Materials and Methods

Animals, human samples, and experimental design: Heterozygous transgenic male mice overexpressing ATP7A from the chicken beta-actin composite promoter (CAG) [1] on C57BL/6J background were weaned at 4 weeks of age and maintained on regular chow. Akt1 KO and Akt2 KO mice (male or female, 6 months old) in a C57BL/6 background were used as described previously [2]. Male db/db and db/+ (16 weeks old) were purchased from Jackson Laboratory (Stock No: 000697). The genetically diabetic mouse (db/db) has a mutation on the chromosome 4 that inhibits the expression of the leptin receptor. Db/db/PTP1B$^{-/-}$ ($K_{db}K_{PTP}$), $H_{db}H_{PTP}$, $K_{db}H_{PTP}$ (H or K indicates heterozygote or knockout mice, respectively. The db and PTP indicate db/db and PTP1B, respectively.) were generated, as previously described[3]. SOD3$^{-/-}$ mice on C57BL/6J background[4]. C57BL/6J mice (male except where otherwise specified) were purchased from Jackson Laboratory (Stock No: 000664). The protocol for animal use was approved by Institutional Animal Care and Use Committee at University of Illinois at Chicago and Medical College of Georgia. Human microvessels (~150-200 um) used for protein isolation were obtained from biopsy samples during bariatric surgery after written informed consent or from mediastinal fat depots during open-heart surgery. The protocol was approved by the Institutional Review Board of the University of Illinois at Chicago and by Institutional Review Board at Medical College of Georgia, respectively. The patient characteristics were shown in Table II in the online-only Data Supplement.

Mice were studied at 6 weeks of age. T2DM mice were generated by a single injection of STZ (Sigma, S0130); 75 mg/kg body weight in 0.05 mol/L citrate buffer, pH.4.5, i.p.) and fed a high-fat (60% kcal) diet (HFD; Research Diet, D12492) from the day of STZ injection as described before [5-7]. The injection of low dose STZ (75mg/kg) in C57BL6 strain does not develop diabetes [8]. HFD for 12-16 weeks only develops obesity, but does not reproducibly develop diabetes. However, combination of low dose STZ with HFD for 16 weeks helps to develop reproducible and advanced degree of type 2 diabetic strain[5]. All data were obtained from male mice 16 weeks after the injection. All reagents were purchased from Sigma-Aldrich (St. Louis, MO), except when specified.

Metabolic characterization: Total cholesterol, LDL, HDL (BioAssay Systems, EHDL-100), triglyceride (Wako Diagnostics, 992-0292;998-0292,), plasma insulin (Crystal Chem Inc, 90080) and nitrate/nitrite level (Cayman chemicals, 780001) were measured with a kit from manufacturer. For a glucose tolerance test, mice were made to fast for overnight, then glucose (2 g/kg body wt) was administrated intraperitoneally, and plasma glucose concentration was measured at time 0 (before glucose administration) and 15, 30, 60 and 120 min after glucose administration. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated by using the following formula: HOMA-IR = 26 x fasting insulin level (ng/ml) x fasting glucose level (mg/dl)/405 as reported previously [9].

Superoxide Dismutase Activity Assays: Tissue harvesting and SOD activity assay were performed as described previously[10]. Briefly, tissues were homogenized in 10 vol of 50 mM potassium phosphate (pH 7.4) containing 0.3 M KBr and a cocktail of protease inhibitors (Roche Applied Sciences, 11836153001). The homogenates were then sonicated and extracted at 48ºC for

30 min. The extracts were then centrifuged at 3,000 g for 15 min. SOD activity was assayed by monitoring inhibition of the rate of xanthine/xanthine oxidase–mediated reduction of cytochrome *c* (Sigma, C7752). Con A-Sepharose chromatography (Amersham Biosciences, GE17-0440-03) was used to isolate SOD3 from vessels of diabetic and control mice.

Measurements of vascular superoxide production: Control or T2DM mice were euthanized by CO_2 inhalation. Vascular $O_2^{\cdot-}$ production was determined using lucigenin-enhanced chemiluminescence as described before[10]. This method has been validated for $O_2^{\cdot-}$ measurements in vascular tissue when low concentrations of lucigenin (5 μmol/L) are used[11].

Vascular reactivity studies: Isometric tension of mesenteric resistance arteries were measured using wire myograph (Model 610M, Danish Myo Technology, Denmark) as described previously[12]. Endothelium-dependent vasorelaxation in response to acetylcholine (Sigma, A2661) and endothelium- independent relaxation to sodium nitroprusside (Sigma, 71778) were studied. Results are expressed as percent relaxation of the phenylephrine (Sigma, P6126) -treated arteries, with 100% relaxation representing basal tension.

Copper Measurements: Copper contents were analyzed by inductively coupled plasma mass spectrometry (ICP-MS) using a PlasmaQuad3, as reported previously[13].

Isolation of VSMC from aorta: Vascular smooth muscle cells (VSMC) were isolated from rat (RASM) or mouse (MASM) thoracic aorta by enzymatic digestion as described previously[14]. Briefly, the thoracic aorta was removed aseptically from male Sprague-Dawley rats or from mouse and cleaned gently of fat in sterile Hanks' balanced salt solution. The vessels were then incubated in Hanks' solution containing 175 units/ml collagenase (Worthington Biochemical Corp, CLS-2) for 30 min at 37°C. Adventitia was then removed, and the endothelium was scraped off with a cotton swab. The tissues were incubated overnight in 10% fetal calf serum in Dulbecco's modified Eagle's medium in a CO_2 incubator. Enzymatic digestion was completed by incubating the vessels in Hanks' solution containing 175 units/ml collagenase and 75 U/ml elastase (Worthington Biochemical Corp, ESFF#LS006365) for 40 min. The reaction was terminated by dilution with 10 ml of 20% calf serum in Dulbecco's modified Eagle's medium (DMEM) and the cells pelleted by centrifugation and plated in dishes. For some experiments, VSMCs from human aortic smooth muscle cells (HASM) was used. HASMs (Clonetics Corp) were cultured in smooth muscle basal medium (Clonetics, M231500) and 5% FBS.

Adenoviral vector and in vivo gene transfer: Adenovirus expressing human SOD3 (Ad.SOD3) was from the adenovirus core at University of Iowa (Iowa City, IA). Ad.SOD3 and Lac Z (0.25 mL of 1.3 X 10^{12} particles/mL in 3% sucrose in PBS) were injected intravenously. Three days after viral injection, mice were killed and vascular tissue collected for further experiments.

siRNA transfection: siRNAs were obtained from Santa Cruz or Origene. VSMCs were seeded into culture dishes one day prior to transfection. VSMCs were grown to 40% confluence and transfected with 30 nM siRNA using Oligofectamine (Invitrogen, 12252011) and Opti-MEM Reduced Serum Medium (Invitrogen, 31985070) for 8 hrs according to manufacturer protocol. In growth-arrested VSMCs, insulin (Sigma-Aldrich, I9278) stimulation was performed 48 h after transfection.

Adenovirus transduction: Nearly confluent VSMCs were infected with adenovirus expressing constitutively active Akt (Ad. myr-Akt) or dominant negative Akt (Ad. Akt-DN) and control adenovirus (Ad. null) with 5 multiplicities of infection in serum-free medium for 24h. In growth-arrested VSMCs, insulin stimulation was performed 24 hrs after transfection.

Immunofluorescence analysis: VSMCs on glass coverslips were rinsed quickly in ice-cold PBS, fixed in freshly prepared 4% paraformaldehyde (Electron Microscopy Sciences, 15712-S) in PBS for 10 min at room temperature, permeabilized in 0.05% Triton X-100 (MP Biomedicals, 194854) in PBS for 5 min, and rinsed sequentially in PBS, 50 μmol/L NH_4Cl (Sigma, A4514) and PBS for 10 min each. After incubation for 1 h in blocking buffer (PBS+3%BSA), cells were incubated with anti-ATP7A (Sigma, GW21023, 5 μg/ml) or anti-SOD3 antibody (Sigma, WH0006649M1, 5 μg/ml) or PBS (for negative controls, Figure XIV in the online-only Data Supplement) for 18 h at 4°C, rinsed in PBS/BSA, and then incubated in Alexa Fluor conjugated goat anti-rabbit IgG (Invitrogen, A11010, 1:1000) or Goat anti-IgY (GenWay, GWB-41BC80, 1:500) for 1 h at room temperature and cells rinsed with PBS. Cells on coverslips were mounted onto glass slides using Vectashield (Vector Laboratories, H-1200) and observed using confocal microscopy.

Immunoprecipitation and Immunoblotting: Growth-arrested cells were stimulated with insulin at 37°C, and cells were lysed with 500 μl of ice-cold lysis buffer, pH 7.4 (50 mM HEPES, 5 mM EDTA, 120 mM NaCl), 1% Triton X-100, 60 mM *n*-Octyl-α-D-glucopyranoside, protease inhibitors (10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin) and phosphatase inhibitors (50 mM sodium fluoride, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate). For immunoprecipitation, cell lysates (700-1000 μg) were precipitated with indicated antibody overnight at 4°C and then incubated with 20 μl of protein A/G-agarose beads (Santa Cruz, SC-2003) for 1.5 h at 4°C. Cell lysates (25 μg) or immunoprecipitates were separated using SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad, 1620115), blocked overnight in PBS containing 5% nonfat dry milk and 0.1% Tween 20 (Fisher, BP337), and incubated for overnight with primary antibodies. Following primary antibodies were used: anti-Atox1, anti-SOD3 (home-made), SOD1 (Abcam, ab16831, 1 μg/ml) anti-p-Akt (Cell signaling, 4060S, 1:1000), anti-p-P44/42 MAPK (Cell Signaling, 9101S, 1:1000), anti- P44/42 MAPK (Cell signaling, 9102, 1:1000), anti-Akt1 (Cell signaling, 2938S, 1:1000), anti-Akt2 (Cell signaling, 3063S, 1:1000), anti-Akt3 (Cell signaling, 3788P, 1:1000), anti-ATP7A (LifeSpan Biosciences, B8162, 2 μg/ml), anti-CCS (Santa Cruz, SC-55561, 0.4 μg/ml), anti-Actin (Santa Cruz, SC-1616, 0.4 μg/ml), anti-Tubulin (Santa Cruz, SC-5286, 0.4 μg/ml), anti-Akt1/2/3 (Santa Cruz, SC-8312, 0.4 μg/ml), anti-Ubiquitin (Santa Cruz, SC-8017, 0.4 μg/ml) or anti-COX17 (Proteintech, 11464-1-AP, 0.6 μg/ml). After incubation with secondary antibodies (Goat Anti-Rabbit IgG-HRP Conjugate, Bio-Rad, 1706515, 1:2000; Goat Anti-Mouse IgG-HRP Conjugate, Bio-Rad, 1706516, 1:2000; Goat IgG HRP-conjugated Antibody, R&D, HAF017, 1:2000), proteins were detected by ECL chemiluminescence.

For protein expression in aortic tissue, mice were perfused with cold phosphate buffer saline. Aorta were harvested and frozen in liquid nitrogen. Aorta were crushed and lysed with RIPA lysis buffer (5 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1%sodium deoxycholate, 0.1% SDS) with protease inhibitor followed by brief sonication. Lysate used for western analysis.

Quantitative real-time PCR: Total RNA from VSMCs or aortic tissue was isolated by using phenol/chloroform and isolated using Tri Reagent (Molecular Research Center Inc.). Reverse

transcription was carried out using high capacity cDNA reverse transcription kit (Applied biosystems, 4368814) using 2 ug of total RNA. Quantitative PCR was performed with the ABI Prism 7000, the SYBR Green PCR kit (Qiagen, 204054) and the QuantiTect Primer Assay (Qiagen) for specific genes. Samples were all run in triplicates to reduce variability. Expression of genes was normalized and expressed as fold-changes relative to GAPDH or HRT.

In-vitro kinase assay: Purified GST proteins (GST-N-terminus ATP7A, GST- middle terminus ATP7A and GST- C-terminus ATP7A) (2 μg) were used for the in vitro kinase assay. Briefly, 2 ug of ATP7A-GST proteins were mixed in 40 μL of kinase buffer (Cell signaling, 9802S) containing 10 μCi [γ-32P]ATP (PerkinElmer) and recombinant active Akt (250 ng) (Upstate, 20-102) and mix carefully by pipetting up and down. Then, transfer the tubes to 30°C in a water bath and incubate for 30 minutes and stop the reaction by adding 20 μL of 3X concentrated sample buffer to stop the reaction. Then, the sample boiled for 5 minutes and centrifuge the samples and electrophoresis the soluble fractions. After running, the gel exposed to X-ray film at -80°C. Kinase activity will be indicated by a band of phosphorylated protein substrate.

Phosphoprotein Purification: A phosphoprotein purification kit (Qiagen, 37101) was applied to enrich phosphoproteins according to the manufacturer's instructions. Briefly cells were lysed in the Lysis Buffer, and the cell lysate was centrifuged at 10,000 × *g* at 4 °C for 30 min to remove insoluble material. After centrifugation, the protein concentration of the cell lysate was quantitated by Bradford assay. The extracted proteins were diluted to a concentration of 0.1 mg/ml with Lysis Buffer, and a total of 25 ml of the extracted proteins was applied to a Lysis Buffer-equilibrated phosphoprotein purification column at room temperature. After washing the column with 6 ml of Lysis Buffer, the phosphoproteins were eluted with 2 ml of Phosphoprotein Elution Buffer. All the buffers and the phosphoprotein purification column were provided in the kit by the manufacturer (Qiagen).

In-vitro phosphorylation and mass spectrometry: 2 ug of purified GST-C-terminus ATP7A protein was mixed in 40 μL of kinase buffer (Cell signaling, 9802S) containing 50μM ATP buffer (Cell signaling, 9804) and recombinant active Akt (250 ng) (Upstate, 20-102) and mix carefully by pipetting up and down. Then, transfer the tubes to 30°C in a water bath and incubate for 30 minutes and stop the reaction by adding 20 μL of 3X concentrated sample buffer to stop the reaction. Then, the sample boiled for 5 minutes and electrophoresis the soluble fractions. The protein gel bands were visualized by Coomassie brilliant blue prior to submission of the bands for mass spectrometry (Taplin Mass spectrometry, Harvard medical School, Boston). Phospho-peptide with Ascore >18 was considered a putative phosphorylation site according to the instructions from the Taplin Mass Spectrometry Facility, Harvard Medical School.

Statistical Analysis: Data are presented as mean ± SEM. Normality of the data (using Shapiro-Wilk test) and the equality of group variance (using Brown-Forsythe test) were performed in all data using SigmaPlot 14. 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 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. Statistical tests were performed using Prism v4 (GraphPad Software, San Diego, CA).

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