Data Supplement

Regulation of Coronary Arterial BK Channels by Caveolae-Mediated Angiotensin II Signaling in Diabetes Mellitus

Running title: BK channel regulation by Ang II in diabetic vessels

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Methods

Type I diabetic animal development and vascular SMC isolation

Sprague-Dawley rats were purchased from Harlan Inc. (Indianapolis, IN). Cav-$1^{(-/-)}$ knockout (KO) mice and cav-$1^{(+/+)}$ (WT) mice were obtained from Jackson Laboratories (Bar Harbor, ME). To develop type I diabetic animals, male Sprague-Dawley rats, cav-1 WT and KO mice at 6 to 8 weeks of age received an injection of STZ (100 mg/kg body weight, ip.) and a second dose was given on the next day. Control animals received vehicle injections. Animals with blood glucose > 300 mg/dl were considered diabetic and were used for experiments in 8 weeks.

Isolated vascular SMC were prepared as we have described [1]. Briefly, rat coronary arteries or mouse aortas were carefully dissected in ice-cold dissociation buffer (in mmol/L): NaCl 145, KCl 4.0, $CaCl_2$ 0.05, $MgCl_2$ 1.0, HEPES 10, glucose 10, pH 7.2. The vessels were placed in dissociation buffer containing 0.1% w/v bovine serum albumin (BSA) and incubated in a shaking water bath at 37 °C for 10 min. The vessels were replaced with fresh 0.1% w/v BSA dissociation buffer containing 1.5 mg/ml papain and 1.0 mg/ml dithiothreitol and incubated in a shaking water bath at 37 °C for another 10 min. This was followed by digestion in fresh 0.1% w/v BSA dissociation buffer containing 1.0 mg/ml collagenase and 1.0 mg/ml of trypsin inhibitor in a shaking water bath at 37 ^{o}C for 10 min. The vessels were then stored in 2 ml dissociation buffer, and gently triturated with a fire-polished glass pipette until the cells were completely dissociated. Dissociated cells were used for patch clamp experiments within 8 h.

Whole-cell patch clamp recording

Whole-cell BK currents were recorded from freshly isolated SMC with a holding potential (HP) of -60 mV and a testing potential (TP) of -40 mV to +160 mV in increments of 10 mV. The pipette solution contained (in mmol/L): KCl 140, HEPES 10, $MgCl_2$ 1.0, Na_2ATP 5.0, Na_2GTP 0.5, EGTA 1.0, and $CaCl_2$ 0.814 (1 μmol/L free Ca^{2+}), pH 7.35. The bath solution contained (in mmol/L): NaCl 145, KCl 4.0, $MgCl_2$ 1.0, $CaCl_2$ 1.0, HEPES 10, and glucose 10, pH 7.40. The effects of chemicals on BK channel activity were determined in the same cell before and after drug superfusion. The K^+ currents inhibited by 0.1 μmol/L iberiotoxin (IBTX) were referred as the IBTX-sensitive BK currents.

Single channel recording

Outside-out single channel recordings were made at +60 mV in freshly isolated SMC of rat coronary arteries. The BK current was identified by IBTX-sensitivity and its unitary conductance. The output signals were filtered with an 8-pole Bessel filter (902 LPF, Frequency Devices, Inc., Haverhill, MA) at 10 kHz and digitized at 50 kHz. Single channel analysis was performed using TAC software (Bruxton, Inc., Seattle, WA) as previously described [1]. The pipette solution and bath solution were the same as those used for whole-cell recording.

Measurement of coronary artery ring tension

Tension in rat coronary artery rings was measured using a multi-wire myograph system (610M, DMT-USA, Atlanta GA) as previously reported [2]. The left anterior descending coronary artery was carefully dissected from the isolated rat heart in ice-cold Krebs' solution that contained (in mmol/L) NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.5 and glucose 5.6 (pH=7.4). The artery was cut into rings of 1.5 to 2 mm in length, and each ring was suspended by two stainless-steel wires and mounted in an 8 ml organ chamber filled with Krebs' solution. Basal tension of the artery ring was maintained at 1.25 fold of the basal diameter of the vessel and equilibrated for 60 min with 96-4% O_2-CO_2. Changes in artery ring tension were recorded using a PowerLab 4/25 Data Acquisition Systems (AD Instruments, Medford, MA) and analyzed by LabChart 5.0 software (AD Instruments, Medford, MA). Changes in coronary artery ring tension

in response to chemicals were expressed as the percentage of maximal contraction produced by 100 mmol/L KCl. NS-1619-mediated relaxation was expressed as the percentage of the changes in the tension produced by 5 nmol/L endothelin-1 (ET-1) .

Sucrose gradient density centrifugation

Cellular distribution of cav-1 and caveolae targeting of the AT_1R-enzyme-channel complex in rat aortas was determined by sucrose density gradient fractionation as previously described [3]. Aortas were homogenized in 500 mmol/L Na_2CO_3 with 2 % protease inhibitors (v/v), and then centrifuged at 5,000 rpm at 4 °C for 10 min. The supernatant was adjusted to 40% sucrose, placed to the bottom of a 10-ml ultracentrifuge tube, layered with 4-ml discontinuous sucrose gradients (40%, 30% and 5%), and centrifuged at 32,000 rpm at 4 °C for 20 h. Ten fractions of 1.2 ml each were collected and analyzed by Western blotting.

To determine the effects of Ang II on caveolae targeting of AT_1R, some rats were infused with Ang II (100 μg/kg, iv.) 1 h prior to the isolation of aortas, which were homogenized and fractionated by ultracentrifugation.

Co-immunoprecipitation and immunoblotting

Immunoprecipitation was performed as described previously [4]. In brief, isolated rat aortas were cut into small pieces and incubated with 200 μl RIPA buffer (in mmol/L): Tris 50, NaCl 150, NaF 2, EDTA 1, EGTA 1, $NaVO_4$ 1, and 1% Triton X-100) and 1 μl protease inhibitor on ice for 1 h, homogenized and then centrifuged at 500 rpm at 4 °C for 10 min. The supernatant (about 200 μg in 200 μl) was incubated with anti-cav-1 (Santa Cruz, CA), anti-phosphotyrosine (Cell Signaling, MA) or anti-3-nitrotyrosine (Upstate, NY) antibodies at a final concentration of 4 μg/ml for each at 4 °C overnight. The samples were then incubated with 20 μl Protein G Plus-Agarose (Santa Cruz, CA) at 4 °C for 2 h with rotation. After centrifugation at 1000 rpm for 7 min and washed twice with RIPA/protease inhibitor buffer, the immunoprecipitates were collected and eluted from Agarose with 30 μl SDS-PAGE loading buffer per tube.

Western blotting was performed as previously reported [5]. Isolated aortas from rats and mice were homogenized, electrophoresed, transferred to nitrocellulose membrane, and immunoblotted against rabbit anti-human BK channel antibodies (1:200, custom made), anti-cav-1 (1:200 Santa Cruz, CA), anti-AT_1R (1:200, Santa Cruz, CA), anti-c-Src (1:200, Santa Cruz, CA), anti-NOX-1 (1:200, Santa Cruz, CA) and anti-$G_{\alpha q/11}$ (1:200, Santa Cruz, CA) antibodies. Blots were probed with mouse anti-β actin antibodies (1:2500, Sigma-Aldrich, MO) as loading control. Following extensive washing, horseradish peroxidase-conjugated secondary antibodies were added. Signals were developed by Immun-Star HRP Chemiluminescent Kit (Bio-Rad, Hercules, CA). Optical density of the bands was analyzed using Scion Image software (Scion, Frederick, MD). Protein expression was expressed as relative abundance normalized to β-actin.

Cav-1 knockdown by small interfering (si)RNA

Cav-1 in SMC was knocked down using human cav-1 siRNA as previously described [6]. Cav-1 siRNA and control scrambled siRNA were obtained from Dharmacon (Lafayette, Co). 48 h after transfection with 100 nM cav-1 siRNA or control siRNA, cells were analyzed for cav-1 abundance.

Confocal immunofluorescence microscopy

Freshly isolated aortic smooth muscle cells from control and diabetic rats, as well as from cav-1 WT and KO mice, were seeded on glass slides, fixed with 4% formaldehyde for 30 min, and then permeated with 0.1% Triton X-100 in PBS for 2 min. After incubation with 10% normal goat serum in PBS for 30 min, cells were incubated with a monoclonal anti-caveolin-1 antibody (1:200 dilution) plus either a polyclonal anti-BK α-subunit antibody (1:100 dilution) or a polyclonal anti-

NOX-1 antibody (1:100). The primary antibodies were detected with fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody (1:1000 dilution) or with Texas Red-conjugated goat-anti-rabbit secondary antibody (1:500 dilution) and mounted in Prolong Anti-fade Reagent (Molecular Probes, Eugene, OR). Samples were washed with PBS after both the primary and the secondary antibody incubations. Cells were visualized using a confocal laser microscope (LSM 510, Zeiss, Germany) with a 63X water immersion lens.

Chemicals

$OONO^-$ was purchased from Upstate Cell Signaling and Solutions (Lake Placid, NY). Lavendustin A (LavA), Lavendustin B (LavB), membrane permeable PKC peptide inhibitor, diphenylene iodonium (DPI) and myxothiazole (MXTZ) were obtained from BIOMOL International (Plymouth Meeting, PA). The other chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO).

Statistical analysis

Data are presented as mean±S.E.M. One way *ANOVA* followed by Tukey's test was employed to compare data from multiple groups. Student's t test was used to compare data between two groups and paired *t* test was used to compare data before and after treatment.

Results

Characterization of STZ-induced type I diabetic animals

8 weeks after the development of hyperglycemia, the average weight and blood glucose level was 223.4±5.7 g and 571.3±6 mg/dl respectively in STZ-induced diabetic rats (n=14) and was significantly different in those of age-matched controls, 316.6±6.7 g and 167.2±7.5 mg/dl respectively (n=17, $p<0.05$ between control and diabetic rats fro both). The average weight and blood glucose level of age-matched non-diabetic WT mice was 24.8±0.6 g and 175.8±28.4 mg/dl (n=18) respectively and 18.8±0.7 g and 490.2±19.1 mg/dl (n=17, $p<0.05$ vs. non-diabetic WT for both) respectively for diabetic WT mice. The average weight and blood glucose level of age-matched non-diabetic KO mice was 24.6±0.8 g and 148.2±10.8 mg/dl (n=11) respectively, and 20.8±0.6 g and 522.4±29.0 mg/dl (n=10, $p<0.05$ vs. non-diabetic KO for both) respectively for diabetic KO mice.

Vascular SMC capacitance

The cell capacitance of SMC isolated from control and STZ-induced diabetic rats was 11.8±0.8 pF (n=41) and 11.1±1.0 pF (n=35) respectively (p=N.S. vs. control). There was also no significant difference in SMC capacitance among cells isolated from control and diabetic cav-1 WT and KO mice (7.1±0.5 pF for non-diabetic WT, n=30; 6.8±0.5 pF for diabetic WT, n=28; 10.6±1.1 pF for non-diabetic KO, n=10 and 8.1±1.2 pF for diabetic KO SMC, n=11, respectively).

Effects of Ang II on single BK channel activity

Single BK currents were recorded at +60 mV from freshly isolated coronary arterial SMC in outside-out configuration. 2 μmol/L Ang II applied extracellularly reduced the channel open probability (Po) by 49.4%, from 0.45±0.06 at baseline to 0.23±0.03 of ($p<0.05$, n=4). For the best fit, the open dwell-time distribution histograms required at least three components: slow (τ-o1), intermediate (τ-o2) and fast (τ-o3) time constants. The closed-dwell time distribution of histograms had four components: very slow (τ-c1), slow (τ-c2), intermediate (τ-c3) and fast (τ-c4) time constants. The representative tracings and kinetic analysis of BK channel was illustrated in Figure I.

Effects of Ang II on BK channel current density of aortic arterial SMC from control and STZ-induced diabetic rats

Figure II shows that 2 μmol/L Ang II reduced aortic SMC BK current density from 206.5±36.2 pA/pF at baseline to 82.6±19.1 pA/pF (TP=+150 mV, n=6, $p<0.05$) in control rats and from 57.4±7.7 pA/pF (TP=+150 mV, n=6, $p<0.05$ vs. baseline of controls) at baseline to 54.2±11.6 pA/pF in STZ-induced diabetic rats (TP=+150 mV, n=6, p=N.S. vs. baseline of diabetic rats). I-V curves of BK channels from control and diabetic rat aortic SMC before and after exposure to Ang II are shown in online Figure II. Hence, the response of BK currents to treatment with Ang II was similar between coronary and aortic SMC from control and diabetic rats.

Co-localization of BK channel and NOX-1 in caveolae of SMC

To corroborate the results of immunoprecipitation, we performed confocal fluorescence microscopy analysis to demonstrate the targeting of BK channel and NOX-1 to the caveolae of SMC. The results showed that the BK channel and NOX-1 are colocalized in the caveolae of SMC, BK channel and NOX-1 targeting to caveolae appeared to be upregulated in diabetes (Figure III).

Role of NAD(P)H and mitochondrial respiratory chain complexes in high glucose-induced ROS generation in coronary arterial SMC

It is well known that culture with high glucose increases ROS generation in SMC. To further determine the source of high glucose-induced ROS generation in coronary arterial SMC, we measured the effects of DPI (a NOX inhibitor) or MXTZ (the mitochondrial respiratory chain complex II inhibitor) on the peroxide level in human coronary arterial SMC after a 2-week culture in 22 mmol/L glucose. Cellular peroxide level was significantly reduced after 1 h incubation with DPI, but not with MXTZ (Figure IV), suggesting that the major source of high glucose-mediated peroxide generation in the cells is from NAD(P)H oxidases, consistent with previous reports.

Ang II treatment increased ROS generation only in the SMC of cav-1 WT, but not in those of cav-1 KO mice (Figure V).

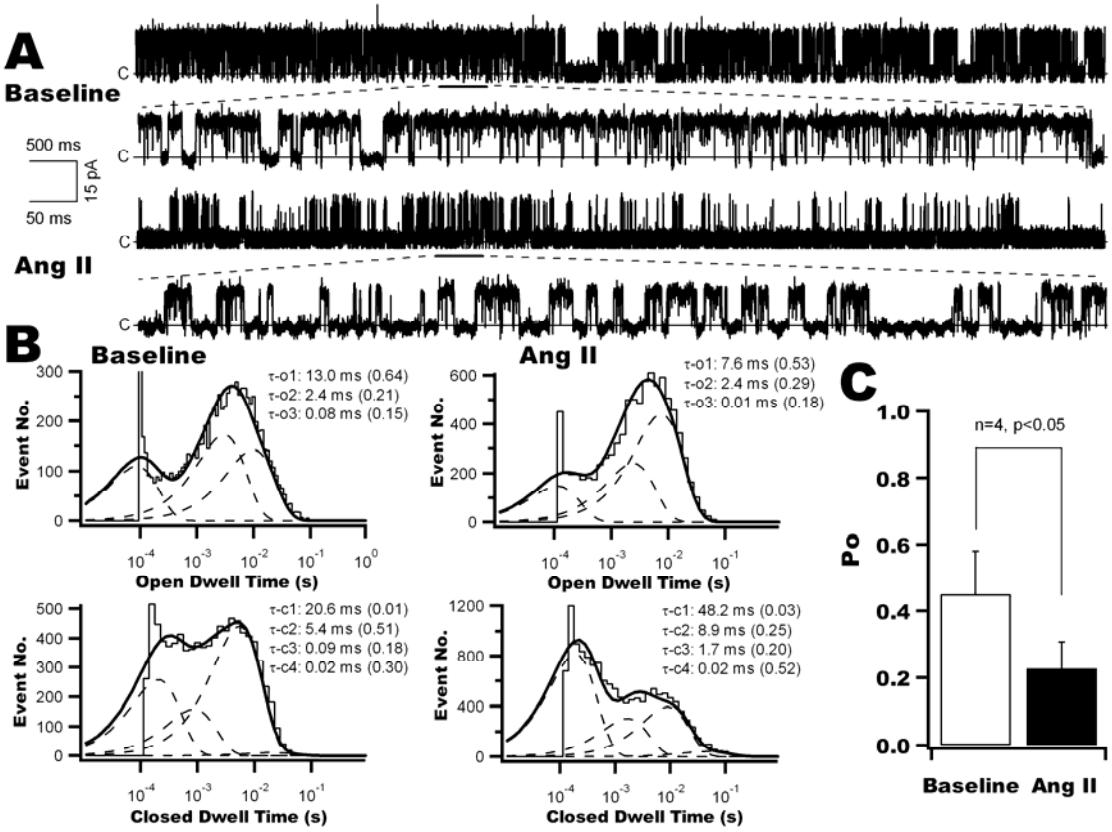

Figure I. Effects of Ang II on single BK channel activity of rat coronary arterial SMC
A: Representative tracings of single BK current were recorded at +60 mV from freshly isolated coronary arterial SMC in outside-out configuration at baseline and after extracellular application of 2 μmol/L Ang II. Selected segments with expanded details (lower tracings) show higher frequency of channel openings in baseline with reduction of channel activity by Ang II. "c" with solid lines indicate the channel closed state. **B:** The histograms of BK channel open- and closed-dwell durations have at least three open-dwell time constant components: τ-o1, τ-o2 and τ-o3; and four closed-dwell time constant components: τ-c1, τ-c2, τ-c3 and τ-c4. Dashed lines represent the distribution of each exponential component of dwell times. The value of each time constant and its relative weight (in parentheses) are given above each histogram. Ang II reduced the τ-o1 and prolonged τ-c1 and τ-c2, compared with those at baseline. **C**: BK channel open probability (Po) was reduced by 49.4% after application of Ang II.

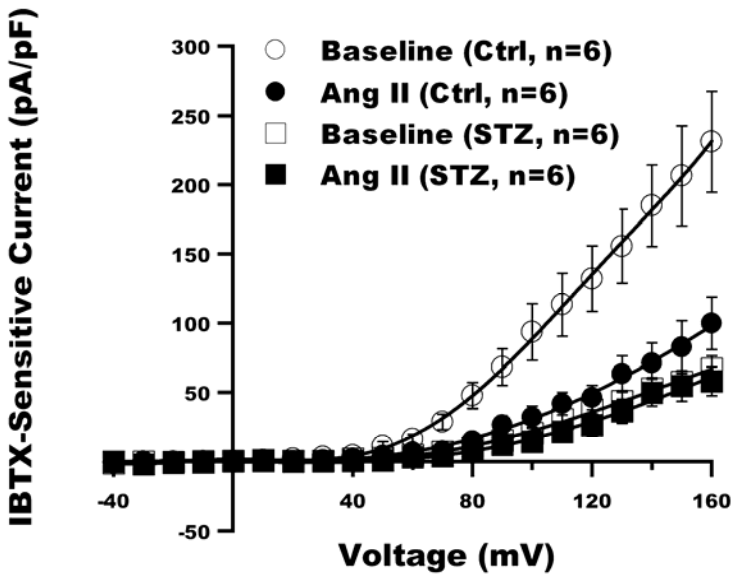

Figure II. Effect of Ang II on aortic arterial SMC BK channel activity of control and STZ-induced diabetic rats

I-V curves of BK current densities in freshly isolated SMC from the aortas of control and STZ-induced diabetic rats. Ang II (2 μmol/L) inhibited BK current density by 50% in control rats. 8 weeks after the development of diabetes, BK current density was significantly reduced at baseline Ang II did not produce any further inhibition, suggesting that the vascular BK channel function was profoundly impaired in diabetes.

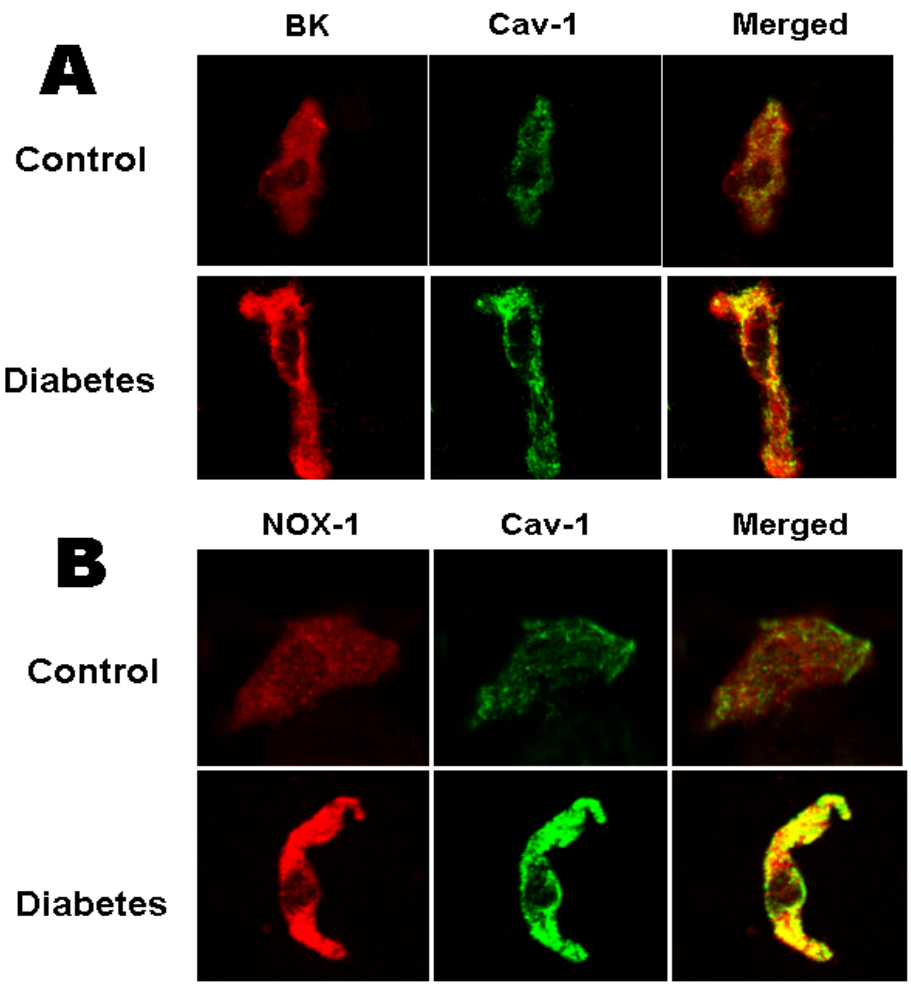

Figure III. Colocalization of BK channel and NOX-1 in the caveolae of coronary arterial SMC from STZ-induced diabetic rats

A: After incubation with anti-BK and anti-cav-1 antibodies, fluorescence images were acquired separately for Texas Red for BK channel (red in left column), fluorescein for cav-1 (green in middle column). BK channel and cav-1 are colocalized on the SMC membrane (yellow in right column) when images are merged. **B**: Similarly, NOX-1 colocalization in the caveolae of diabetic SMC is increased.

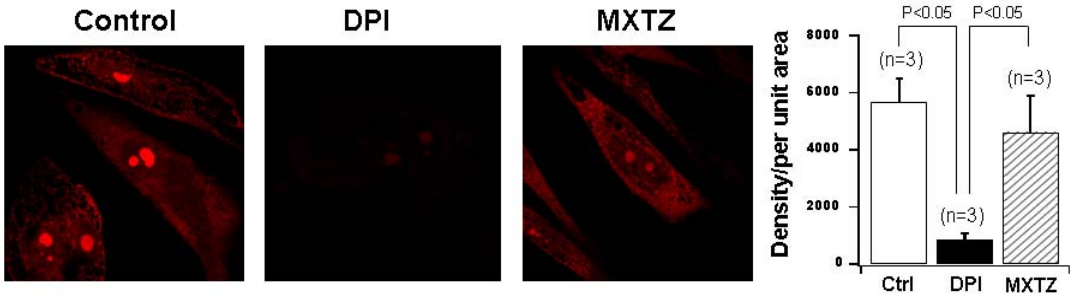

Figure IV. Inhibition of high glucose-mediated ROS generation by NOX and mitochondrial electron transport complex inhibitors in human coronary arterial SMC

Human coronary arterial SMC were cultured in 22 mmol/L glucose for two weeks. After pretreatment with DPI (20 μmol/L) for 1 h or with MXTZ (10 μmol/L) for 10 min, cells were incubated with 2 μmol/L dihyroethidium (DHE) for 30 min and then examined by confocal laser scanning microscopy. DHE is oxidized by $O_2^{\bullet -}$ and intercalates DNA, producing a bright red fluorescence. DHE was excited at 488 nm and fluorescence emission was detected with a 585- to 615-nm band-pass filter. Laser settings were identical for acquisition of all images. Bar graphs showing a significant reduction of fluorescence in cells after pretreated with DPI, but not with MXTZ.

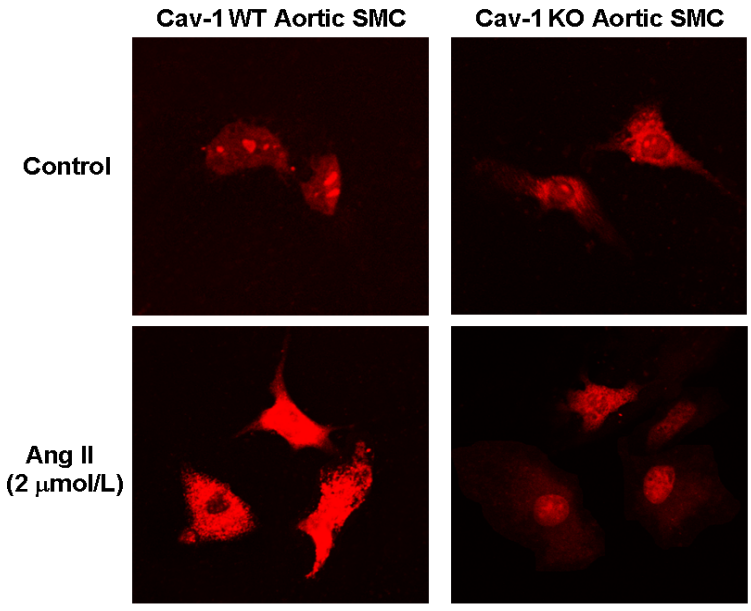

Figure V. Increased ROS production in aortic arterial SMC of WT but not cav-1 KO mice by Ang II

Freshly isolated aortic SMC from WT and cav-1 KO mice were incubated with 0 (control) or 2 μmol/L Ang II for 1 h, followed by incubation with DHE for 30 min. Ang II enhanced the intensity of bright red fluorescence in SMC from WT mice, but not in those from KO mice.

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