ONLINE SUPPLEMENT

IMPAIRED cAMP-MEDIATED SIGNALING IN IEX-1-DEFICIENT VASCULAR SMOOTH MUSCLE CELLS

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Running title: ***$G\alpha_{i2}$ in hypertension induced by IEX-1 deficiency***

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SUPPLEMETAL MATERIALS

Extended Methods

Treatments of hypertensive IEX-1 KO mice with anti-oxidants: IEX-1 KO mice and WT mice at 3 weeks (wks) of age were provided with daily drinking water supplemented with 10 g/L N-acetylcysteine (NAC, Sigma) for the indicated periods and SBP was measured every week. In a second series of experiments, a role for mitochondrion-derived superoxide (O_2^-) in IEX-1 deficiency-induced hypertension was determined by treatment of the mice with either MnTBAP or apocynin. MnTBAP (AG Scientific Inc, San Diego, CA) was first dissolved in 0.1 M NaOH and then diluted with sterile water, followed by intra-peritoneal (i.p.) injection of 5 mg/kg every day for two wks. Apocynin (Sigma, St Louis, MO) was solubilized in dimethyl sulfoxide (DMSO) and added to the drinking water at a final concentration of 1.5 mM apocynin with 0.05% DMSO. Drinking water containing only 0.05% DMSO was supplied to control mice[1]. In a third series of experiments, arterial BP was monitored by radiotelemetry in 12-wk-old IEX-1 KO and WT mice before and after i.p. treatment with either vehicle or pertussis toxin (PTX; List Biological Laboratories, Inc., Campbell, CA) at a dose of 20 μg/kg of body weight once a week.

Measurement of BP in mice: SBP was measured noninvasively in awake mice after a training period of 3 days with a tail-cuff pressure-recording device (Kent Scientific, Torrington, CT). Alternatively, arterial BP was monitored by radiotelemeters implanted in the carotid artery as described previously[2].

Measurement of vascular reactivity: Vascular reactivity of thoracic aortic rings was measured using a wire myograph as previously described[3]. Some of the rings were incubated with either 1 μM rotenone or 5 mM NAC for 2 hrs or with 100 ng/ml PTX for 1 hr before the cumulative dose-responses to isoproterenol (ISO) were measured[4]. These drugs previously have been demonstrated to reach and act effectively on VSMCs of ex vivo vascular preparation with intact endothelium of different species.

Angiotensin II (Ang II)-induced hypertension
Male mice at 12 wks of age were anesthetized and implanted subcutaneously with an Ang II-filled osmotic minipump (Alzet 1002, Durect Corporation). Ang II was infused at a dose of 0.7 mg/kg/day for 2 weeks[5].

Detection of vascular superoxide (O_2^-) production
Ethidium bromide fluorescence: Dihydroethidine (DHE), an oxidative fluorescent dye, was used to localize vascular O_2^- production *in situ* as previously reported[6]. Briefly, thoracic aortas were dissected, cut into 3-4 mm long rings, and incubated with DHE (5 μM, Invitrogen) at 37 °C for 45 min in dark followed by three washes with phosphate buffer saline (PBS). Some of the rings were preincubated with either rotenone (1 μM), MnTBAP (10 μM), or apocynin (100 μM and 500 μM) for 45 min before addition of DHE to determine the source of superoxide production. The resultant rings were then cut opened longitudinally to expose endothelial layer and analyzed under an inverted confocal laser scanning microscope (Zeiss Pascal LSM 5, Thornwood, NY, USA). The en face images were captured with the endothelial layer facing up using excitation wavelength 488 nm and emission 568 nm. Images were analyzed using the Zeiss Axionvision (Thornwood, NY, USA) imaging software.

Lucigenin chemiluminescence: Superoxide production was also measured by lucigenin chemiluminescence as described previously[7]. Briefly, cleaned aortas were cut into 5-mm ring segments, with care taken not to injure the endothelium. After 30 minutes of equilibration at 37°C, scintillation vials containing 1 mL Krebs/HEPES buffer with 5 μM lucigenin (Sigma) were placed into a scintillation counter switched to out-of-coincidence mode. Background counts were recorded, a vascular segment was added to the vial, and the chemiluminescence was recorded as scintillation counts per seconds (scps). The vessels were then dried at 60°C overnight, and the counts were normalized with background and dry tissue weight.

Detection of mitochondrial localized O_2^- production

Thoracic aortas were dissected, cleaned, cut into 3-4 mm long rings, and incubated with MitoSox Red (1 μM, Invitrogen CA) at 37 °C for 20 min in dark followed by three washes with prewarmed HBSS[8]. The rings were then immediately embedded in OCT medium, and cryosectioned. Some of the aortic rings were preincubated with a mitochondrial specific antioxidant MitoQ (100 nM) for 2 hrs before staining with MitoSox. Images were captured using an inverted confocal laser scanning microscope using excitation wavelength 510 nm and emission 580 nm. Images were analyzed using the Zeiss Axionvision imaging software.

Levels of mitochondrion-localized O_2^- production were also measured in cultured VSMCs isolated from the aorta using MitoSox Red [9]. Briefly, VSMCs were starved overnight with DMEM containing 2% fetal bovine serum (FBS) followed by three washes with HBSS. The cells were then loaded with MitoSox Red (1 μM) for 20 min at 37 °C in dark followed by three washes with prewarmed HBSS. MitoSOX Red fluorescence was immediately captured using a confocal laser scanning microscope as above. To confirm mitochondrial localization of MitoSOX Red, cells were loaded with 50 nM MitoTracker Green[10], a mitochondrial marker dye (Invitrogen/Molecular Probes) for 30 min at 37 °C before staining with MitoSox. The cells were washed three times with prewarmed HBSS buffer before loading with MitoSox Red. MitoTracker Green fluorescence was captured separately at excitation wavelength 490 nm and emission 516 nm.

RT-PCR for semi-quantifying vascular $G\alpha_{i2}$ and $G\alpha_{i3}$ mRNA expression

Aortas dissected from 10~12-wk-old mice were cleaned free of connective and adipose tissues and snap frozen in liquid nitrogen followed by homogenization. Total RNA was extracted from the resultant homogenate using a Trizol reagent (Sigma) per the manufacture's instructions. The RNA was reverse transcribed using SuperScript reverse transcriptase (Invitrogen) and amplified with primers specific for $G\alpha_{i2}$ and $G\alpha_{i3}$. The primers were $G\alpha_{i2}$, GAAGAGCACCATCGTCAAGC (forward) and CACAGGACAGGGCGAACAGC (reverse); $G\alpha_{i3}$, GCAGGTCCAGGGAATATCAG (forward) and CAGCCAGAACAAGGTCGTAA (reverse), and β-actin, CTTCTTTGCAGCTCCTTCGTTG (forward) and TCGTCCCAGTTGGTAACAATGC (reverse). PCR products were separated by 1.5% agarose gel electrophoresis and densitometry of individual bands was analyzed by NIH software Image J and normalized to the band density of control β-actin.

Immunoblotting for detection of $G\alpha_{i2}$ protein

Aortic cell membrane was prepared as described with some modifications[11]. Briefly, aortas were isolated and snap frozen in liquid nitrogen as described above, powdered with a pre-cooled pestle mortar, and placed in lysis buffer (50 mM Tris-HCl, 300 mM NaCl, 5 mM EDTA, 1% Triton 100x, pH 7.5, 1X protease inhibitor mixture, and 1 mM PMSF), followed by homogenization with a polytron. Disrupted cells were centrifuged at 500 x *g* at 4°C for 10 min to remove nuclei and unbroken cells. Supernatant was collected and the pellet was resuspended in lysis buffer and

centrifuged again as above. The supernatant was pooled, and spun at 60,000 x *g* for 25 min at 4 °C. The pellet containing plasma membrane was washed twice with lysis buffer. The membrane preparation equivalent to 10 µg protein was analyzed by western blotting using anti-$G\alpha_{i2}$ monoclonal Ab (Chemicon, Temecula, CA).

VSMC culture

VSMCs were isolated from mouse descending aorta by enzymatic digestion as described previously[12]. Briefly, the aortas were removed, cut open longitudinally, cleaned free of connective tissue, fat and endothelium, and digested with collagenase type II (175 units/ml) and elastase (1.25 units/ml) to remove the adventitia and to dissociate VSMCs. Single cell suspension was plated in a culture flask, and grown in DMEM medium supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin and fungizone. Cells were passaged by trypsinization, and passages 3 to 6 were used for experiments. To treat the cells with NAC, rotenone or MitoQ 10, confluent cell cultures were starved by incubation for 3 h in DMEM containing 0.1% FBS at 37^{o} C, after which the cells were incubated in the absence or presence of indicated concentrations of NAC, rotenone or MitoQ 10 for 24 h. The treated cells were washed and harvested, and the total RNA was extracted as above. The mRNA expression level of $G\alpha_{i2}$ was analyzed by semi-quantitative RT-PCR with β-actin as a control as described above. The cell viability was checked by tryptan blue exclusion. There was 90-95 % viability in each case and no significant differences between WT and IEX-1 KO cells after treatment.

Vascular wall and kidney morphometry

The kidney, aortic and second order mesenteric artery segments were obtained and processed as described[3]. Mice were initially perfused with saline, and then with 10% formalin. The fixed tissues were embedded, cut at 5 µm, and stained with hematoxylin and eosin (H&E). The kidney sections were also separately stained with Periodic Acid Schiff's reagent (PAS) or Masson Trichrome (MTr). The stained sections were analyzed using an Axiosphot system and AxioVision 4.6 software (Zeiss MicroImaging, Inc.). Arterial wall thickness was measured from the internal to the external elastic lamina at 5 evenly spaced sites around the segment.

Measurements of intracellular cAMP levels in cultured VSMCs

The intracellular cAMP levels were measured in cultured aortic VSMCs using a commercially available ELISA kit (Assay Design, MI). Confluent cell cultures were plated into 12-well plates at a density of $3x10^5$ cells/well and starved overnight with DMEM containing 0.1% FBS. The cells were then incubated with different concentrations of ISO or forskolin for 10 min. Some cells were also preincubated with PTX (100ng/ml) for 1 hr before addition of ISO or forskolin to the culture. After stimulation, cells were lyzed using 0.1 M HCl and centrifuged. The intracellular cAMP levels in supernatents were measured using the Acetylation method of a Direct cAMP Immunoassay kit (Assay design, MI) according to the manufacturer's instructions.

EXTENDED RESULTS

Generation of IEX-1-KO mice

The 7.5 Kb genomic DNA fragment of mouse IEX-1 was subcloned into p*Bluesscript* II using *Xba1* and *SacI* restriction enzyme sites, into which the pNTR-Lacz-PGK-neo fragment excised from the pNTR plasmid was inserted at the two Xho1 sites in the IEX-1 coding region, resulting in the removal of the most intron sequence and more than half of exon 2's sequence. The resulting plasmid with 3 Kb flanking sequence on each arm was ligated with the HSV-TK gene at the 5'-end for negative selection as illustrated in Figure 1. The linearized construct was introduced into embryonic stem (ES) cells followed by positive and negative selection in medium containing both G418 and ganciclovir. Genomic DNA from individual clones was screened for homologous DNA recombination by southern blot using probe A (P-A) or B (P-B) after digestion with either *stu1* and *EcoR1* or *BamH I*, as shown in the diagram (Figure 1A). ES clones with expected homologous DNA recombination such as clones 14 and 40 shown in Figure 1B were used to generate homozygous IEX-1-KO mice that were identified by PCR of tail DNA samples using specific primers (Fig. 1C).

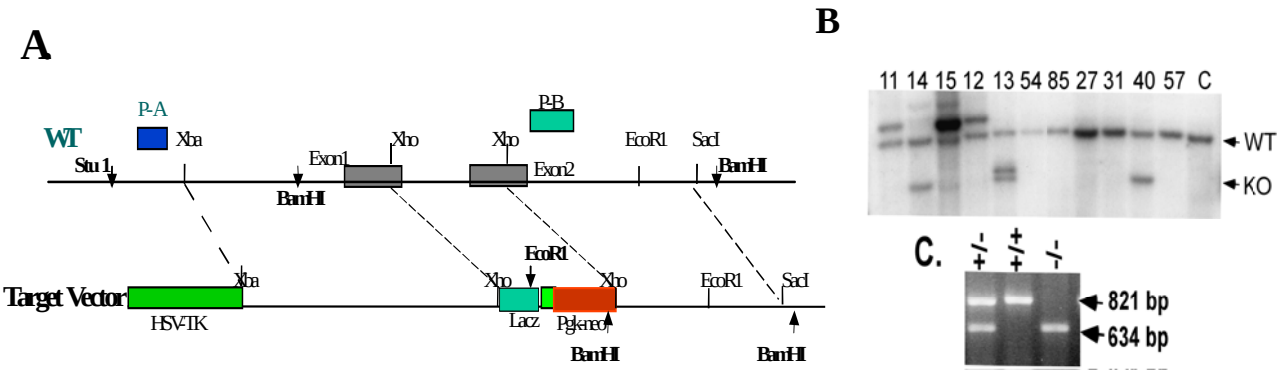

Figure S1. Diagram of IEX-1 genomic and target vector maps **(A)**, selection of IEX-1 recombinant ES clones by Southern blot (**B**), and genotyping of IEX-1 KO mice by PCR (**C**). **B** shows one of representative Southern blots, where a 5 kb DNA band results from IEX-1 DNA recombination (KO); and a 7 kd DNA band is yielded from WT IEX-1 genomic DNA (WT); the numbers on the top of the blot are the identities of individual ES clones; and the blot is hybridized with probe A. **C** shows one of the genotyping samples of IEX-1 KO mice where 821 bp and 634 bp PCR product are derived from WT and recombinant IEX-1 DNA, respectively.

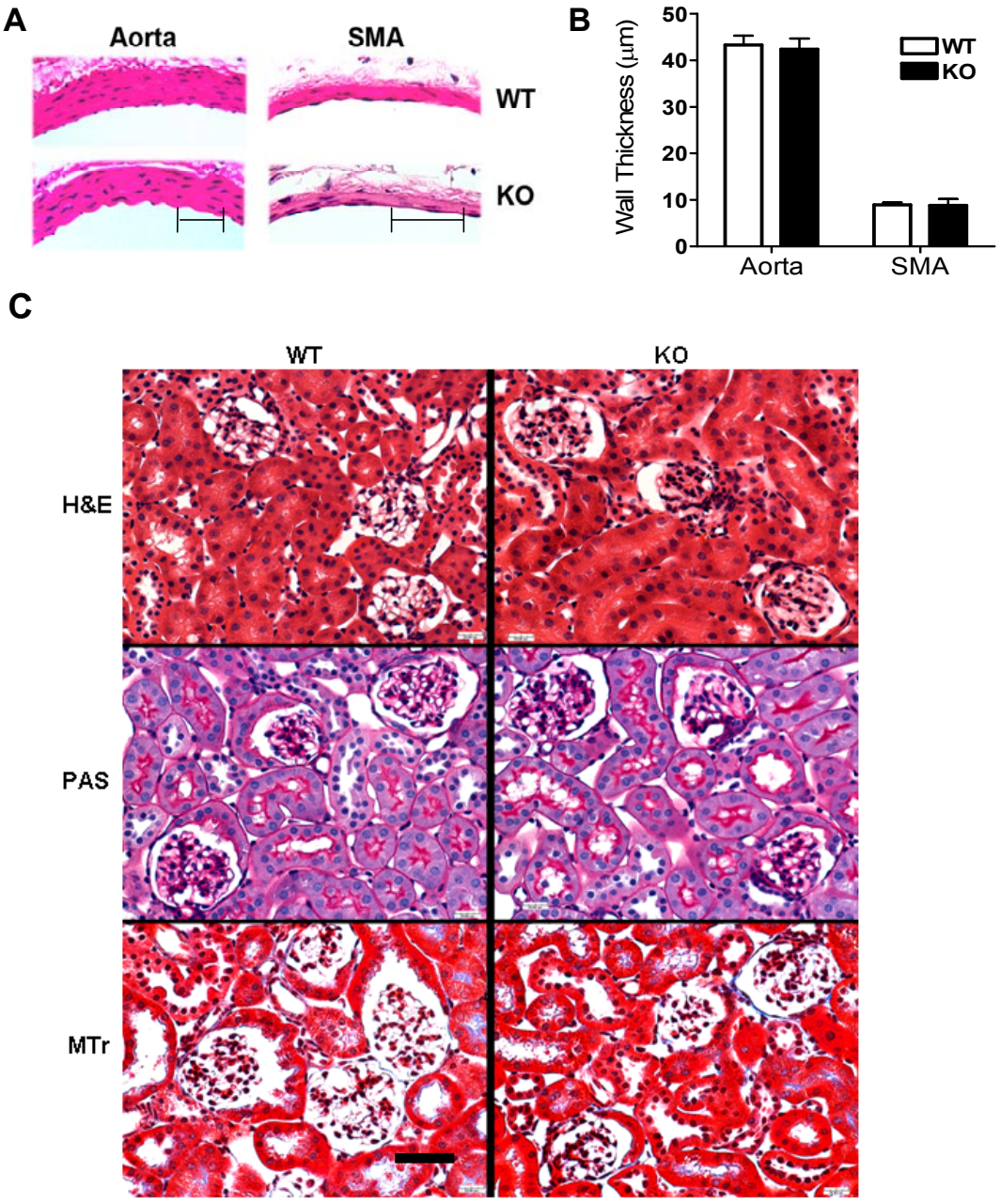

Figure S2. Similar morphology of aorta, second order mesenteric artery (SMA) or renal tissues. **A**. Hematoxylin and Eosin (H&E) -stained sections of aorta and SMA segments of IEX-1 KO (KO) and WT mice (WT). **B**. Planimetric analysis of wall thickness of aorta and SMA (n=3) in H&E-stained sections. **C**. Kidney tissue section stained with H&E (top), Perodic Acid Schiff (PAS) (middle) and masson trichrome (MTr) (bottom). Data are expressed as mean ±SEM of wall thinkness (µm) of three samples. Bar in **A** and **C**, 50 µm.

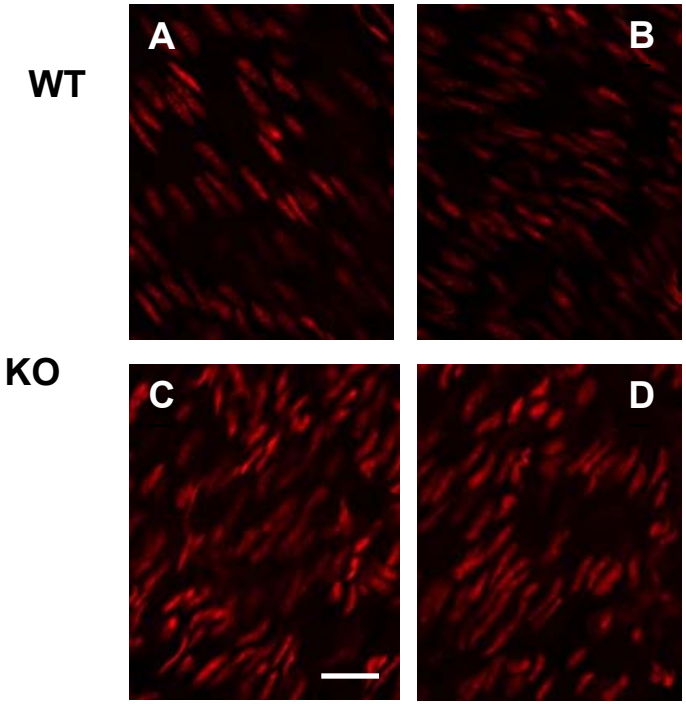

Figure S3. Increased superoxide production in vasculature of 4-wk-old IEX-1 KO mice. Dihydroethidium fluorescence intensity was stronger in smooth muscle cells in aorta of 4-wks-old IEX-1 KO mice (C & D) than WT control mice (A & B). Magnification: 40X. Bar = 25 μM

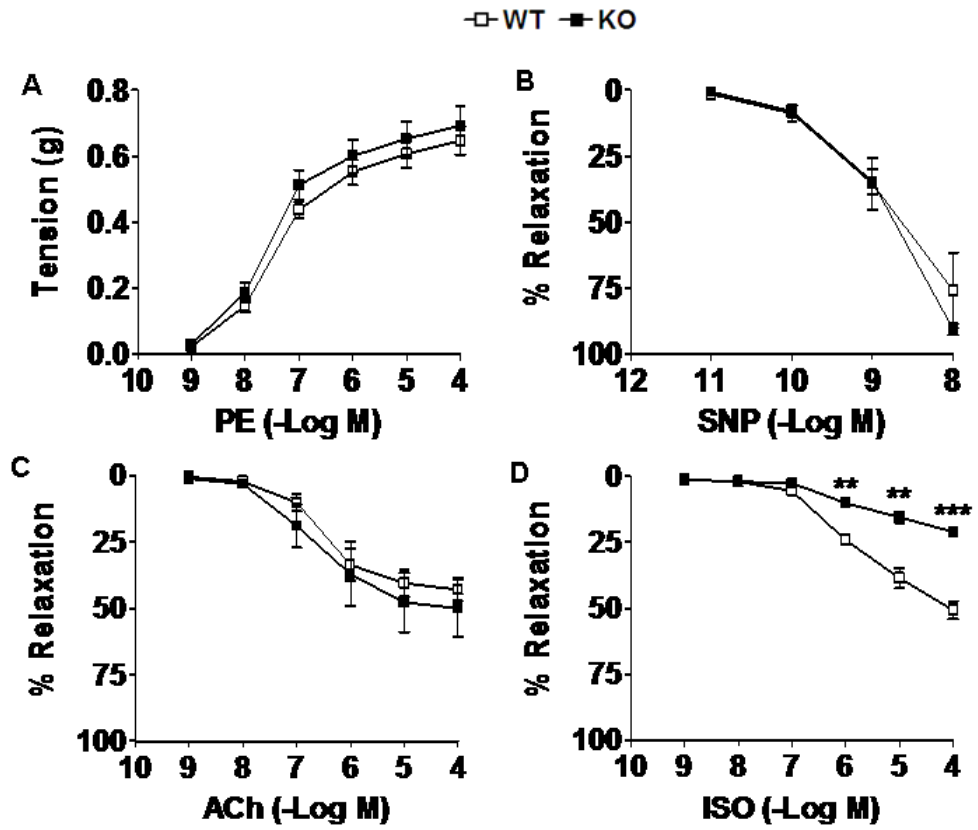

Figure S4. Cumulative concentration responses to phenylephrine (PE, **A**), sodium nitroprusside (SNP, **B**), acetylcholine (ACh, **C**), and isoproterenol (ISO, **D**) in aortic rings prepared from indicated mice. Vascular reactivity of thoracic aortic rings prepared from 3~4-wk-old mice was measured using a wire myograph containing Krebs-Henseleit buffer maintained at 37°C, pH 7.4 as described. Vasorelaxation responses were obtained on PE (10^{-6} M)-induced precontracted rings and was expressed as mean percentages ±SEM of the contraction induced by PE. * or **, $p<0.05$ or $p<0.01$, respectively, in the presence or absence of IEX-1.

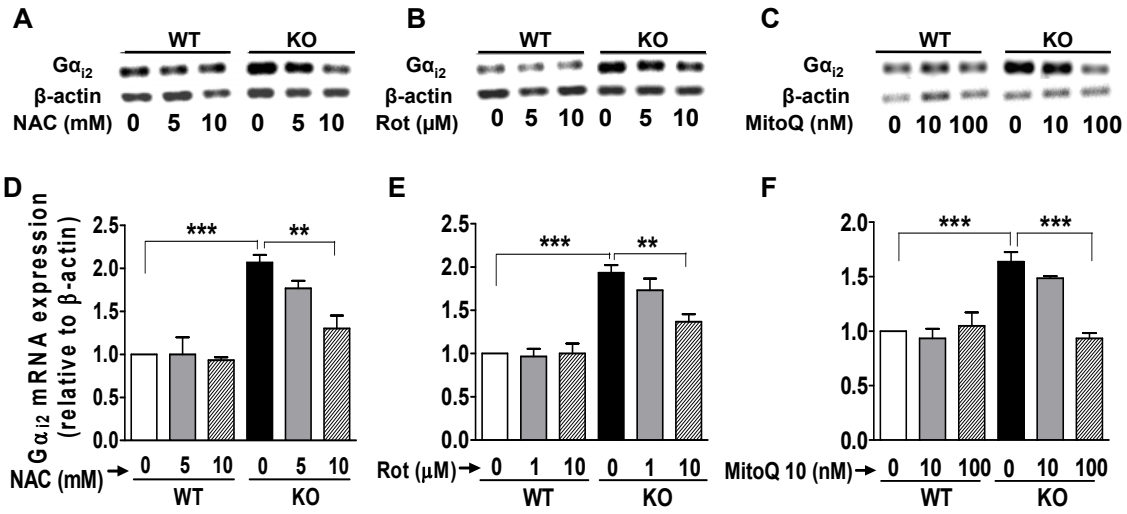

Figure S5. Gαi2 mRNA expression in IEX-1 deficient VSMCs depends mROS. The expression levels of Gαi2 were assessed by RT-PCR in cultured VSMCs isolated from the aorta of IEX-1 KO and WT mice and treated with NAC **(A),** rotenone **(B)** or MitoQ 10 **(C)**. One representative result of three independent experiments with similar results is shown. Relative intensity of individual bands was analyzed using β-actin as a control (D, E and F). Data are expressed as mean ± SEM of arbitrary units; *n* = 3. * p<0.05, ** P<0.01 and *** p<0.001 respectively in the presence or absence of IEX-1, NAC, rotenone or MitoQ 10.

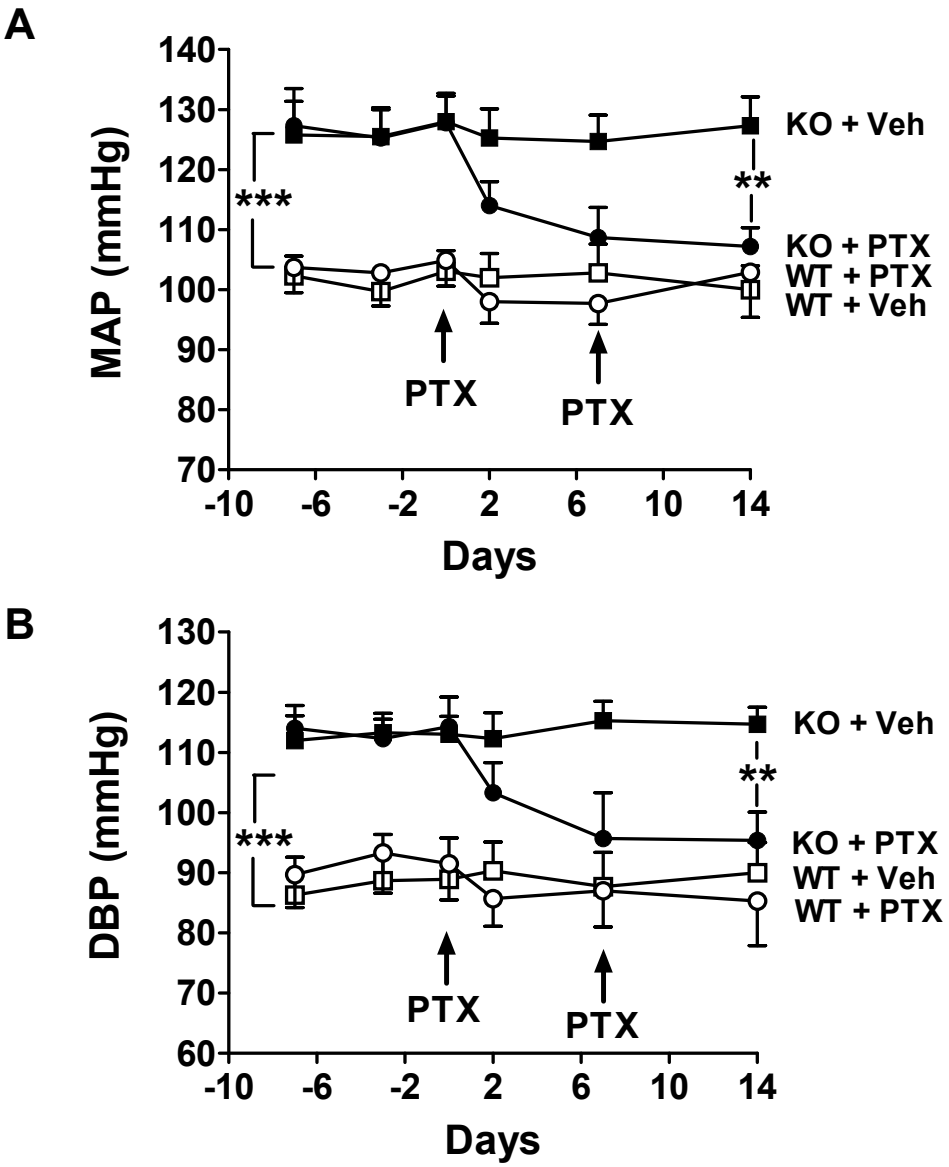

Figure S6. Mean arterial pressure (MAP) and diastolic blood pressure (DBP) of IEX-1 KO and WT control mice at 12 wks of age treated once a week with PTX (20 μg/kg intraperitoneally) or vehicle (veh) for two wks (arrows) (E). Arterial blood pressure was monitored for two wks by radiotelemetry. Data are expressed as mean ± SEM of MAP or DBP (mmHg); *n* = 5. ** $p<0.01$ and *** $p<0.001$ in the presence or absence of IEX-1 or PTX treatment.

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