On-line Supplement

The β3 Subunit Contributes to Vascular Calcium Channel Upregulation and Hypertension in Angiotensin II-infused C57BL/6 Mice

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Methods

Animals

All procedures involving animals were approved by the Institutional Animal Care and Use Committee at the University of Arkansas for Medical Sciences (UAMS). Initial studies used C57BL/6 mice obtained at 10 weeks of age from Charles River Laboratory (Wilmington, MA). The mice were maintained in a temperature controlled room in a 12:12-hour light:dark cycle with free access to food and water. A pair of heterozygous ($Ca_v\beta3^{+/-}$) mice was obtained from Dr. William Guido at the Virginia Commonwealth University with permission of the original designer, Dr. Hee-Sup Shin from the Korea Institute of Science and Technology. Colonies of mice were established in the Division of Laboratory Animal Medicine at UAMS. Genotyping was done by the Extract-N-Amp™ Blood PCR kit (Sigma-Aldrich, St. Louis, MO) using blood drawn from tails of 4 week-old mice and PCR was performed using primer sets and conditions described earlier.[1]

Blood pressure measurement and minipump implantation

Systolic blood pressure (SBP) was recorded using tail-cuff plethysmography (IITC Life Science, Woodland Hills, CA). Briefly, 10 to 12 week-old mice were trained for one week (once daily) before recording baseline SBP for 3 consecutive days. For implanting osmotic minipumps, mice were anesthetized with 5% isoflurane and maintained with 2% isoflurane inhalation in 100% O_2. Osmotic minipumps (Alzet 2004, Durect Corporation, Cupertino, CA) containing angiotensin II (Ang II, Bachem, Torrance, CA, infusion rate: 2 ng/g/min), or an equal volume of vehicle (0.9% saline) were implanted subcutaneously. After recovery from anesthesia, mice were housed in individual cages and allowed free access to food and water. The SBP recordings were continued on days 4, 7, 10 and 14 following minipump implantation.

Vascular reactivity assays

Second order mesenteric arteries (MA) were isolated from saline and Ang II-infused mice. After cleaning of adhered fat and connective tissue, arteries were placed in physiological salt solution (PSS) containing (in mmol/L): NaCl (119), KCl (4.7), $MgSO_4$ (1.17), $NaHCO_3$ (24), EDTA (0.026), NaH_2PO_4 (1.17), glucose (5.55) and $CaCl_2$ (1.6) in a heated (37°C) chamber bubbled with a 95% O_2-5% CO_2 gas mixture. MA were cannulated on both ends with tapered glass micropipettes in a microvessel perfusion system (Living Systems, St. Albans, VT).[2] Arteries were perfused with PSS at an intraluminal pressure of 80 mm Hg with no outflow. The PSS in the chamber was exchanged every 15 minutes during equilibration and after application of reagents. After equilibration for 1 hr, the viability of MA was verified by observing a contractile response to 60 mmol/L KCl. Internal diameter changes were recorded using an upright microscope/Spot RT camera operated with MetaVue acquisition software (Molecular Devices, Inc. Sunnyvale, CA) and analyzed with an automated edge-detection script in IPlab software (BD Biosciences, San Jose, CA).

Patch clamp studies

VSMCs were enzymatically isolated from 2nd order MA branches and whole-cell Ca^{2+} channel currents were recorded using standard pulse protocols, recording solutions and a patch-clamp station described previously.[2,3] Briefly, recordings were obtained at 25°C using 10 mmol/L $BaCl_2$ as a charge carrier to limit current rundown. With the use of -70 mV as the

constant inter-pulse holding potential, whole-cell currents were elicited in response to progressive 8 mV voltage steps from -70 mV to +58 mV. The identity of $Ca_v1.2$ channel currents was verified by their block by 1 µmol/L nifedipine in a subset of cells. The pipette solution consisted of (in mmol/L): cesium glutamate (145), $MgCl_2$ (1), HEPES (10), EGTA (10), and Na_2ATP (3). The bath solution (pH 7.3 with TEA-OH) contained (in mmol/L): $BaCl_2$ (10), TEA·Cl (135), $MgCl_2$ (1), HEPES (10), and glucose (10). Voltage-dependent activation and inactivation curves were fit by the Boltzmann equation: $I/I\text{max} = A_2 + (A_1 - A_2)/(1 + \exp((V - V_{1/2})/s)$, where V is the command voltage, $V_{1/2}$ is the half-maximal potential, s is the slope factor, A_1 is the maximum value of the ratio I/Imax (fixed at 1), and A_2 is the minimum value of the ratio I/Imax. Data analysis was performed using Clampfit (Molecular Devices, Inc. Sunnyvale, CA) and Origin (OriginLab Corp., Northampton, MA).

Quantitative real-time PCR

Total RNA was isolated using RNeasy Plus Mini kit (Qiagen Inc., Valencia, CA) from 2nd order branches of MA pooled from two mice. RNA concentration and purity was measured using a Nanodrop spectrophotometer (ND1000, Thermoscientific, Wilmington, DE). The cDNA was synthesized from DNase-treated RNA samples using High Capacity RNA-to-cDNA Kit (Applied Biosystems, Carlsbad, CA). After reverse transcription, quantitative real-time PCR was performed using an iCycler iQ® System (Bio-Rad, Hercules, CA) and primers specific for α_{1C} (F = 5'-TGTCCCTCTTCAACCGCTTTGACT-3' R = 5'-GTTCAGCAAGGATGCCACAAGGTT-3', β3 (F = 5'-TGAGGTCACGGACATGATGCAGAA-3' R= 5'-TGATGGTCCTCTTGCCAG GATTGT-3'), or GAPDH (F = 5'-TGGCAAAGTGGAGATTGTTG-3', R = 5'-CATTATCGG CCTTGACTGTG-3') sequences. In some experiments, PCR was paused on the steep slope of the amplification curve and amplified products were loaded on a 2% agarose gel to verify a single band of expected size and visualize their abundance using the Molecular Imager Gel Doc system (Bio-Rad, Hercules, CA).

Western blotting

Lysates were prepared by pooling MA from two mice in tissue lysis buffer containing (in mM): HEPES (50), NaCl (150) EGTA (1) $MgCl_2$ (1.5), PMSF (0.01), 1% Triton X-100, 10% glycerol and 5 µg/mL of the following protease inhibitors: leupeptin, aprotinin,and antipain. The protein concentration was measured using the Bradford protein assay. Forty micrograms of the total protein was subjected to gel electrophoresis under reducing conditions on 7% tris acetate gels and electrophoretically transferred to a PVDF membrane using a XCell *SureLock*™ Mini-Cell Western blotting kit (Invitrogen, Carlsbad, CA). The membranes were then blocked with 10% non-fat dry milk (Bio-Rad, Hercules, CA) prepared in tris-buffered saline containing 0.5% Tween-20 (TBST) and incubated overnight at 4°C with primary antibodies reconstituted in 5% milk. Specific polyclonal antibodies raised against α_{1C} (1:200, Alomone) and $Ca_v\beta3$ (1:2500, Abcam), or monoclonal anti-GAPDH (Sigma-Aldrich, St. Louis, MO) were used for protein detection. After incubation with primary antibodies, the membranes were incubated for one hour at room temperature in HRP linked secondary anti-rabbit (1:5000) or anti-mouse (1:20,000) antibodies (GE healthcare, Piscataway, NJ). Membranes were washed with TBST (3 times, 10 minutes per rinse), and bands were identified by chemiluminescence on X-ray film.

Data analysis and statistics

Densities of immunoreactive bands developed on X-ray films were measured using Scion Image software and normalized to GAPDH. The threshold cycle (C_T) values for the $Ca_v1.2$ channel α_{1C} and $Ca_V\beta3$ subunits were normalized using GAPDH as an internal standard and analyzed by the $\Delta\Delta C_T$ method for relative quantification of each subunit transcript. Calcium channel current (pA) was normalized for whole-cell capacitance (pF) and reported as current density (pA/pF). Changes in diameter were calculated as percent of the initial constrictor response to KCl (60 mmol/L). Data represent mean ± standard error of the mean for the number (n) of animals indicated in each figure. Student's *t*-test was used to compare two data sets and one-way ANOVA with Bonferroni's post-hoc test was used for multiple group comparisons. $P \leq 0.05$ was considered significant.

Reference List

1. Namkung Y, Smith SM, Lee SB, Skrypnyk NV, Kim HL, Chin H, Scheller RH, Tsien RW, Shin HS. Targeted disruption of the Ca^{2+} channel β3 subunit reduces N- and L-type Ca^{2+} channel activity and alters the voltage-dependent activation of P/Q-type Ca^{2+} channels in neurons. *Proc Natl Acad Sci USA.* 1998;95:12010-12015.

2. Thakali KM, Kharade SV, Sonkusare SK, Rhee SW, Stimers JR, Rusch NJ. Intracellular Ca^{2+} silences L-type Ca^{2+} channels in mesenteric veins: mechanism of venous smooth muscle resistance to calcium channel blockers. *Circ Res.* 2010;106:739-747.

3. Pesic A, Madden JA, Pesic M, Rusch NJ. High blood pressure upregulates arterial L-type Ca^{2+} channels: is membrane depolarization the signal? *Circ Res.* 2004;94:e97-e104.

Supplemental Figures

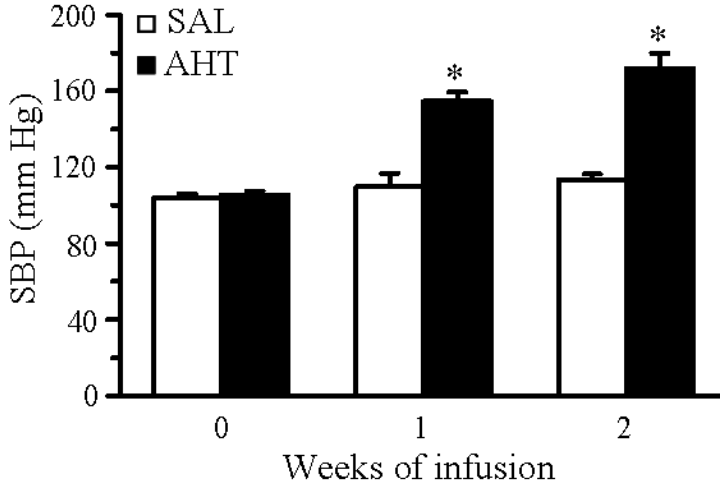

Figure S1. The SBP profiles of C57BL/6 mice infused with isotonic saline (vehicle, SAL) or Ang II (2 ng/g/min, AHT) respectively, for two weeks. Values of SBP at weeks 1 and 2 in SAL mice were not significantly different from baseline at week 0 (n=10). However, SBP was significantly increased at weeks 1 and 2 after the start of Ang II infusion in AHT mice (n=10). * = $p<0.05$.

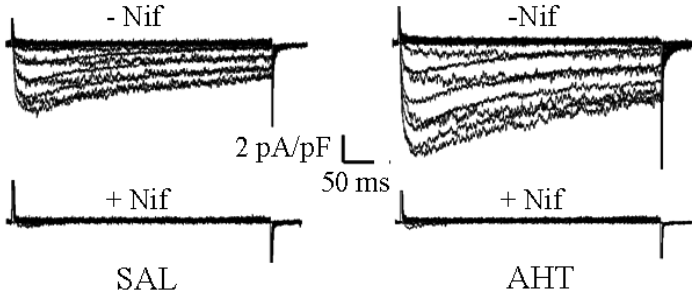

Figure S2. Representative traces of families of $Ca_v1.2$ channel currents elicited by 8 mV steps from -70 mV to +58 mV in mesenteric VSMCs of SAL and AHT mice. The control currents (top traces) were blocked by 1 μmol/L nifedipine (lower traces).

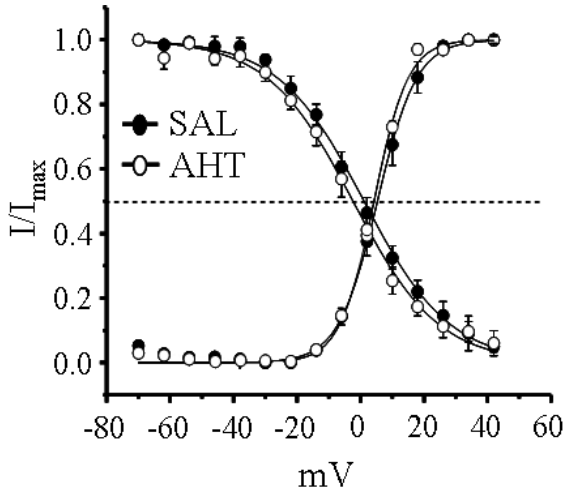

Figure S3. Boltzmann fits to voltage-dependent activation and inactivation relationships. There was no difference in half-activation and inactivation voltages between SAL and AHT mice. The $V_{1/2}$ values for activation were 5.2±0.4 mV (SAL, n=11) and 4.0±0.4 mV (AHT, n=10). The $V_{1/2}$ values for inactivation were -2.4±0.7 mV (SAL, n=8) and -4.6±0.7 mV (AHT, n=7).

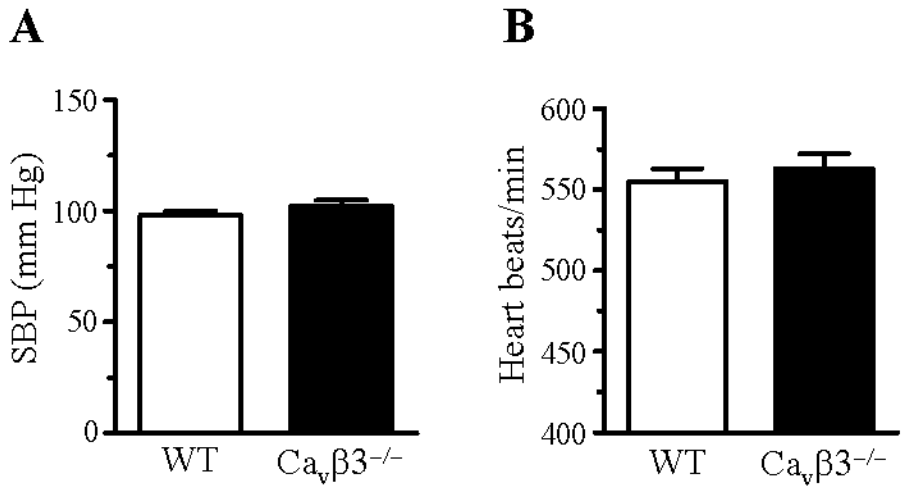

Figure S4. Resting SBP was similar between WT and $Ca_v\beta3^{-/-}$ mice (n=10 each). **B.** Resting heart rate also was not significantly different between WT and $Ca_v\beta^{-/-}$ mice (n=10, 11).