-mediated Akt and eNOS phosphorylation are intact in Akt1$^{-/-}$ mice[22]. Although impaired Akt-eNOS pathway in endothelial cells from T2DM has been reported[17–19], the role of Akt in VSMCs for T2DM-induced endothelial dysfunction and its relationship with SOD3 remain unclear. In the present study, we show that ATP7A protein is markedly downregulated in microvessels isolated from adipose tissues of T2DM patients as well as mesenteric arteries and aorta of T2DM (high fat diet-induced, and db/db) mice. Using various genetically approach (gene delivery of SOD3 as well as SOD3$^{-/-}$, Akt1$^{-/-}$, Akt2$^{-/-}$, and ATP7A transgenic (ATP7A-Tg) mice), we provide the novel evidence that Akt2 phosphorylates ATP7A and plays an important role in insulin-induced ATP7A protein stabilization and plasma membrane translocation in VSMCs, which contributes to full expression of SOD3 activity. Thus, restoring the Akt2-ATP7A-SOD3 axis is potential therapeutic strategy for treatment of diabetes vascular complications.

Materials and Methods

Materials and methods are available in the online-only Data Supplement

Results

Selectively impaired Akt signaling contributes to decrease in ATP7A expression in T2DM vessels

To determine the role of ATP7A-SOD3 axis in T2DM mice with compensatory hyperinsulinemia and selectively impaired insulin-Akt pathway, we used T2DM mice that were induced by a high-fat diet feeding (HFD) combined with a single injection of low dose streptozotocin (STZ). This is a well-established animal model of T2DM which show insulin resistance and compensatory hyperinsulinemia[23–25]. Combination of low dose STZ with HFD helps to develop reproducible and advanced degree of type 2 diabetic strain[23–25]. After 4 months of HFD, body weight, plasma levels of triglycerides, total cholesterol, HDL and LDL were significantly higher in T2DM than control mice (Table I in the online-only Data Supplement). T2DM mice exhibited a significant increase in the epididymal, retroperitoneal, mesenteric fat, and brown adipose tissue fat pads over control mice (Figure I in the online-only Data Supplement). Plasma insulin level was significantly higher in T2DM than control

mice (Figure 1A), which was associated with a significant decrease in insulin sensitivity (Figure 1B). We also confirmed that insulin resistance assessed by HOMA-IR (calculated by using the fasted blood glucose and insulin levels)[26] was significantly increased in T2DM mice (Table I in the online-only Data Supplement) which show impaired insulin-induced vasorelaxation and reduced NO_2^-/NO_3^- level in vascular tissue of T2DM mice compared to control mice (Figure IG and IH in the online-only Data Supplement). These findings indicate that we successfully generated T2DM mice.

Insulin resistance in T2DM has been characterized by selective impairment of Akt pathways without altering other branches, including MAP kinase pathways[17–19]. Consistent with this, phosphorylation of Akt, but not that of ERK, was significantly decreased in aortas of T2DM mice (Figure 1C). In these T2DM vessels, protein expression of ATP7A, but not other Cu transport proteins Atox1, CCS and COX17, was markedly decreased (Figure 1D). Similar results were also obtained in mesenteric arteries and aortas from genetically-induced T2DM db/db mice (Figure IIA and IIB in the online-only Data Supplement). Of note, ATP7A protein expression was also significantly downregulated in microvessels from adipose tissue of diabetic patients (Figure 1E). Furthermore, decreased ATP7A expression in aortas and cultured VSMCs from T2DM mice was rescued in T2DM mice crossed with $PTP1B^{-/-}$ mice which enhances insulin-Akt signaling (Figure 1F) or by overexpression of constitutive-active Akt (Figure 1G). These results suggest that impaired Akt signaling contributes to decrease in ATP7A expression in T2DM vessels or VSMCs.

Cu transporter ATP7A expression is decreased in T2DM vessels, which results in decreased SOD3 activity and endothelial dysfunction

Figure 2A shows that SOD3 activity was decreased while SOD3 protein level was increased in the aorta of T2DM mice compared to control mice. By contrast, SOD1 activity and protein were not changed. Thus, the specific activity of SOD3, as determined by the ratio of activity to protein, was markedly decreased in T2DM vessels. These results suggest that decrease in ATP7A protein contributes to increase in inactive SOD3 protein (Figure 2A). In addition, decrease in SOD3 activity in T2DM vessels was associated with a marked increase in $O_2^{\bullet-}$ production (Figure 2B) and impaired acetylcholine (ACh)-induced endothelium-dependent vasorelaxation (EDR) (Figure 2D, 2E, 2G and 2H). Importantly, endothelial dysfunction in T2DM mice was rescued by SOD mimetic Tempol (Figure 2D), or adenoviral gene transfer of SOD3 (Figure 2H), and further enhanced in $SOD3^{-/-}$ T2DM mice (Figure 2G). Furthermore, decreased SOD3 total or specific activity as well as impaired EDR in T2DM mice were significantly restored in diabetic ATP7A-Tg mice (Figure 2C and 2E). We confirmed overexpression of ATP7A in diabetic ATP7A-Tg mice (Figure III in the online-only Data Supplement). Of note, sodium nitroprusside (SNP)-induced endothelium-independent vasorelaxation was not different between T2DM vessels and control groups (Figure 2F and Figure IVA and IVB in the online-only Data Supplement). Given that decreased SOD3 activity results in $O_2^{\bullet-}$ production in vessels[10, 11, 27, 28], these results suggest that decreased ATP7A expression in T2DM vessels resulted in decreased SOD3 activity, thereby promoting $O_2^{\bullet-}$ production and endothelial dysfunction.

Insulin increases ATP7A protein stability by inhibiting ubiquitination and degradation in a PI3K/Akt-dependent manner in VSMCs

We then examined the mechanism by which insulin increases ATP7A protein expression and found that insulin stimulation in VSMC increased ATP7A protein expression without affecting ATP7A mRNA in a dose- and time-dependent manner (Figure V in the online-only Data Supplement). Furthermore, insulin-induced ATP7A protein expression was blocked by specific PI3K or Akt inhibitors, or dominant negative Akt, but not by MEK or JNK inhibitors (Figure 3A, Figure VI in the online-only Data Supplement). These results suggest that Akt mediates insulin-induced ATP7A expression in VSMCs and that decreased ATP7A protein expression in T2DM vessels is partly due to the impaired insulin-Akt pathway. We next examined whether insulin regulates ATP7A protein stability and found that the half-life of ATP7A was significantly prolonged by insulin in the presence of protein synthesis inhibitor cycloheximide (Figure 3B). Proteasome inhibitor MG132 prevented the PI3K inhibitor-induced attenuation of insulin-induced increase in ATP7A protein expression (Figure 3C). Furthermore, insulin-induced attenuation of ATP7A ubiquitination was reversed by the PI3K or Akt inhibitors (Figure 3D). These results suggest that insulin stimulation increases ATP7A protein stability by inhibiting ATP7A protein degradation/ubiquitination in an Akt-dependent manner.

Insulin promotes ATP7A translocation to the plasma membrane where SOD3 colocalizes with ATP7A in an Akt- and Cu-dependent manner in VSMCs

We next examined effects of insulin stimulation on subcellular localization of ATP7A in VSMCs and found that insulin promoted ATP7A translocation from the TGN to the plasma membrane within 30 min with a peak at 2 hrs in VSMCs (Figure 4A). Insulin-induced ATP7A translocation was inhibited by dominant negative-Akt or cell permeable Cu chelator TTM, but not by cell impermeable Cu chelator BCS (Figure 4B, 4C, and 4D). Immunofluorescence and co-immunoprecipitation analysis revealed that insulin increased ATP7A binding to SOD3 at the plasma membrane in VSMCs (Figure 4E). Furthermore, insulin-induced ATP7A translocation was associated with a decrease in intracellular Cu contents, which might be at least due to increased Cu export or increased Cu-bound SOD3 section (Figure VII in the online-only Data Supplement). These results suggest that Akt is also involved in insulin-stimulated ATP7A translocation from TGN to the plasma membrane where SOD3 might obtain catalytic cofactor Cu from ATP7A.

Insulin promotes Akt2-dependent phosphorylation of ATP7 in VSMCs

Since Akt-dependent phosphorylation is shown to stabilize Akt substrates by decreasing ubiquitination[29], we next examined if Akt directly phosphorylates ATP7A. Figure 5A shows that insulin stimulation increased phosphorylation of ATP7A, which was inhibited by Akt or PI3K inhibitors in VSMCs. Furthermore, insulin significantly increased binding of p-Akt to ATP7A within 5 min, which continued at least for 2 hrs (Figure 5B). Furthermore, insulin significantly increased binding of Akt2, but not Akt1, to ATP7A in VSMCs (Figure 5C), suggesting that insulin induces Akt2-dependent phosphorylation of ATP7A in VSMCs.

Akt directly phosphorylates ATP7A in vitro

To identify the Akt phosphorylation sites in ATP7A, we performed an in vitro kinase assay using recombinant active Akt and the GST-tagged various ATP7A protein fragments [N-terminal (N), Middle (M), C-terminal (C)], which are based on in silico analysis of the amino acid sequence and Cu sensitive phosphorylation sites in ATP7A (Figure 5D)[15]. We found that Akt phosphorylated ATP7A at C-terminus, but not at N- or middle-terminus (Figure 5E). Mass spectrometry analysis identified three Akt-dependent serine phosphorylation sites in C-terminus ATP7A: S1424, S1463 and S1466 with ascore value higher than 18, which was confidently assigned (Figure 5F, Figure VIII in the online-only Data Supplement). There was significant reduction in Akt-induced phosphorylation of ATP7A mutants (S1424A, S1463A, S1466A) compared with WT-ATP7A-C (data not shown). Furthermore, alignment of the vertebrate ATP7A C-terminal region shows complete conservation of these three phosphorylation sites (Figure VIIIC in the online-only Data Supplement). Thus, these results indicate that insulin increased Akt binding to ATP7A to induce phosphorylation at S1424/S1463/S1466.

Akt2 is involved in ATP7A protein expression and ATP7A translocation to membrane in VSMCs, which contributes to SOD3 activity

Since it is shown that role of Akt1 and Akt2 in insulin resistance and blood vessel function is different, we next examined the role of Akt isoform in VSMCs for endothelial dysfunction and its relationship with SOD3. Consistent with our results that Akt2 induces phosphorylation of ATP7A, insulin-induced ATP7A protein expression in VSMCs was inhibited by Akt2 siRNA, but not Akt1 siRNA (Figure 6A). We then examined the relationship among ATP7A, Akt2, and SOD3 activity and found that insulin-induced specific activity of SOD3 secreted in VSMCs culture medium was decreased in Akt2-depleted VSMCs, which was restored in ATP7A-Tg VSMCs (Figure IX in the online-only Data Supplement). These results suggest that Akt2-mediated ATP7A protein expression contributes to full activation of SOD3 in VSMCs. Next, we examined the role of Akt isoforms in ATP7A translocation and found that insulin-induced ATP7A translocation to the plasma membrane was markedly inhibited by Akt2 siRNA-treated VSMCs or in $Akt2^{-/-}$ VSMCs, but not by either Akt1 siRNA-treated VSMCs or $Akt1^{-/-}$ VSMCs (Figure 6B and Figure X in the online-only Data Supplement). These results suggest that Akt2 is also involved in insulin-induced ATP7A translocation to the membrane in addition to ATP7A protein expression. To examine in vivo role of Akt2 for the ATP7A-mediated SOD3 activity, we used female $Akt2^{-/-}$ mice which were neither insulin-resistant nor diabetic, in contrast to male $Akt2^{-/-}$ mice which have T2DM phenotype[21]. We found that ATP7A protein expression, but not ATP7A mRNA, was significantly decreased in aorta from female $Akt2^{-/-}$ mice, but not $Akt1^{-/-}$ mice (Figure 6C, Figure XI in the online-only Data Supplement). We confirmed these findings using mesenteric arteries (data not shown). Figure 6D showed that the activity of SOD3, but not that of SOD1, was significantly decreased in female $Akt2^{-/-}$ mice compared to WT mice. By contrast, protein expression of SOD3 and SOD1 was not changed. Thus, the specific activity of SOD3, but not SOD1, was markedly decreased in vessels of female $Akt2^{-/-}$ mice (Figure 6D). We also confirmed these findings in male $Akt2^{-/-}$ mice (Figure XII in the online-only Data Supplement). Thus, these results suggest

that Akt2-mediated ATP7A protein stabilization contributes to full activation of SOD3 in blood vessels.

DISCUSSION

In this study, we provide the novel evidence that: 1) ATP7A protein is markedly downregulated in microvessels isolated from adipose tissues of T2DM patients as well as mesenteric arteries and aorta of T2DM (high fat diet-induced, and db/db) mice; 2) insulin-induced Akt2 activation, which is selectively impaired in T2DM, is required for ATP7A stabilization via preventing ubiquitination/degradation as well as ATP7A trafficking to plasma membrane in VSMCs, which induces full activation of SOD3 that protects against endothelial dysfunction in T2DM; 3) downregulation of ATP7A in vessels or VSMCs from T2DM is rescued by constitutive active Akt or PTP1B$^{-/-}$ T2DM mice which enhance insulin-Akt signaling; 4) Akt2 activated by insulin binds to ATP7A to induce phosphorylation of C-terminus at Ser1424/1463/1466. Thus, these new findings provide novel mechanistic insights into how SOD3 activity is decreased in T2DM blood vessels via impaired Akt2-ATP7A pathway, which contributes to endothelial dysfunction.

The functional significance of impaired ATP7A-SOD3 axis in endothelial dysfunction in T2DM is demonstrated by the results that the reduced ACh-induced endothelium dependent vasorelaxation (EDR) in T2DM vessels was rescued by SOD mimetic tempol or gene transfer of SOD3, while it was enhanced in SOD3$^{-/-}$ mice. In contrast, sodium nitroprusside (SNP)-induced endothelium-independent vasorelaxation was not different between T2DM vessels and control groups. EDR of mesenteric arteries in mice depends on not only NO but also endothelium-dependent hyperpolarizing factor including H_2O_2[30] which is resistant to NOS inhibitor L-NAME but sensitive to calcium-activated K^+ channel inhibitors (both Apamin (small conductance calcium-activated K-channel inhibitor) and Charybdotoxin (intermediate and large conductance calcium-activated K-channel inhibitor)[30]. Relative contribution of NO- and EDH-mediated component to EDR is inversely proportional to mesenteric arterial diameter[31]. In the current study, we used 1st order (~230 μm internal diameter) mesenteric arteries showing that L-NAME markedly inhibits ACh-induced EDR. Indeed, we verified that NO dependent component is predominant in the 1st order mesenteric arteries compared to the 2nd order ones, while EDH component is larger in the 2nd order mesenteric arteries compared to the 1st order ones (Data not shown), confirming our methodology to measure EDR. Of note, Stepp and colleagues reported that NO component, but not EDH component, is impaired in the 2nd order mesenteric arteries from T2DM db/db mice, which is rescued by SOD mimetic[32–34]. Taken together, our study suggests that reduced SOD3 activity is associated with increased $O_2^{\bullet-}$ production, which results in reduced NO bioavailability and impaired NO-dependent vasorelaxation in the T2DM mice. The potential sources of $O_2^{\bullet-}$ in vessels from T2DM will include mitochondrial electron transport chain and NADPH oxidases[24, 35]. Thus, whether disrupted balance between the antioxidant systems through the ATP7A-SOD3 pathway and $O_2^{\bullet-}$ generating systems may contribute to EC dysfunction in T2DM should be clarified in future study.

The present study also shows that phosphorylation of Akt, but not that of ERK, is selectively reduced in T2DM blood vessels or VSMCs and that decrease in ATP7A expression and Akt

phosphorylation in T2DM mice are rescued by constitutive-active Akt or PTP1B$^{-/-}$ T2DM mice which enhance Akt phosphorylation compared to T2DM[32]. Akt isoforms consist of three families including Akt1, Akt2, and Akt3 which have distinct roles[36], and vascular tissues mainly express Akt1 and Akt2 with below limit of Akt3 as shown in our study and previous reports[20]. Note that Akt2 is expressed in aorta and femoral artery of mice[20] and plays a key role in insulin signaling[21]. In this study, both male and female Akt2$^{-/-}$ mice which are insulin resistant and not insulin resistant, respectively[21], but not Akt1$^{-/-}$ mice, show significant decrease in ATP7A protein expression and SOD3 activity, which are rescued in ATP7A-Tg mice. Thus, these results suggest that insulin-Akt2 pathway plays an important role in increasing the ATP7A-SOD3 axis in blood vessels, which protects against diabetic endothelial dysfunction.

Mechanistically, using Akt2 siRNA and Akt2$^{-/-}$VSMCs or vessels, we demonstrate that Akt2 activated by insulin, which is impaired in T2DM, is required for stabilizing ATP7A protein by preventing ubiquitination/degradation as well as for ATP7A trafficking from TGN to plasma membrane where it binds to SOD3. Co-immunoprecipitation, in vitro kinase assay and LC-MS/MS analysis reveal that insulin promotes Akt2 binding to ATP7A, thereby phosphorylating C-terminus of ATP7A at S1424/S1463/S1466. Previous reports suggest that Akt-mediated phosphorylation of substrate proteins prevents ubiquitination/proteosomal degradation, thereby enhancing stabilization[29, 37], and that phosphorylation of ATP7A regulates its trafficking required for its Cu transport function[15, 38, 39]. Furthermore, it is shown that Cu-dependent serine phosphorylation of ATP7B is required for preventing its degradation as well as for Cu-induced trafficking[40]. We also found that cell-permeable Cu chelator, TTM, but not cell-impermeable Cu chelator BCS, blocked insulin-induced ATP7A trafficking. Consistently, previous reports show that Cu-induced ATP7A or ATP7B phosphorylation and trafficking require Cu binding to cytoplasmic regions of these proteins at N-terminus[15] in a BCS-independent manner[41]. Taken together, these findings indicate that Akt2 directly binds to ATP7A to induce its phosphorylation, which may prevent ubiquitination/degradation of ATP7A and promote translocation of ATP7A to plasma membrane where SOD3 might obtain Cu via ATP7A, thereby increasing the full SOD3 activity.

Since excess Cu is toxic[13, 42], not only Cu transporter function for secretory Cu enzymes but also Cu exporter function of ATP7A plays an important role in regulating bioavailable intracellular Cu levels[13]. The physiological role of Cu exporter ATP7A in other systems is not merely the elimination of excess cellular Cu but the supplying adequate Cu to the developing fetus as gestation progresses[41, 43] or neuronal protective mechanism[42]. In placental cells, insulin-induced ATP7A relocalization is proposed to supply adequate Cu to the developing fetus as gestation progresses[41, 43]. Thus, downregulation of ATP7A by impaired insulin-Akt2 pathway may impair Cu delivery to fetus. Indeed, it is reported that Cu metabolism is abnormal in diabetes[44, 45], and that Cu chelation therapy mitigates various complications of diabetes[44]. The present study shows that intracellular Cu content is increased in T2DM vessels (Figure XIII in the online-only Data Supplement), which may be due to a decrease in Cu exporter function of ATP7A. This excess Cu is not bioavailable, because Cu is not properly transported to secretory Cu enzymes, and thus resulting in decreased specific SOD3 activity. Indeed, accumulated Cu caused by Cu importer CTR1

deficiency in intestinal cells do not activate Cu-dependent enzymes[46]. However, since some reports indicate that Cu supplementation rather restores diabetic phenotype[45], role of Cu homeostasis in diabetes may differ, depending on severity or genetic background.

We and others previously reported that decrease in SOD3 activity in blood vessels contributes to endothelial dysfunction, hypertension, impaired ischemia-induced neovascularization[4, 47] by overproduction of $O_2^{\bullet-}$ levels[10, 11, 28]. Moreover, SOD3 gene transfer prevents neointima formation in vascular injury model[48]. In addition to SOD3, Cu transporter function of ATP7A is required for activating various secretory Cu enzymes, including lysyl oxidases[13], which promotes collagen cross-linking involved in vascular remodeling and wound repair. Thus, our finding that the impaired Akt2-ATP7A-SOD3 or other Cu enzymes axis may apply to other cardiovascular diseases such as hypertension, atherosclerosis, vascular remodeling and aging. Addressing this issue is important subject of future study.

In summary, the present study provides compelling evidence that ATP7A, a Cu transporter for SOD3, is markedly downregulated in T2DM vessels due to impaired insulin-Akt2 pathway in VSMCs, which contribute to decrease in SOD3 activity, excess $O_2^{\bullet-}$ production and subsequent impaired NO-dependent EC function. Mechanistically, Akt2 activated by insulin phosphorylates C-terminus of ATP7A, thereby promoting ATP7A protein stabilization via preventing ubiquitination/protein degradation as well as ATP7A trafficking to plasma membrane to deliver Cu to SOD3. Thus, enhancing and restoring Akt2-ATP7A-SOD3 axis is novel therapeutic strategy for treatment of diabetic vascular complications and various cardiovascular diseases which are associated with oxidative stress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Mr. Ronald D. McKinney for editorial assistance. We thank Taplin Mass Spectrometry's facility for assistance with phosphorylation site determinations. This research was supported by 5NIH R01 HL070187-14 (to T.F.), Department of Veterans Affairs Merit Review grant 2I01BX001232-05 (to T.F.), R01HL133613, R01HL116976 (to T.F., M.U.-F.), NIHR01 HL077524, HL077524-S1, R21HL112293 (to M.U.-F.), HL085614, HL095701, HL130513 (to S.A.P.), 11POST5740006 and 15SDG25700406 (to V.S.).

Non-standard Abbreviations and Acronyms

Akt-DN	Akt-dominant negative
BCS	Bathocuproine disulfonate
C/LR	Caveolin enriched lipid raft
CHX	Cycloheximide
ATP7A	Copper transporting/exporting ATPase
Cu	Copper

EDR	Endothelium-dependent vasorelaxation
HFD	High fat diet
IB	Immunoblot
IP	Immunoprecipitation
ICP-MS	Inductively coupled plasma mass spectrometry
IRS	Insulin receptor substrate
LC – MS/MS	Liquid chromatography tandem-mass spectrometry
LOX	Lysyl oxidase
MS	Mass spectrometry
MASMs	Mouse aortic smooth muscle cells
RASMs	Rat aortic smooth muscle cells
RT-PCR	Reverse transcription-polymerase chain reaction
SOD	Superoxide dismutase
TTM	Tetrathiomolybdate
TGN	Trans-Golgi network
VSMC	Vascular smooth muscle cell

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Highlights

- Protein expression of ATP7A, a Cu transporter for SOD3, is downregulated in the vessels from type2 diabetic patients and mice with impaired insulin-Akt signaling.
- Akt2 activated by insulin phosphorylates ATP7A, thereby promoting ATP7A protein stabilization via preventing ubiquitination/protein degradation as well as ATP7A trafficking to plasma membrane in VSMCs., which contributes to full activation of SOD3 that protects against endothelial dysfunction in T2DM.
- Decreased ATP7A expression in type2 diabetic vessels or VSMCs are rescued by constitutive active Akt or PTP1B$^{-/-}$ diabetic mice which enhance insulin-Akt signaling.
- Restoring the newly identified Akt2-ATP7A-SOD3 axis is novel therapeutic strategy for treatment of diabetic vascular complications which are associated with oxidative stress.

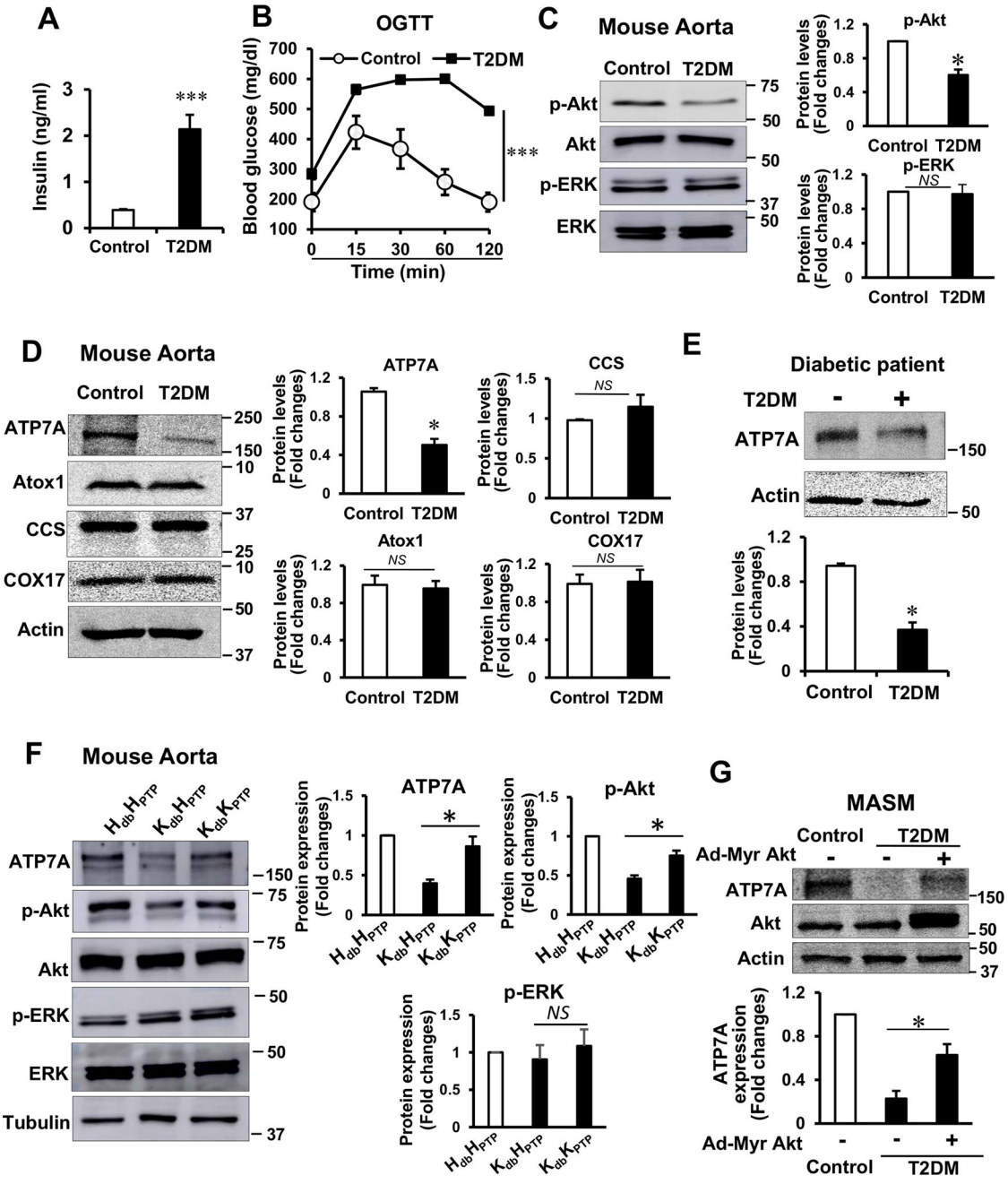


Figure 1. Selectively impaired Akt signaling contributes to the decrease in ATP7A expression in T2DM vessels

T2DM was induced by 16 week of high fat diet combined with single injection of low dose streptozotocin (STZ). **A**, plasma insulin concentrations in non-fasting T2DM and control mice (n=20). **B**, Blood glucose levels in control and T2DM mice fasted overnight before OGTT (n=6). **C**, pAkt, pERK levels and their total proteins in T2DM and control mouse aorta (n=4). **D**, ATP7A protein, but not other copper trafficking proteins, is decreased in T2DM mouse aorta (n=5). **E**, ATP7A protein expression is decreased in microvessels from adipose tissue of diabetic patient (n=7–8). The blot is representative of 7 control and 8

diabetic independent patients. Densitometric analysis are shown below. **F**, decrease in ATP7A protein expression in aortas from genetically-induced T2DM db/db mice is rescued in those from T2DM crossed with PTP1B−/− which enhances insulin Akt signaling (n= 4). H or K indicates heterozygote or knockout mice, respectively. The db and PTP indicate db/db and PTP1B, respectively. **G**, Mouse aortic SMC (MASM) isolated from control and T2DM mice were transfected with Adenovirus-myristoylated Akt. Lysates were used to measure ATP7A, Akt and actin protein expression (n=4). Results are presented as mean ± SEM. *p<0.05, ***p<0.001, *NS*, not significant.

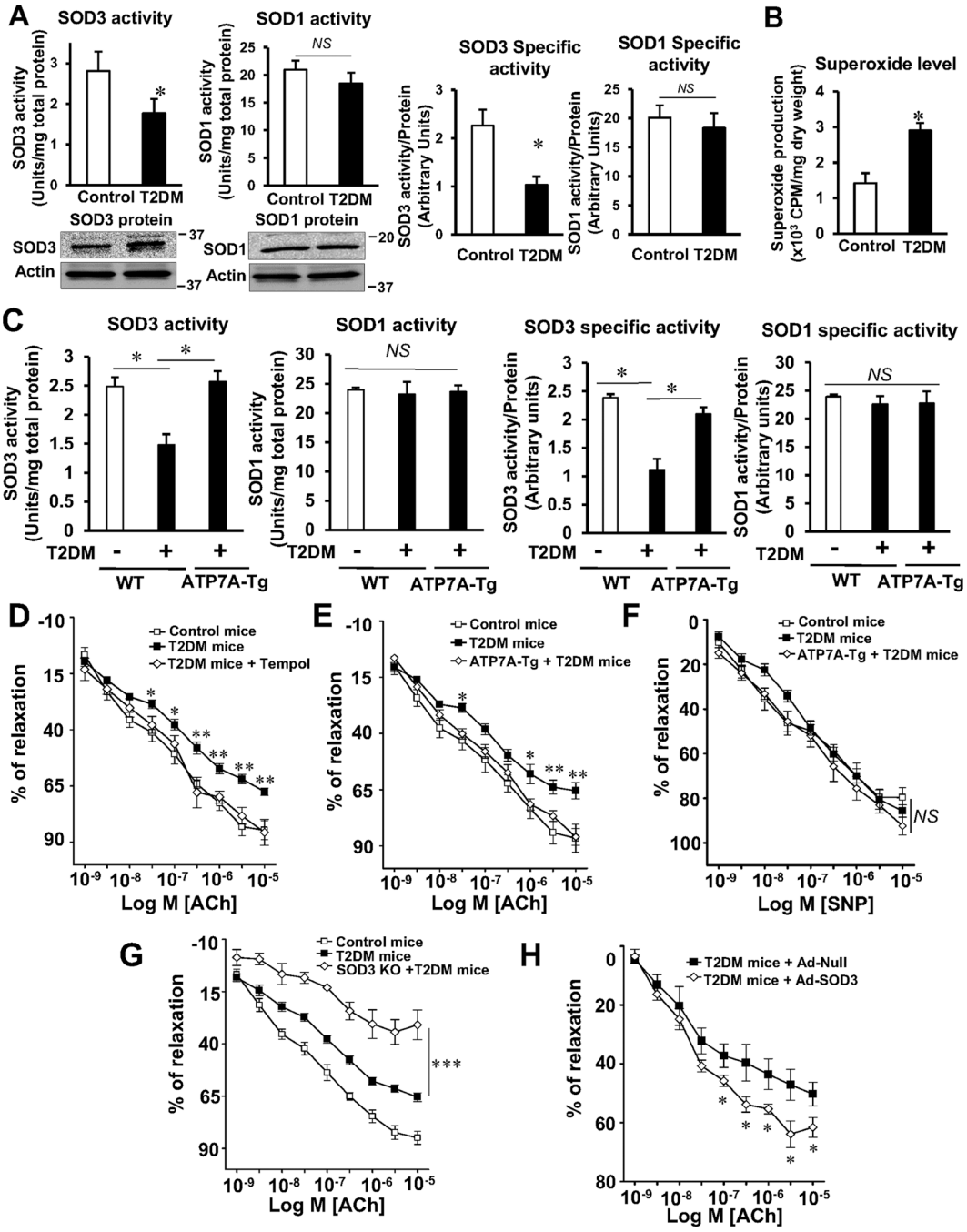


Figure 2. Decreased ATP7A expression in T2DM vessels contributes to reduced SOD3 activity, resulting in impaired endothelium-dependent relaxation

A, Activities of SOD3 and SOD1 in T2DM or control mice aorta were measured by inhibition of cytochrome c reduction by xanthine/xanthine oxidase. Con A-Sepharose chromatography was used to isolate SOD3 from tissue homogenates. Protein levels of SOD1, SOD3 and actin (bottom). Specific activity of SOD1 and SOD3 were determined by the ratio of activity to relative amount of protein (n=4). **B**, Aortic superoxide production in T2DM and control mice was measured by a lucigenin-enhanced chemiluminescence (5

μmol/L) (n=6). **C**, Decreased SOD3 activity in T2DM mice aorta is restored in T2DM ATP7A overexpressing mice. Activity and specific activity of SOD3 and SOD1 were assayed, as described (n=4). **D, E, F, G, and H**, Isometric tension of mesenteric resistance arteries from T2DM with SOD mimetic or SOD3 gene transfer (**D** and **H**), transgenic mice overexpressing ATP7A with T2DM (**E** and **F**), SOD3KO with T2DM (**G**), or control mice was measured 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) (**D**), or after injection of adeno-SOD3 or adeno-null (control) (**H**) (n=5–8). Results are presented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, *NS*, not significant.

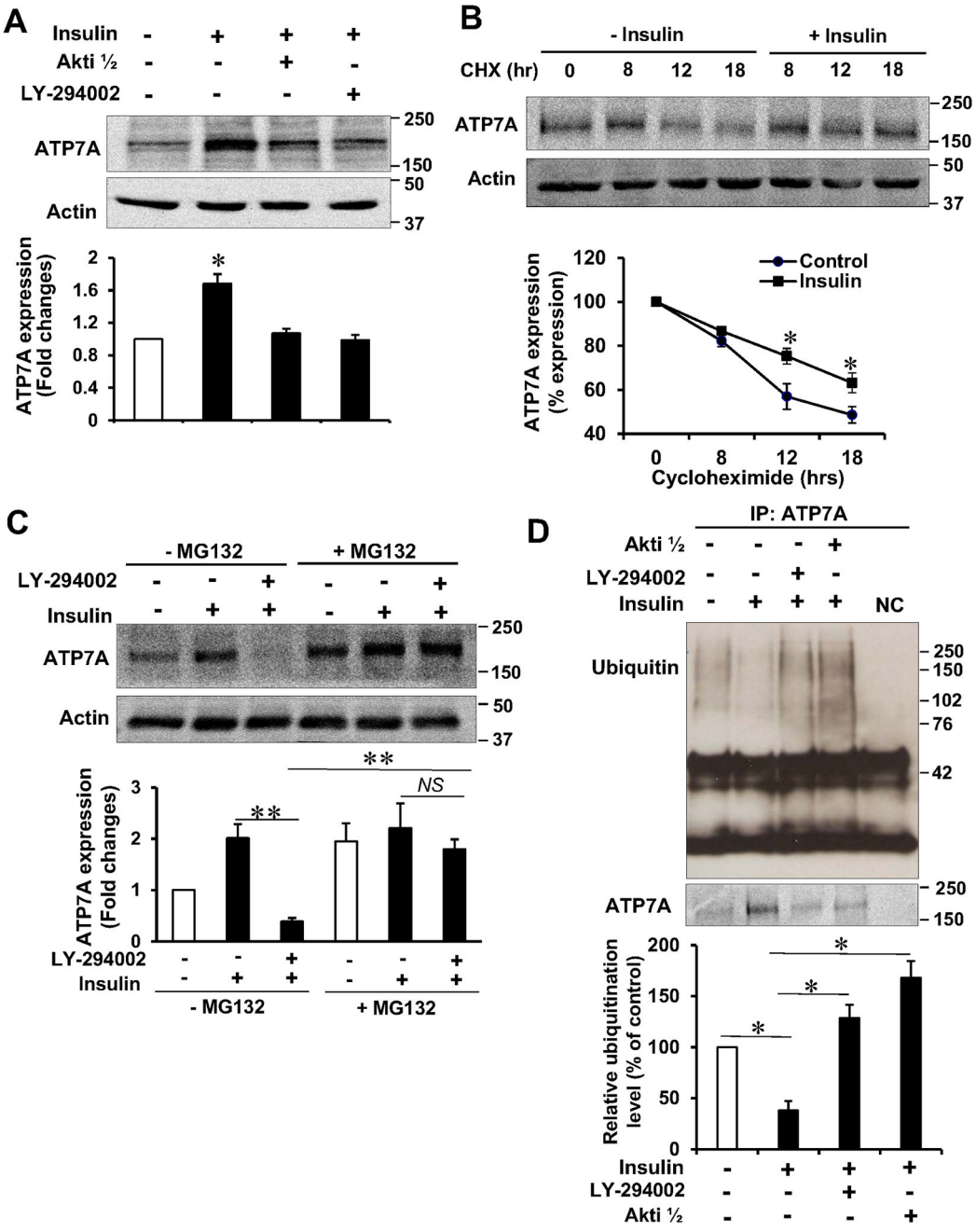


Figure 3. Insulin increases ATP7A protein stability by inhibiting ubiquitination and degradation in a PI3K/Akt-dependent manner in VSMCs

A, rat aortic SMCs (RASMs) were incubated with insulin (10 nM, 16 hrs) in the presence of PI3K inhibitor, LY-294002 (20 μM) or Akt inhibitor, Akti$^{1/2}$ (5 μM) and then used to measure ATP7A protein expression (n=4). **B**, RASMs were incubated with insulin (10 nM) or cycloheximide (CHX, 10 nM) and then used to measure ATP7A protein expression (n=3). **C**, RASMs were incubated with insulin (10 nM, 16 hrs) with or without LY-294002 (20 μM) or inhibitor of proteosomal degradation, MG132 (20 μM) and then used to measure ATP7A protein exression (n=4). **D**, RASMs were incubated with insulin (10 nM, 16 hrs) with or

without LY-294002 (20 μM) or Akt inhibitor, Akti 1/2 (5 μM) and immunoprecipitated with anti-ATP7A, followed by immunoblotted with anti-ubiquitin antibody (n=4). Bottom, averaged data for ATP7A ubiquitination (n=4). Results are presented as mean ± SEM. *p<0.05, **p<0.01, NS, not significance.

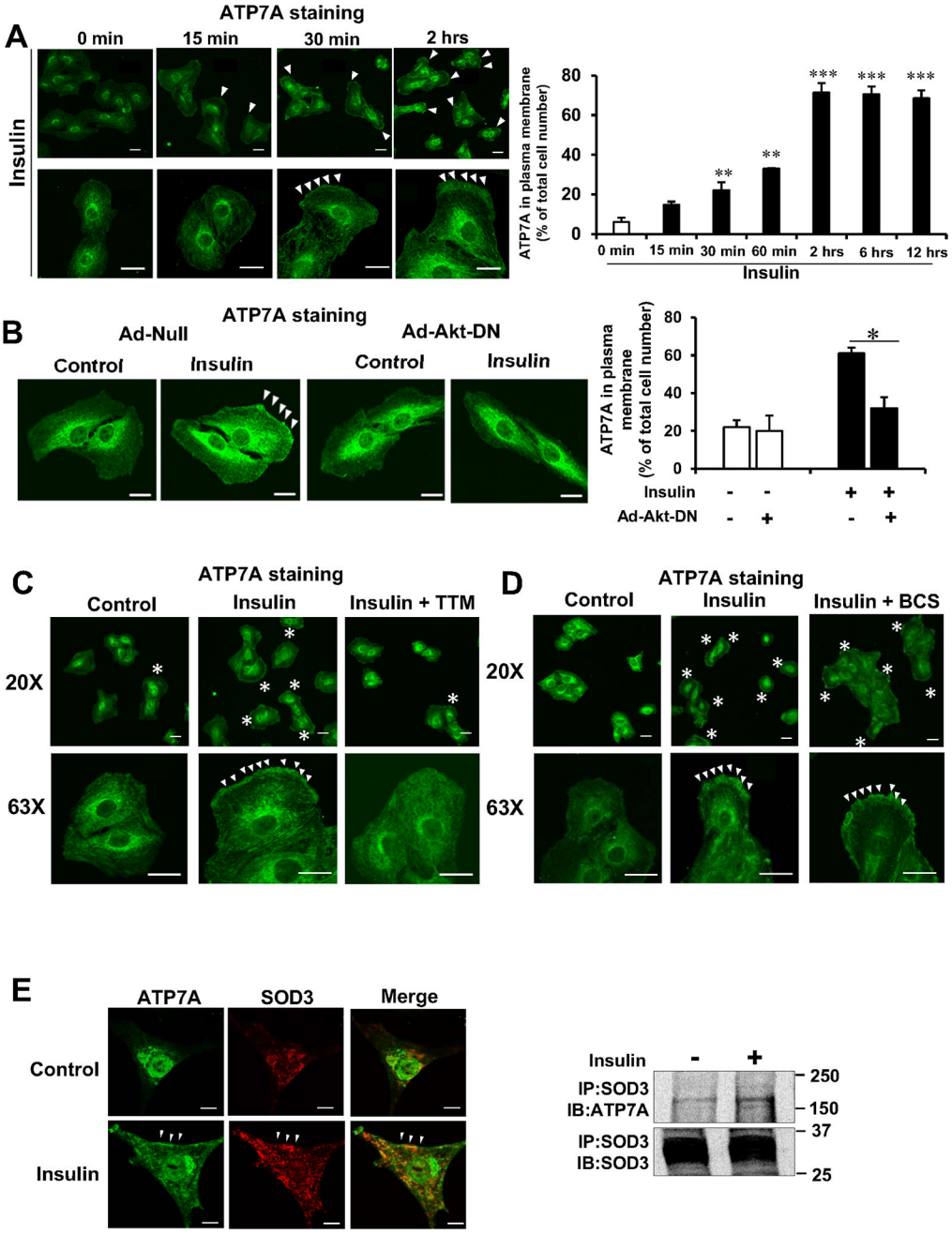
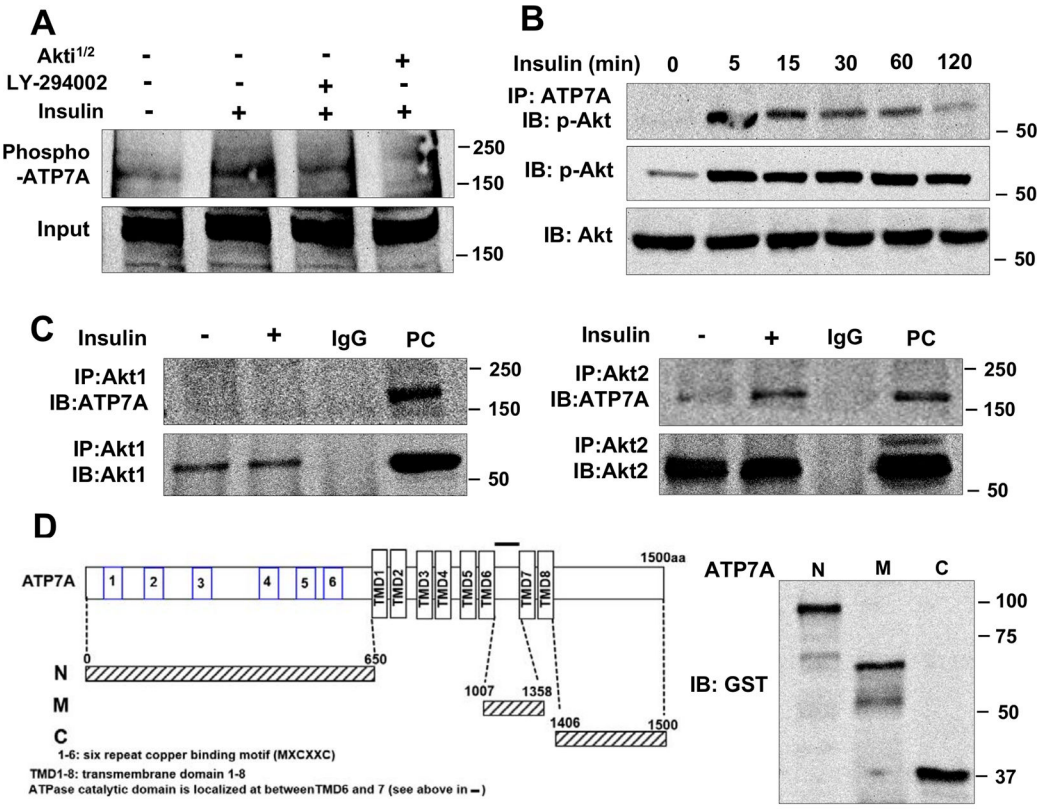


Figure 4. Insulin increases ATP7A translocation to the plasma membrane, where SOD3 colocalizes with ATP7A in an Akt- and Cu-dependent manner in VSMCs

A–D, RASMs were stimulated with 10 nM of insulin for indicated time (**A**). RASMs infected with adenovirus expressing Akt-dominant negative (Ad-Akt-DN) or Ad.null (control)(**B**) or treated with the cell permeable Cu chelator TTM (10 nM, 24 hrs)(**C**) or treated with cell impermeable Cu chelator, BCS (200 μM, 72 hrs)(**D**) were stimulated with 10 nM insulin for 2 hrs. These cells were stained with anti-ATP7A antibody, and images were taken at 5 different fields/well from 3 different experiments. Graph represents quantification of plasma membrane ATP7A positive cells. Results are presented as mean±

SEM. *p<0.05, **p<0.01, ***p<0.001. **E**, Immunofluorescence analysis showing the co-localization of ATP7A and SOD3 at plasma membrane (left) or co-immunoprecipitation of SOD3 and ATP7A (right) in human aortic smooth muscle cells (HASMs) stimulated with or without insulin (10 nM, 2 hrs) (n=4). Negative control staining lacking the primary antibodies in each staining is shown in Figure XIV in the online-only Data Supplement. Bar represents 20 μm.

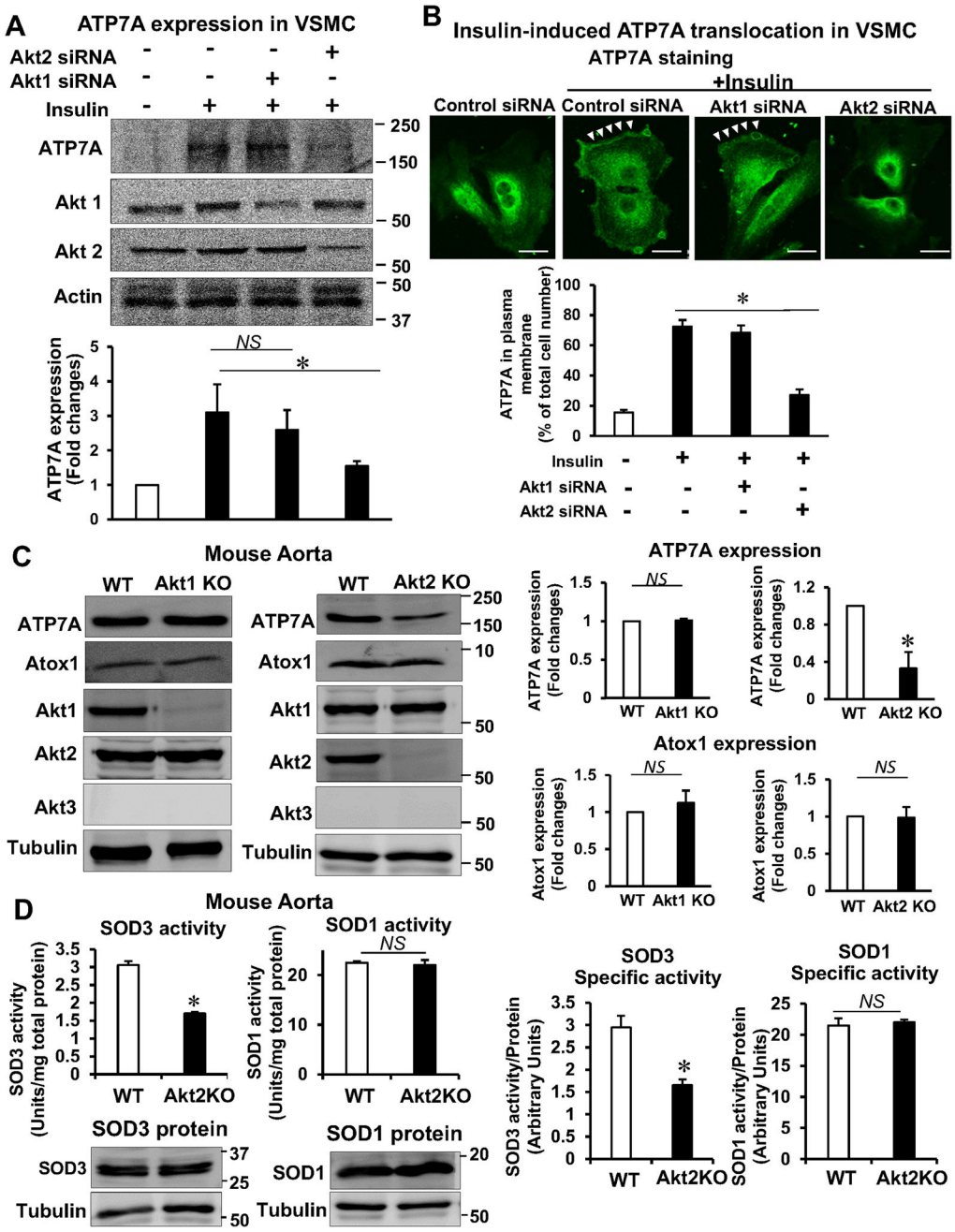


Ascore Seq_A	Ascore 1A	Ascore Seq_B	Ascore 1B
KPTYESYELPAR**S** (1424) #QIGQK	46.8	KPTYESYELPAR**S** (1424) #QIGQK	66.6
A**S**(1463)#INSLLSDK	30.7	A**S**(1463)#INSLLSDK	39.5
ASIN**S** (1466)#LLSDKR	26	ASIN**S**(1466)#LLSDKR	35.8

Figure 5. Insulin increases Akt2 binding to ATP7A as well as ATP7A phosphorylation in VSMCs

A, RASMs treated with PI3K inhibitor, LY-294002 (20 μM) or Akt inhibitor, Akti 1/2 (5 μM) and were stimulated with 10 nM insulin for 2 hrs. Phosphoprotein were purified by column and then immunoblotted (IB) with anti-ATP7A-antibody (n=3). **B**, RASMs treated with 10 nM insulin for indicated time were immunoprecipitated (IP) with anti-ATP7A antibody, followed by IB with p-Akt and total Akt antibody (n=3). **C**, RASMs stimulated with insulin (10 nM) for 2 hrs were IP with anti-Akt1 or anti-Akt2, followed by IB with anti-ATP7A antibody (n=3). **D**, Domains contained in the GST-ATP7A fusion construct (left). Molecular masses of various construct in kDa (right). **E**, Various GST-ATP7A construct (N-,

Middel (M)- C-Terminus ATP7A-GST) (2 ug) were incubated with 10 μCi [γ-32P] ATP with or without recombinant Akt. [γ-32P]-incorporated GST-ATP7A protein was visualized by autoradiography. **F**, Peptides identified in C-Terminus ATP7A by mass spectrometry containing phosphorylated serine amino acids. Three phosphorylation sites were identified.



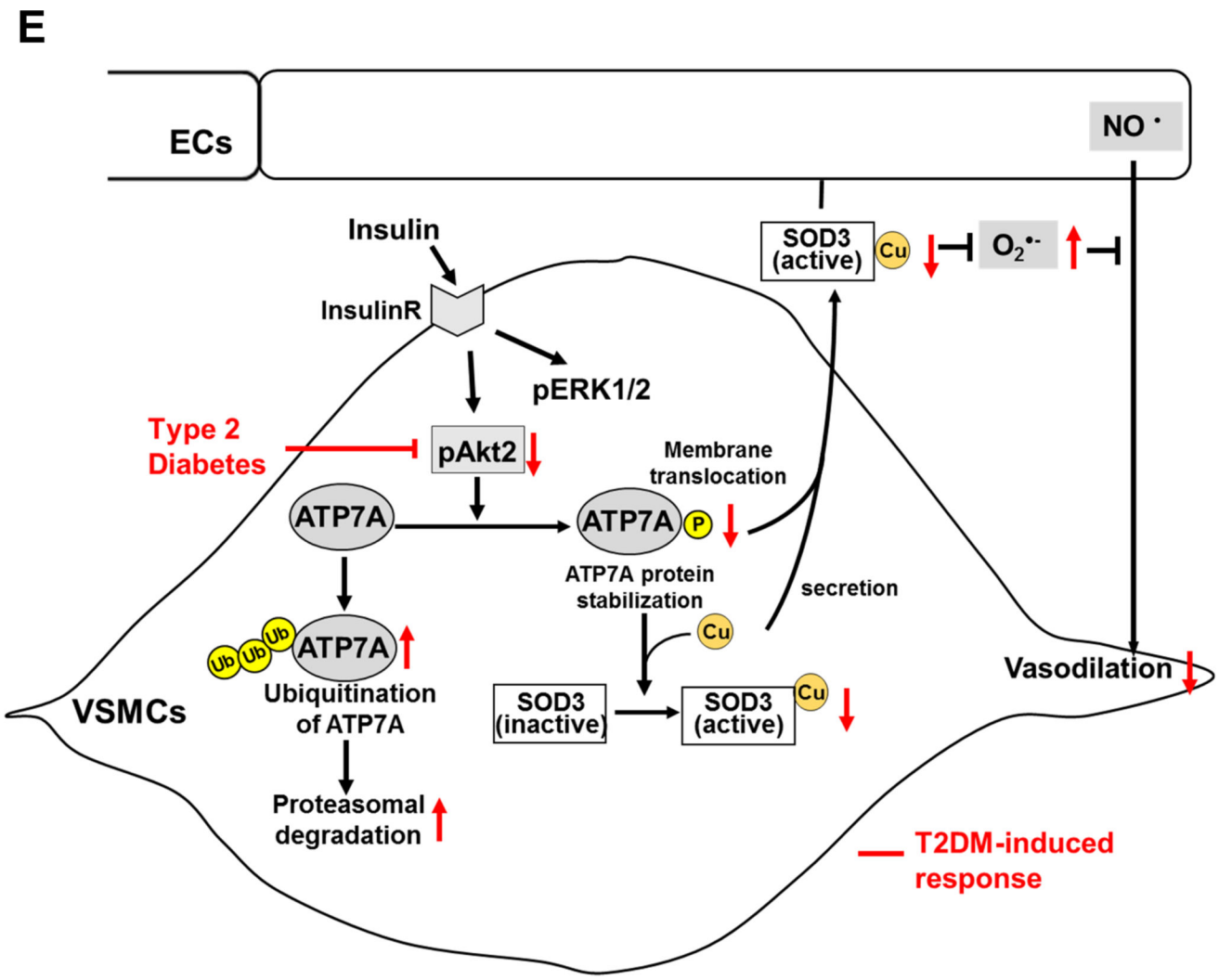


Figure 6. Akt2 is involved in ATP7A protein expression and ATP7A translocation to membrane in VSMCs, which contributes to SOD3 activity

A and B, RASMs transfected with control or Akt1 or Akt2 siRNA were stimulated with 10 nM insulin for 2 hrs (B) or 16 hrs (A). In **A**, lysates were used to measure ATP7A protein expression (n=4). In **B**, cells were stained with anti-ATP7A. Small white stars point to the plasma membrane. Fluorescence images were analyzed as described in Figure 4. Negative control staining lacking the primary antibodies in each staining is shown in Figure XIV in the online-only Data Supplement. **C**, Protein expression for ATP7A and Atox1 in aortas of female Akt1 or Akt2 KO mice (n=4). **D**, Specific activity of SOD1 and SOD3 was determined by the ratio of activity to relative amount of protein (n=5). Activities and protein levels of SOD3 and SOD1 in homogenates from female Akt2 KO or WT mice aorta were assayed as described in Figure 2. Results are presented as mean ± SEM. *$p<0.05$. NS, not significant. **E**, Proposed model for the protective role of the insulin-Akt2-ATP7A-SOD3 pathway against T2DM-induced endothelial dysfunction. Decreased ATP7A expression in vessels from T2DM with selective impairment of insulin/Akt signaling contributes to decreased SOD3 activity, resulting in increased $O_2^{\bullet -}$ production and endothelial dysfunction. Mechanistically, insulin increases Akt2 binding to ATP7A to induce

phosphorylation, which may increase ATP7A protein expression via preventing proteasomal degradation as well as ATP7A translocation to the plasma membrane, which contributes to full activation of SOD3 in VSMC and preserves endothelial function.