K_{2P}6.1 Disruption Produces Hypertension

ONLINE SUPPLEMENT

DISRUPTION OF K_{2P}6.1 PRODUCES VASCULAR DYSFUNCTION AND HYPERTENSION IN MICE

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Methods

All studies were approved by the Institutional Animal Care and Use Committee at the Baylor College of Medicine.

Generation of *$K_{2P}6.1^{Tm(TG0009)Tigm}$* mice (herein referred to as *K_{2P}6.1* for the background strain)

Mice used in this study were made available on a collaborative basis through the Texas A&M Institute for Genomic Medicine (Houston, TX). The knockout strategy was to replace exon 1 of *K_{2P}6.1* with a selection cassette. Exon 1 encodes the first ~120 aa of the 313 aa protein and contains the coding sequence for the first transmembrane domain, the first pore region, and the site for dimerization (Figure S1A). Briefly, a gene replacement targeting vector, pKOS-30, containing the β-galactosidase/neomycin phosphotransferase fusion gene flanked by sections of DNA homologous to the 5' untranslated region and 5' intronic region to exon 1 of K_{2P}6.1 (NM_001033525.1) was created. This targeting vector was electroporated into the ES cells (derived from the 129S5/SvEvBrd mouse substrain) for selection of neomycin resistant clones. The ES cells containing the portion of the disrupted *K_{2P}6.1* gene were confirmed by Southern Blot. The ES cell was introduced into a blastocyst and implanted into a pseudopregnant mouse to create a chimeric mouse. This chimeric mouse was bred on a mixed C57/129 background and used for all K_{2P}6.1 studies. Figure S1B details the targeting strategy. Male mice were used in this study.

Genotyping

DNA from a 2-mm tail clipping of each mouse at 6 weeks of age was isolated using Qiagen's Blood & Tissue Kit (cat 69506) according to the manufacturer's instructions. 50 ng of purified DNA was used in a 20-μl PCR reaction to identify *$K_{2P}6.1^{+/+}$* (WT) and *$K_{2P}6.1^{-/-}$* (KO) male littermate pairs. The PCR reaction consisted of 10X PCR buffer, 5X Q solution (used with WT primers) (Qiagen), 1.5 mmol/L $MgCl_2$ (3 mmol/L was used with WT primers), 0.5 mmol/L dNTP mix, 2 units recombinant *Taq* DNA polymerase (Invitrogen cat 10342053), and 0.5 mmol/L forward and reverse primers. Primers used were *$K_{2P}6.1^{+}$* forward primer, *$K_{2P}6.1^{-}$* forward primer, and *K_{2P}6.1* reverse primer for both alleles as well as LacZ forward and reverse primers (see below for more details). PCR was performed on an Eppendorf thermocycler with the conditions as follows: initial 5 min denaturation step at 95°C followed by 35 cycles of denaturation for 45 sec at 94°C, annealing for 30 sec at 55°C, and extension for 1 min at 72°C. A final 5 minute extension step at 72°C followed the cycling. Individual PCR products were pooled and analyzed by gel electrophoresis. Briefly, 10 μl of PCR product with 6X loading buffer (Invitrogen) were loaded onto a 2% agarose gel with TBE buffer and ethidium bromide, run at 100V for 60 min, and visualized in a UV light box. The expected sizes of amplicons were 465 bp for the *$K_{2P}6.1^{+}$* allele, 537 bp for the *$K_{2P}6.1^{-}$* allele and 389 bp for the lacZ insert within the *$K_{2P}6.1^{-}$* allele. Presence of only the 465 bp band represents the *$K_{2P}6.1^{+/+}$* whereas both the 537 and 389 bp bands represent the *$K_{2P}6.1^{-/-}$*. Heterozygotes (*$K_{2P}6.1^{+/-}$*) possess all three bands. Figure S1C shows results from PCR genotyping.

The following genotyping primers were designed by TIGM and synthesized by Invitrogen:
$K_{2P}6.1^{+}$ fwd 5'-GAGCTGCCAACGCTTCGG, $K_{2P}6.1^{-}$ fwd 5'-GCAGCGCATCGCCTTCTATC, and K_{2P}6.1 rev 5'-CAAGGAAAGCCCTACCAAGG. Primers for lacZ were previously reported[1]:
lacZ fwd 5'-GTTGCAGTGCACGGCAGATACACTTGCTGA and lacZ rev 5'-CGGTGACCACACCCGGTATTAAGTTAAGCG.

RNA isolation and real-time PCR
To isolate RNA from the thoracic aorta, F1N1 littermate pairs were deeply anesthetized with a ketamine-xylazine-acepromazine combination followed by administration of 50 units of heparin. Under sterile conditions, the abdominal aorta was opened and the thoracic aorta was flushed with ice-cold HBSS via cardiopuncture. The thoracic aorta was carefully removed, placed into a dish containing ice-cold HBSS, and cleaned of connective tissue and fat. The thoracic aorta was blotted on a Kimwipe® and flash frozen in liquid nitrogen. RNA was isolated using the RNeasy Micro System (Qiagen) according to the manufacturer's instructions. Briefly, the aorta was ground with a mortar and pestle under liquid nitrogen, thawed to room temperature with buffer containing guanidine thiocyanate, bound to a silica-based membrane, treated with DNAse to remove trace genomic DNA, and eluted into water for real-time PCR analysis. To isolate RNA from heart, the heart was quickly excised and flushed of blood with ice-cold HBSS. TRIzol LS reagent (Invitrogen) was used with a Dounce homogenizer to disrupt the tissue and lyse the cells. The RNA fraction was isolated and processed according to the manufacturer's instructions, followed by DNAse treatment (Invitrogen). RNA purity was assessed by spectrophotometry with A260/A280 > 1.9. Reverse transcription using random hexamers and Superscript III was carried out using 0.1 μg and 1 μg total RNA for the aorta and heart respectively.

To isolate RNA from endothelial and smooth muscle cell fractions (4) Tie2-GFP mice were used at 8-12 weeks. Tie2 is expressed on endothelial cells and is not expressed on VSMC. Using fluorescent labeled cell sorting on aortic digests (aortas previously flushed of blood) allowed separation of endothelial and VSMC compartments. Thoracic aortas were dissected and digested as described under Electrophysiology Methods. A BD Biosciences FACS Aria II was used to sort GFP^{+} and GFP^{-} fractions directly into collection tubes for RNA extraction. Cell viability determined by propidium iodide uptake was >95%. Post-sort analysis revealed >95% purity in the fractions. RNA was extracted as described above with the exception of using a Qiagen Shredder for homogenization.

Primers were designed using Primer Express 2.0 software. The gene abbreviation is followed by the NCBI accession number and resulting amplicon size:
Gapdh [NM_008084 (111 nt)],
fwd 5'-AGCCTCGTCCCGTAGACAAAA and rev 5'-TGGCAACAATCTCCACTTTGC.
K_{2P}1.1 [NM_008430 (106 nt)],
fwd 5'-CCCAGAATTGCTACCACAAGC and rev 5'-TGGTCCTACACCGTTGCAAGA.
K_{2P}2.1 [NM_010607 (115 nt)],

fwd 5'-GAAAATGCCTACCCGTGCA and rev 5'-AATGAGAGCCTCGGTTTGGA.
$K_{2P}3.1$ [NM_010608 (135 nt)],
fwd 5'-ACATGGACTCCCCTTTGCTGT and rev 5'-CAAATGAATACGGAGGTGGCA.
$K_{2P}4.1$ [NM_008431 (100 nt)],
fwd 5'-CCACTGTAGGCTTTGGCGATT and rev 5'-GGCTAGGCCAAACAAGATCCA.
$K_{2P}6.1$ [NM_001033525 (119 nt)],
fwd 5'-AGGCATCGAAACCAGACGTGT and rev 5'-TCCCCCGTGTGACTTTCTACA.
$K_{2P}10.1$ [NM_029911 (105 nt)],
fwd 5'-GATTCAGCATGCACTCGATGC and rev 5'-CCCAGCAAAGAAGAAGGCACT.
$K_{Ca}1.1/\beta1$ [NM_031169 (103 nt)],
fwd 5'-GCCATGCTGTATCACACGGAA and rev 5'-TCTTCACATCTGCCAAGGCTGT.
$K_{Ca}3.1$ [NM_008433 (108 nt)],
fwd 5'-CGTGCACAACTTCATGATGGA and rev 5'-TCCTTCCTTCGAGTGTGCTTG.
eNOS [NM_008713, 125 nt),
fwd 5'-TGCAAACCGTGCAGAGAATTC and rev 5'-GCGCAATGTGAGTCCGAAAA.
sm22α [NM_011526.3, (133 nt)],
fwd 5'-TCCAGGTGTGGCTGAAGAATG and rev 5'-TTGAGCCACCTGTTCCATCTG.

The efficiency of each primer set was determined to be > 95% using high quality RNA from pooled heart and aorta samples. Real-time PCR using SYBR green and GAPDH-normalized expression was performed on WT and KO samples using identical conditions and processed simultaneously for each target and GAPDH. The PCR products were validated by direct sequencing methods (SeqWright, Houston TX).

Electrophysiology
For electrophysiology studies, freshly dispersed aortic VSMC were obtained by enzymatic dissociation using a papain/dithiothreitol cocktail, followed by a collagenase H /hyaluronidase cocktail (Sigma). Electrophysiological studies were measured using an Axopatch 200B integrated patch clamp amplifier and pClamp version 9.2 software. Membrane potential was determined using current clamp (i=0 pA) in the whole cell mode using perforated patches (Amphotericin B). The currents were normalized to individual whole cell conductance and expressed as current density (pA/pF) where noted.

For voltage clamp studies Ba^{2+} currents in ruptured patches were measured and used as a surrogate for Ca^{2+}. Pipette buffer consisted of (in mmol/L): 140 NMDG-Cl, 1 $MgCl_2$, 5 EGTA acid, 1 HEPES, and 3 Na_4ATP with the pH adjusted to 7.2 with HCl. Bath buffer consisted of (in mmol/L): 135 TEACl, 10 $BaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose with the pH adjusted to 7.4 with TEAOH as previously described[2]. P/4 leak subtraction was used with calcium channel currents. Currents during step potentials and voltages during current clamp mode were averaged when the traces were in a steady state condition. Pipettes were pulled and polished to resistances of 5-9 MΩ. Pipette buffer for current clamp studies consisted of (in mmol/L): 100 K-gluconate, 4.3 KCl, 1 $MgCl_2$, 0.1 EGTA, 10 HEPES with the pH adjusted to 7.1 with NaOH. Bath buffer consisted of (in mmol/L): 140 NaCl, 3 $NaCO_3$, 4.2 KCl, 1.2 KH_2PO_4, 0.1 $CaCl_2$, 10 glucose, and 10 HEPES with the pH adjusted to 7.4 with NaOH.

Body Composition Analysis
A Lunar PIXImus (GE Medical Systems) was used to non-invasively measure bone and tissue composition of 6 littermate pairs of each genotype by a blinded observer.

Non-invasive blood pressure determination by tail plethysmography
Blood pressure was measured on 6 littermate pairs of each *K_{2P}6.1* genotype using tail cuff plethysmography, a six-channel NIBP system, and BPMonWin software. The NIBP system was calibrated to known pressures using an inline mercury bulb sphygmomanometer. The mice were 8-12 weeks in age and weighed from 20 to 32 g. Prior to making the measurements, each mouse was acclimatized to the measuring chambers for 30 min on four consecutive days. Systolic, mean arterial blood pressure and heart rate were recorded by the BPMonWin software automatically based on the flow waveforms and collated by a blinded observer.

Cardiovascular phenotyping of *K_{2P}6.1* mice
Six littermate pairs of *K_{2P}6.1* mice of each genotype were anesthetized with Nembutal (50 mg/kg, i.p.) (Sigma). Body temperature was monitored and maintained at 37° C. Electrocardiogram was taken from electrodes attached to the paws. Blood flow velocity was measured in the aortic arch and the abdominal aorta using pulsed Doppler ultrasound[3,4]. Arterial and intraventricular pressures were measured using a Millar catheter/pressure transducer (SPR1000, 1F ~ 0.33mm [Millar Instruments Inc, Houston TX]) inserted into the right carotid artery and advanced into the left ventricle. Cardiac function and coronary flow reserve were assessed from these measurements as previously described[4-7].

Histology
Aortic rings were fixed in 10% neutral buffered formalin overnight and embedded in paraffin, cross-sectioned at 4 μm, and stained with Verhoeff's Elastic and Masson's Trichrome stain. Images from two consecutive sections were obtained from four equally –spaced locations of the thoracic aorta using a Zeiss Axioplan I microscope with a 5x objective by a blinded observer. The perimeter of the luminal (P_{Lu}) and abluminal (P_{Ab}) boundaries of each section of the aorta were measured using Adobe Photoshop CS3 Enhanced. Lumen diameter was calculated by P_{Lu}/π. Wall thickness was calculated by $P_{Ab}/2\pi - P_{Lu}/2\pi$.

Isomeric force from aortic rings
Changes in isometric force were measured in aortic ring segments from KO mice and WT littermates (8-12 weeks). Thoracic aortas were harvested as described above and cleaned of connective tissue and visceral fat. The aorta's were cut into 2-mm rings and mounted on metal stirrups attached to a myography (ChuelTech, Houston, TX). Isometric force was measured at 37°C in Krebs buffer (in mmol/L): 119 NaCl, 4.7 KCl, 1 $MgSO_4$, 1.2 KH_2PO_4, 24 $NaHCO_3$, 11 glucose, 2.5 $CaCl_2$. The buffer was equilibrated with a gas consisting of CO_2 5% / O_2 20% / N_2 balance for pH=7.4. Krebs buffer was refreshed every 15-20 min. Changes in force in each ring were measured using a Research Isometric Transducer (Harvard Apparatus, Holliston, MA). Data were

acquired and analyzed using Powerlab/8sp (AD Instruments, Colorado Springs, CO) at 10 Hz by LabChart v4.2.4 (AD Instruments). Aortas were allowed to equilibrate for 60 minutes under minimal force followed by slowly increasing the force to a resting point of 15 mN (except where noted). Rings were then contracted with 40 mmol/L KCl in Krebs buffer and washed with normal Krebs buffer (KCl 5.4 mmol/L). Rings were repeatedly contracted with 40 mmol/L KCl in Krebs buffer until the force developed was constant from 2-consecutive contractions. Only one experiment was performed on each aortic ring. For some studies rings that were treated with LNAME were incubated with 100 μmol/L LNAME for 60 minutes.

Proliferation and Migration Studies

Migration and proliferation were studied in cultured VSMC from KO and WT mice using the Electric Cell-Substrate Impedance Sensing (ECIS) method (Applied Biophysics)[8,9]. Each well of the ECIS array contained gold electrodes. The technique involves passing very weak currents across ten gold electrodes. As cells proliferate and/or migrate to cover the electrode, electrical resistance and impedance increase while capacitance decreases. As a result the ECIS acts as a biosensor to determine the dynamics of proliferation or migration.

VSMC from WT and KO littermates were cultured from the thoracic aorta[10], passed three times, and plated in wells of an ECIS eight-well array (8W10E, Applied Biophysics). Cells from a single aorta were plated at a density of 30K cells per well (300 μL total per well) in each of four wells. Resistance or conductance measurements of these four wells were averaged to obtain a single observation. For proliferation studies, the capacitance at 64 kHz was measured every 10 min after seeding VSMC until a plateau was reached indicating confluence. The percent of the electrode area covered by VSMC at each time point was calculated by the following equation[8]: % of the electrode covered by cells = 100 X (C_{open}-C_{cells})/ (C_{open} -$C_{confluence}$) where C_{open} = capacitance of electrode without cells, C_{cells} = capacitance at different time points after seeding VSMC, and $C_{confluence}$ = capacitance upon the plateau.

For migration studies, VSMC were allowed to grow to confluence in wells of the ECIS array. After reaching confluence, proliferation was inhibited by changing to serum-free medium for 24 hours. Cells over the circular electrodes were killed by applying a potential of 5V delivered at 40 kHz for a duration of 30 seconds[8]. Electrical resistance was measured at 4 kHz every ten minutes as the VSMC migrated over the electrode to replace the killed cells. The migration rate was calculated from the time taken for the electrical resistance measure to plateau; i.e., the time required for cells to migrate inward from the perimeter of the round electrode to the center (125 μm radius).

Cell Volume Regulation

Osmotic volume regulation was studied in freshly dispersed thoracic aorta VSMC obtained from littermate pairs of WT and KO mice[11,12]. Cell volumes were measured before and at 1, 2, 4, and 6 min after reducing the osmotic pressure in the bath buffer by 50%. The isoosmotic bath buffer consisted of (in mmol/L): 145 α-mannitol, 54.8 NaCl, 3 $NaCO_3$, 4.2 KCl, 1.2 KH_2PO_4, 0.1 $CaCl_2$, 10 glucose, and 10 HEPES with the pH

adjusted to 7.4 with NaOH (osmolality 290 mOsm/kg). The hypoosmotic bath buffer was identical to the isoosmotic bath but without α-mannitol (osmolality contribution of 145 mOsm/kg). The osmolality of all buffers was assessed using a Wescor Vapro 5520. The average area of cells in each well was determined using Adobe Photoshop CS3 Enhanced perimeter detection and area calculation by automated analysis of all image files with identical conditions. Advanced edge detection algorithms were used to delineate each cell boundary. A single cell was tagged and followed through the experimental conditions generating 5 total image files (baseline, 1, 2, 4, and 6 minutes after changing to the hypoosmotic buffer). On average 11 cells from a single aorta were considered a single observation from each experimental group.

Apoptotic volume decrease was measured in cultured VSMC on their 3rd passage from WT and KO mice[11,13-15]. A single cell was tagged and followed through the experimental conditions generating 5 total image files (baseline, 1, 2, 4, and 6 hours after initiation of apoptosis with 1 μmol/L staurosporine). On average 4 cells from a single aorta were considered a single observation from each experimental group. Cell volumes were measured as described above.

Data analysis:
The data were reported as mean ± standard error of the mean (SEM) or mean ± standard error of the least mean squares (SELMS) as noted. Data was analyzed using the two-way repeated measures analysis of variance followed by post hoc Holm-Sidak analysis when appropriate. Other data was analyzed using the Students T-test with a Bonferroni correction. *P* values of 0.05 or less were considered significant.

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Tables and Figures:

Table S1. Body composition of K_{2P}6.1 KO and WT mice. n=6 per experimental group. BMD (bone mineral density), BMC (bone mineral content).

Body composition analysis	**Units**	**WT**			**KO**			**T-test**
Weight	gms	28.6	±	0.9	27.5	±	1.4	0.49
Length	cm	9.4	±	0.1	9.5	±	0.1	0.45
BMD	mg/cm^2	0.05	±	0.01	0.09	±	0.05	0.34
BMC	mg	0.46	±	0.02	0.45	±	0.02	0.87
Surface area	cm^2	9.1	±	0.3	9.1	±	0.3	0.93
Lean	gms	18.8	±	0.6	18.6	±	0.7	0.83
Fat	gms	4.4	±	0.3	4.6	±	0.5	0.61

Table S2. Cardiovascular indices measured by catheter and pulsed Doppler ultrasound in WT and KO mice. Peripheral vascular resistance was calculated from mean arterial pressure and mean aortic velocity. Coronary flow vascular reserve was calculated by the maximum coronary flow velocity (induced by 2.5% isoflurane) over the basal flow velocity. n=6 per experimental group. *p<0.05. PkLVP (peak left ventricular pressure), LVEDP (left ventricular end-diastolic pressure), IVCT (isovolumic contraction time), IVRT (isovolumic relaxation time).

Cardiac phenotyping	Units	WT			KO			T-test
Aortic pressure								
Systolic	mmHg	98	±	3	115	±	6	0.05*
Diastolic	mmHg	64	±	5	80	±	5	0.05*
Mean arterial	mmHg	81	±	4	98	±	6	0.04*
Pulse	mmHg	34	±	3	35	±	2	0.73
Heart rate	beats/min	415	±	35	517	±	49	0.43
Ventricular pressure								
PkLVP	mmHg	105	±	3	115	±	6	0.16
LVEDP	mmHg	6	±	0.1	5	±	10	0.43
+dP/dtmax	mmHg/s	8303	±	811	10891	±	1367	0.14
-dP/dtmax	mmHg/s	-9150	±	849	-11642	±	991	0.09
Tau	msec	8	±	1	7	±	1	0.21
Mitral inflow analysis								
E-A peak velocity ratio	----	1.3	±	0.1	1.3	±	0.1	0.76
IVCT	msec	15	±	2	13	±	1	0.37
IVRT	msec	18	±	1	23	±	3	0.12
Pulse wave velocity	cm/sec	570	±	144	667	±	92.3	0.39
Aortic outflow analysis								
Mean velocity	cm/sec	29.2	±	1.2	25.9	±	2.3	0.24
Mean acceleration	cm/sec^2	8476	±	645	6746	±	526	0.89
Peak acceleration	cm/sec^2	15336	±	646	13324	±	758	0.91
Coronary flow								
Peak diastolic velocity	cm/sec	33.0	±	4.6	29.4	±	4.2	0.58
Vascular reserve	----	2.56	±	0.28	2.30	±	0.29	0.56
Calculated indexes								
PVR	$dyne\text{-}s/cm^3$	954	±	79	1304	±	96	0.02*
Ejection time	msec	45	±	1	57	±	4	0.11
C Tei index	----	0.78	±	0.02	0.74	±	0.05	0.59
M Tei index	----	0.72	±	0.03	0.67	±	0.04	0.36

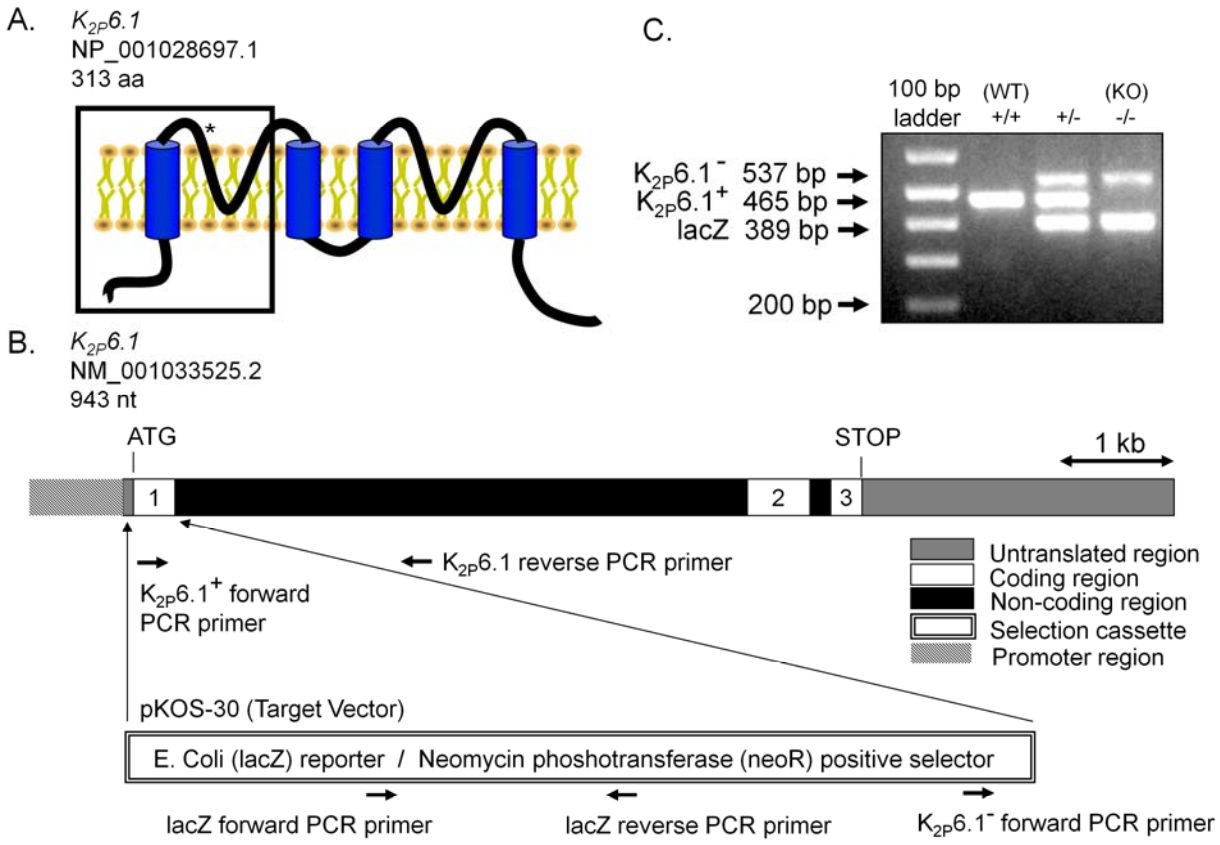

Figure S1.
(A) Predicted protein structure of *K_{2P}6.1* showing the translation product of the first exon in a white square; the dimerization domain is marked by an asterisk. (B) Strategy for gene targeting and generation of *K_{2P}6.1* $^{-/-}$ mice with the location of primers for genotyping and PCR products for $K_{2P}6.1^{+}$ and $K_{2P}6.1^{-}$. (C) Agarose gel analysis of genomic DNA shows *K_{2P}6.1* $^{+/+}$ (WT) animals possess the 1st exon/intron junction and *K_{2P}6.1* $^{-/-}$ (KO) animals have the first exon replaced by the selection cassette and the lacZ gene. The heterozygotes carry a copy of both the *K_{2P}6.1* $^{+}$ and *K_{2P}6.1* $^{-}$ gene forms (*K_{2P}6.1* $^{+/-}$).

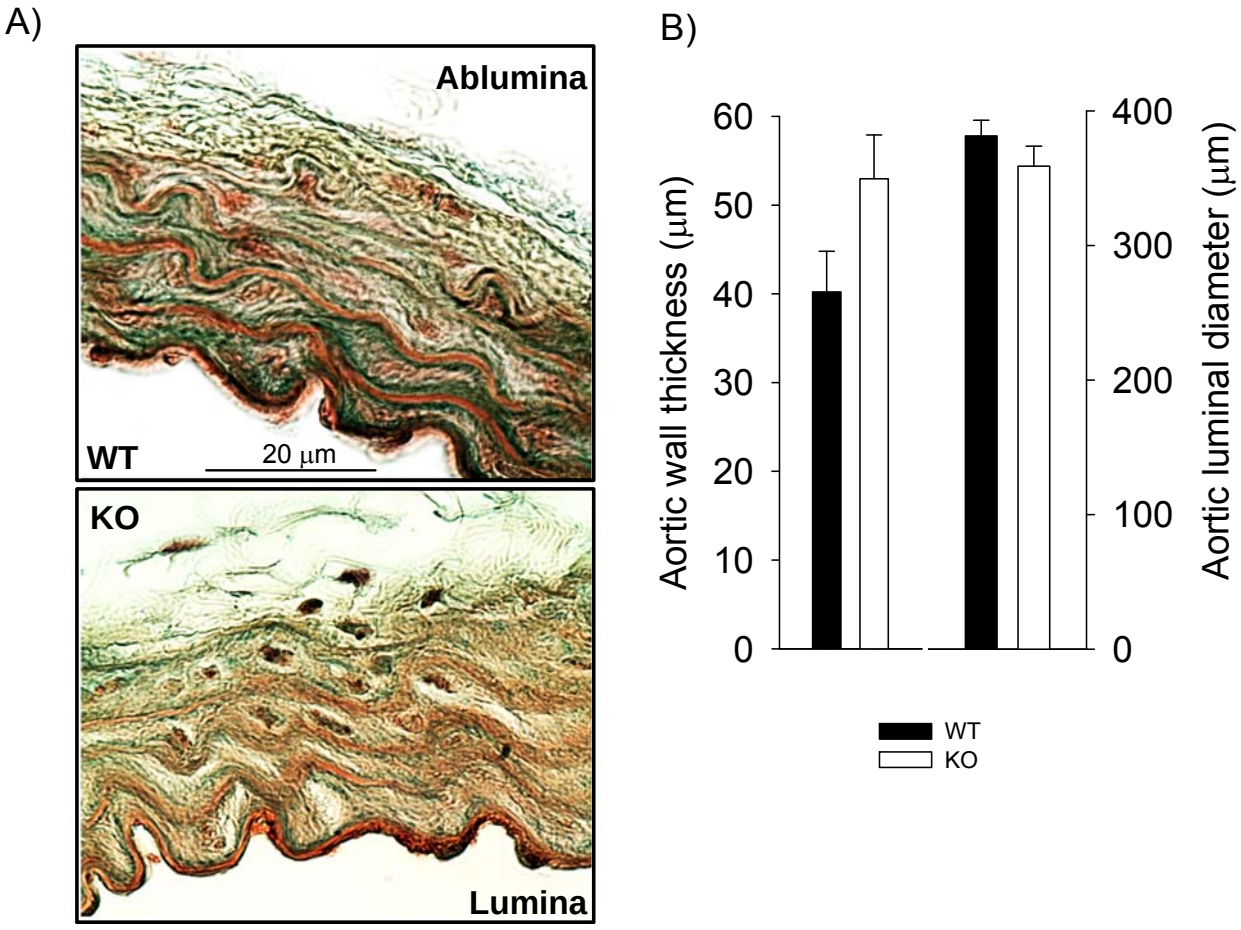

Figure S2. (A) Histological cross section of the aortic wall in WT and KO mice at 8-12 weeks. (B) Luminal diameter and wall thickness of aorta in WT and KO mice. Images from 3-6 serial sections per ring were averaged, with 2-4 rings per thoracic aorta. n=6 per experimental group.

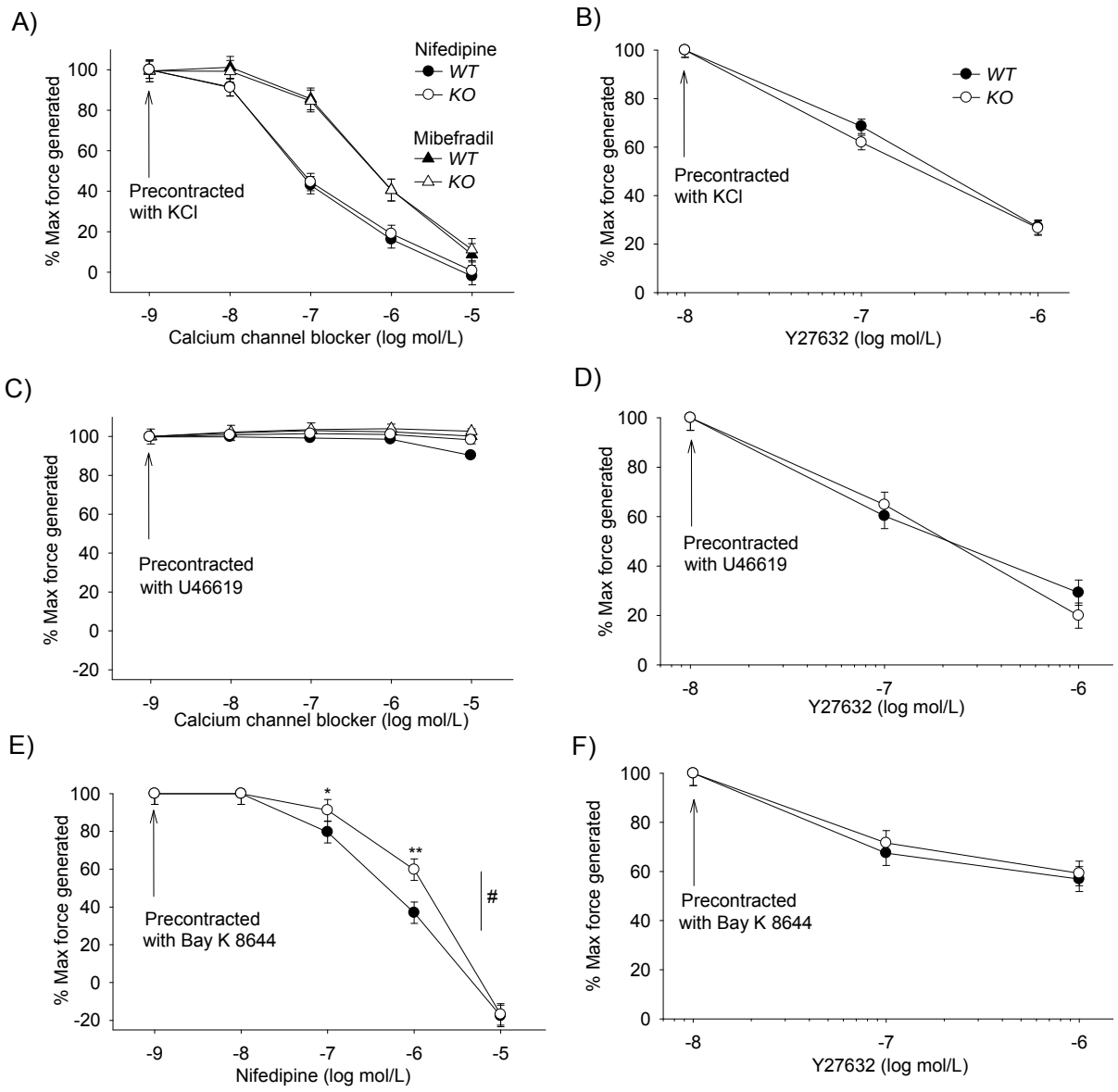

Figure S3. Relaxations of aortic rings to (A) nifedipine, an L-type, or mibefradil, a T-type calcium channel blocker, and (B) Y27632, a rho kinase inhibitor, when precontracted with 60 mmol/L KCl. Relaxations of aortic rings to (C) nifedipine or mibefridil and (D) Y27632, when precontracted with 10^{-6} mol/L U46619. Relaxations of aortic rings to (E) nifedipine and (F) Y27632 when precontracted with the L-type calcium channel activator, BAY K 8644. n=5-8 for each experimental group. $^{\#}p<0.05$ using 2-way RM-ANOVA, *p<0.05 and **p<0.001 using the Holm-Sidak method for multiple comparison.

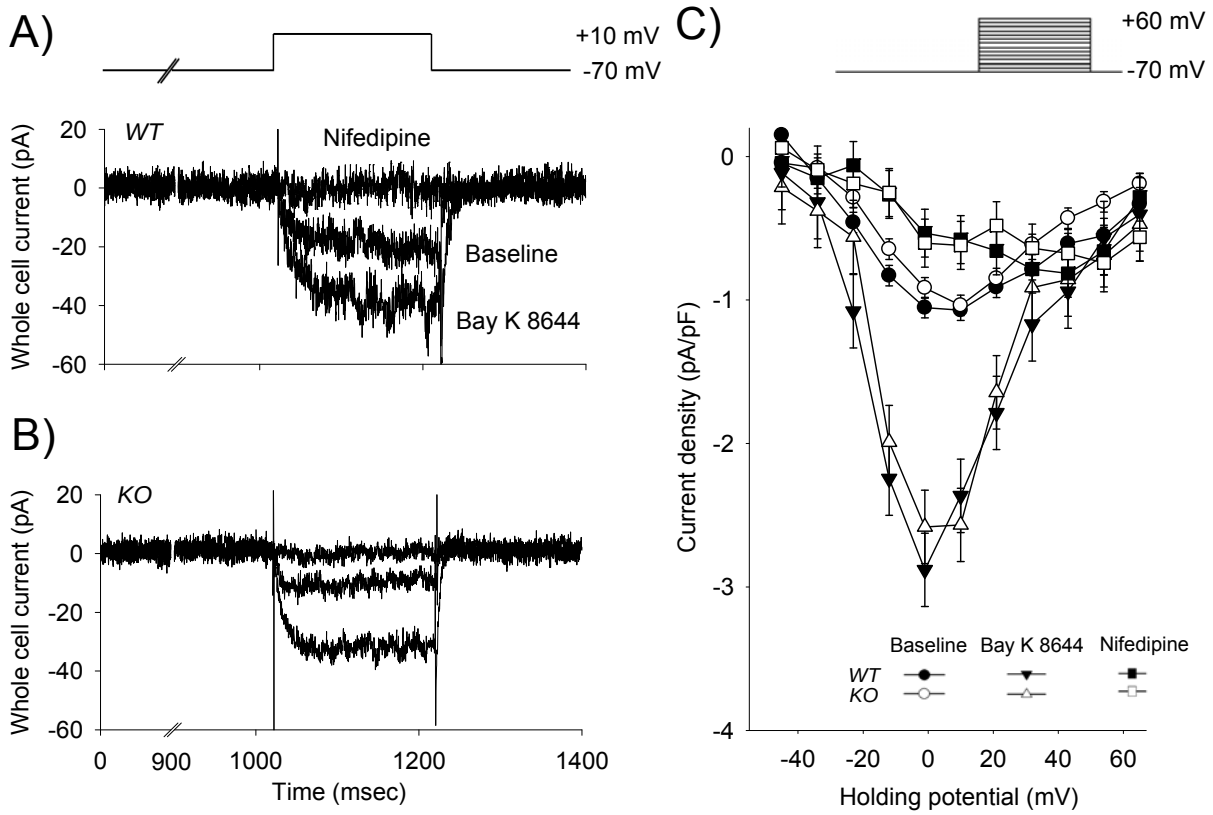

Figure S4. Ba^{2+} currents in freshly dispersed aortic VSMC. Ba^{2+} was used as a surrogate for Ca^{2+}. (A) and (B) shows WT and KO raw traces at baseline, in response to 10^{-5} mol/L BAY K 8644, and in response to BAY K 8644 plus 10^{-6} mol/L nifedipine, respectively. (C) Summary data for pulse protocol. n=5 per experimental group.

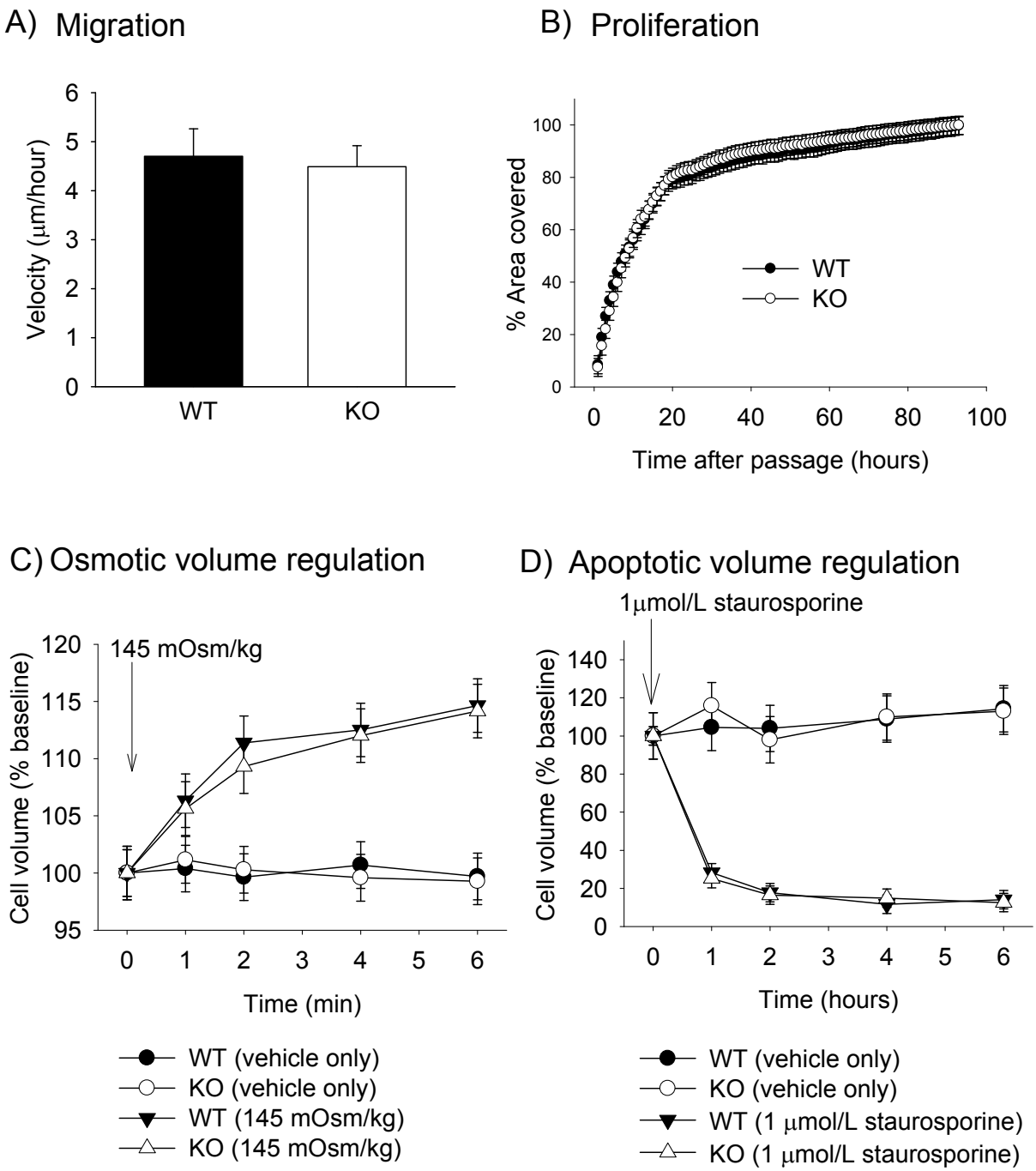

Figure S5. (A) Migration rate of cultured aortic VSMC. (B) Cell proliferation, expressed as percent of confluence (100%), in cultured aortic VSMC over time. (C) Percent change in cell volume of freshly dispersed aortic VSMC over time after decreasing osmotic force in the bath by 50%. (D) Percent volume decrease during apoptosis resulting from the addition of 1 μmol/L staurosporine to cultured aortic VSMC. Data are not significant between genotype. n=4-8 per experimental group.