Detailed Methods for:

Pannexin 1 channels as unexpected new target of the anti-hypertensive drug spironolactone

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Detailed Methods

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Chemicals and reagents

Most of the chemicals used were obtained from Sigma-Aldrich except when stated otherwise. Annexin V-PE and Annexin V-Pacific Blue were obtained from Biolegend and Life Technologies, respectively. Other reagents were obtained as follows: 0.4 cm electrode cuvettes (Bio-Rad), 10X annexin V binding buffer (eBioscience), 7-aminoactinomycin D (7-AAD) and TO-PRO-3 iodide (TO-PRO-3) were obtained from Thermo Fisher Scientific. Finerenone was purchased from Synovel Laboratory.

Mammalian cell culture and transfection

HEK293T cells were cultured at 37 °C, with humidified air containing 5% CO2, in Dulbecco's Modified Eagle Medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS, Gibco), penicillin, streptomycin, and sodium pyruvate and transfected using Lipofectamine2000™ (Invitrogen). Constructs used for expressing Pannexin 1 channels and mouse α1D-adrenergic receptors (MR222643; OriGene) have been previously described1, 2. To activate human PANX1 via a caspase-independent C-terminal cleavage, Tobacco Etch Virus protease (TEVp) was co-transfected with human PANX1(TEV), in which caspase recognition sequence was replaced by TEV protease recognition sequence (ENLYFQG)1. Phenylephrine-induced Panx1 currents were recorded from HEK293T cells heterologously co-expressing mouse α1D-adrenergic receptors, with either wild type mouse Panx1 or human PANX1(TEV). Rat connexin 43 (Cx43) was subcloned into pcDNA3.1 by Dr. Scott Johnson and was verified by DNA sequencing, as previously reported3. Mouse Panx2 was a generous gift from Dr. Dale Laird4. Jurkat T cells (E6.1) were maintained at a density of 0.5-1.5 cells ml⁻¹ in RPMI 1640 (Corning) supplemented with 10% FBS, penicillin, streptomycin, and L-glutamine. Apoptosis of Jurkat cells were induced by anti-Fas antibody (250 ng/ml) for 2 hr at 37 °C.

Electrophysiology

All voltage-clamp analyses were carried out at room temperature. Borosilicate glass patch pipettes of 3-5 and 7-10 megaohm were used in whole-cell recording and inside-out patch recording, respectively. Whole-cell recordings were performed using apoptotic Jurkat cells or
transiently transfected HEK293T cells with an Axopatch 200B amplifier and pCLAMP10 software (Molecular Devices). The bath solution contained (in mmol/L): 140 NaCl, 3 KCl, 2 MgCl2, 2 CaCl2, 10 HEPES, and 10 glucose (pH 7.3); and the pipette solution contained (in mmol/L): 30 tetraethylammonium chloride, 100 CsMeSO4, 4 NaCl, 1 MgCl2, 0.5 CaCl2, 10 HEPES, 10 EGTA, 3 ATP-Mg, and 0.3 GTP-Tris (pH 7.3). Ramp voltage commands were applied by using the pCLAMP software and a Digidata 1322A digitizer (Molecular Devices). The same bath solution was used to obtain the inside-out patch configuration; after patch excision, the bath solution was exchanged to a solution composed of (in mmol/L) 150 CsCl, 5 EGTA, 10 HEPES, and 1 MgCl2 (pH 7.3). Spironolactone or derivatives were applied in bath solutions after acquisition of steady-state Panx1 activities induced by removal of C-terminal tails or by phenylephrine (20 µmol/L in bath solution)-mediated activation of α1D-adrenergic receptors. Data were filtered at 5 kHz using an 8-pole low-pass Bessel filter (LPF-8, Warner Instruments) and digitized at a sampling rate of 20 kHz using a Digidata 1322A digitizer (Molecular Devices). Data were analyzed using pCLAMP software. Results were presented as mean ± s.e.m. The dose-response curve was fitted and IC50 was determined using Prism 7 software.

**TO-PRO-3 and ATP release by apoptotic cells**

For TO-PRO-3 uptake assay, human Jurkat T cells were treated with 500 ng/ml anti-Fas (clone CH11) in RPMI/1% BSA for 4 hr at 37°C, 5% CO2 to induce apoptosis. Cells were then treated with the pan caspase inhibitor Q-VD-OPH (50 µmol/L), and the indicated concentrations of drugs for 20 minutes at 37°C, 5% CO2. Cells were stained with TO-PRO-3, 7-AAD and annexin-FITC (to distinguish apoptotic cells from viable and necrotic cells) and analyzed on a FACSCanto flow cytometer, with the resultant data analyzed by FlowJo software. For the ATP release assay, human Jurkat T cells were induced to undergo apoptosis (500 ng/ml anti-Fas) in the presence of the indicated concentrations of drugs at 37°C, 5% CO2. ATP in apoptotic cell supernatants was measured by CellTiter-Glo (Promega) according to manufacturer's instructions.

**NR3C2 Knockdown in Jurkat T cells and Apoptosis Induction**

Jurkat T cells (E6.1) were maintained in RPMI 1640, 10% FBS, penicillin/streptomycin/L-glutamine at a density of 0.5-1.5 cells ml\(^{-1}\). Transfections were performed using the BTX Electro Square Porator T820. Jurkat cells (5-10 million), cells washed and resuspended in 400 µl plain RPMI 1640. Cells were electroporated at 250mV/25msec/1 pulse with 200 nmol/L control or NR3C2 siRNA (GE Dharmacon) and used 60-72 hours after transfection. After 60-72 hours, the
cells were induced to undergo apoptosis in RPMI (1%BSA) with 250 ng/ml of anti-Fas (clone CH11) and ATP release/TO-PRO-3 dye uptake was measured after 4 hours. Knockdown efficiency was measured using Taqman probes for NR3C2 and qRT-PCR normalized to HPRT.

Human Samples

Under IRB-HSR Study protocol #17194, human volunteers with resistant hypertension (elevated blood pressure above 140 mm Hg systolic) had an approximate 40 mm x 20 mm biopsy of adipose tissue removed from the abdomen (no additional procedures were performed), which was immediately placed in ice-cold KREBS buffer, and the arterioles dissected manually within 30 minutes. The arterioles (approximately 50-90 µm) were cannulated, pressurized, and subject to phenylephrine cumulative dose-response curves described below. All subjects were males (N = 4). Metrics of the hypertensive human subjects are listed below.

<table>
<thead>
<tr>
<th>Metric</th>
<th>Mean</th>
<th>SD</th>
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<tr>
<td>Age</td>
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<td>Body mass index</td>
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<tr>
<td>Systolic pressure</td>
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<td>5.67</td>
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<tr>
<td>Diastolic pressure</td>
<td>84.75</td>
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</tbody>
</table>

Immunohistochemistry

Arterioles isolated from hypertensive patients were fixed in 4% paraformaldehyde and embedded in paraffin for sectioning. Sections were stained for Panx1 antibody directed against the carboxyl terminal tail (1:500) and the α-smooth muscle actin (Sigma #A5228; 1:200) followed by secondary antibodies Alexa Fluor® 568 Donkey Anti-Rabbit IgG (H+L) (Invitrogen A10042; 1:500) and Alexa Fluor 633 Donkey Anti-Goat IgG (H+L) (Invitrogen A21082; 1:500), respectively. The sections were then coated in mounting medium containing DAPI (Invitrogen Cat# P36931). All images were obtained with an Olympus Fluoview 1000 and are representative images of composite z-stacks. The internal elastic lamina separating the SMC and endothelial cell layer was visualized via its autofluorescence in the 488 nm channel.

Mice
All mice and procedures were approved by the University of Virginia School of Medicine Animal Care and Use Committee. All mice were male as the SMMHC Cre is located on the Y chromosome, and used between 10-20 weeks of age. The genotypes include: C57Bl/6; Panx1^fl/fl^ / SMMHC CreER^{T2+} (SMC-Panx1, 2); Panx1^fl/fl^ / Cdh5 CreER^{T2+} (EC-Panx1, 7); Nr3c2^fl/fl^ / SM α-actin CreER^{T2+} (SMC-Nr3c2, 8); hypertensive BPH/2 (9); and global deletion of Panx1 (Panx1^−/−, 10).

**Pressure myography**

Freshly isolated human arterioles or mouse thoracodorsal arteries were placed into ice-cold Krebs-HEPES solution containing (in mmol/L) NaCl 118.4, KCl 4.7, MgSO_{4} 1.2, NaHCO_{3} 4, KH_{2}PO_{4} 1.2, CaCl_{2} 2, Hepes 10, glucose 6. The vessels were then mounted in a pressure arteriography (Danish MyoTechnology) with the lumen Krebs-HEPES solution supplemented with 1% BSA (for thoracodorsal arteries only). The vessels were pressurized to 60 mmHg (human arterioles) or 80 mmHg (thoracodorsal arteries) and heated to 37°C for 20 min. The bath and lumen solutions were then replaced with Krebs-HEPES containing 5µmol/L PanX^{2}, 0.1% DMSO, or the corresponding concentration (10-80 µmol/L) of spironolactone (dissolved in DMSO) and the vessels were allowed to equilibrate for 30 min. Cumulative concentrations of phenylephrine were applied to the circulating bath and vessel lumen diameters were measured. Acetylcholine (10µmol/L) following phenylephrine constriction, serotonin (1µmol/L) or KCl (40 mmol/L) was added to verify spironolactone had no effect on other vasodilators and vasoconstrictors. Maximal diameter was measured after washing with a Ca^{2+} free Krebs-HEPES solution supplemented with 1 mmol/L Ethyleneglycol-O, O′-bis(2-aminoethyl)-N,N,N′,N′-tetraacetic acid (EGTA) and 10 µmol/L sodium nitroprusside. Values calculated as percent of maximal diameter. The dose-response curve was fitted and IC50 was determined using Prism 5 software.

**TO-PRO3-uptake in arteries**

Thoracodorsal arteries were isolated and perfused with Krebs-HEPES to remove red blood cells and placed in Krebs-HEPES solution with 0.1% DMSO or 80 µmol/L spironolactone (in DMSO) and incubated for 30 min. Phenylephrine (10 µmol/L) was used to activate Panx1 channels and TO-PRO3 was added to the vessels for 5 min. The vessels were then rinsed three times with Krebs-HEPES and placed immediately onto a slide, coated with mounting media containing DAPI, and imaged using an Olympus Fluoview 1000. Representative images are composite z-stacks.
Radiotelemetry

Mice were implanted with radiotelemetry units [Data Sciences International (DSI)]. Briefly, while under isoflurane anesthesia, the catheter of the radiotelemetry unit was placed in the left carotid artery and the radiotransmitter was placed in a subcutaneous pouch on the right flank. Mice that were injected with peanut oil or tamoxifen underwent implantation at least 10 days after last injection. Mice were allowed to recover for five days.

Blood pressure was continuously recorded. At the same time of day, mice were injected intraperitoneally with either DMSO (50 µL; vehicle for all drugs), spironolactone (40 or 90 mg/kg in a volume of 50 µL), trovafloxacin (40 mg/kg in a volume of 50 µL), or finerenone (10 or 40 mg/kg in a volume of 50 µL). Baseline pressure was calculated 2 hr prior to injection. Average post-injection pressure was calculated 15 minutes after injection for 30 minutes. Injections were at least 72 hr apart to allow for blood pressure to return to normal levels. Order of injection was varied between mice. Change in blood pressure was measured by comparing the 2 hr baseline mean arterial pressure (MAP) prior to injection to the average MAP after injection.


channels regulate leukocyte emigration through the venous endothelium during acute inflammation. *Nat Commun.* 2015;6:7965


Supplemental Figure I: Spironolactone, but not several other steroidal compounds, inhibits PANX1 channel function. TO-PRO-3 uptake by apoptotic Jurkat T cells treated with cholesterol, β-estradiol, hydrocortisone, aldosterone, spironolactone, dexamethasone and CBX (n=3). Data are presented as mean ± s.e.m. and analyzed using one-way ANOVA, followed by Bonferroni’s test for multiple comparisons. **** p<0.0001; n.s.: p>0.05.
Supplemental Figure II: Spironolactone antagonizes phenylephrine-induced vasoconstriction in a caspase-independent and Panx1-dependent manner. (A) Representative whole-cell recording from a HEK293T cell co-expressing human PANX1(TEV) channels and α1D adrenergic receptors. Phenylephrine-induced currents (cyan shading) were inhibited by spironolactone addition (20 μmol/L), and further by CBX (50 μmol/L). Inset: whole-cell current at +80 mV in the presence of phenylephrine, and the indicated inhibitors. (B) Inhibition of phenylephrine-induced Pannexin 1 currents by spironolactone (50 μmol/L). PE-induced and CBX-sensitive whole-cell currents were assessed from HEK293T cells expressing α1D-adrenergic receptors and human PANX1(TEV) or mouse wild type Panx1 channels at a membrane potential of +80 mV. Percent inhibition was calculated by dividing spironolactone-
sensitive current over the CBX-sensitive currents from the same cell (n=8, mean ± s.e.m.). (C) Vasodilation induced by 10 μmol/L acetylcholine following phenylephrine-induced constriction. Thoracodorsal arteries pretreated with spironolactone displayed vasodilation, with over 70% of the maximal inner diameter (grey dashed line) in response to acetylcholine. (D) Vasoconstriction induced by 1 μmol/L serotonin (which is independent of Panx1) and treatment with spironolactone resulted in vasoconstriction, evident by <50% of the maximal inner diameter (grey dashed line). In both C and D, N≥4 for each group. In C57Bl6 control mice, a dose range of spironolactone was used, while 80 μmol/L was used for Panx1fl/fl/EC CreERT2+ with tamoxifen injection, Panx1fl/fl/SMC CreERT2+ with tamoxifen injection, and Panx1fl/fl/SMC CreERT2+ with vehicle injection (peanut oil). One-way ANOVAs found no differences between genotypes for either acetylcholine dilation or serotonin constriction.
Supplemental Figure III: Spironolactone inhibits Pannexin 1 function in a mineralocorticoid receptor-independent manner. (A) Representative histograms showing TO-PRO-3 uptake in the presence or absence of spironolactone from Fas-induced apoptotic Jurkat cells. Spironolactone reduced TO-PRO-3 uptake in cells transfected with scramble siRNA or NR3C2 siRNA. (B) PCR of thoracic aortas and thoracodorsal arteries from tamoxifen injected Nr3c2f/f/SMC CreERT2+ mice revealing two bands, representing the floxed and excised alleles. The continued detection of the floxed allele is because both smooth muscle cells and endothelial cells express Nr3c2; however, mice lacking the SMC-Cre (Cre-) but injected with
tamoxifen have only the floxed gene present. (C) With treatment of spironolactone, vasodilation (left) and vasoconstriction (right) were induced by 10 μmol/L acetylcholine, following phenylephrine constriction, or 1 μmol/L serotonin (independent of Panx1) (grey dashed line). N=5 vessels from C57Bl6 or smooth muscle cell-specific Nr3c2 deleted (SMC Nr3C2-KO) mice. Student’s t-tests revealed no significant differences between control and SMC Nr3c2-KO mice. (D) Night mean arterial pressure (MAP) for C57Bl6 control, SMC Nr3c2-KO, SMC Panx1-KO, and hypertensive BPH/2 mice. Note that SMC Panx1-KO mice have significantly reduced MAP while, conversely, the BPH/2 mice have significantly increased MAP. (E) C57Bl6 and SMC Nr3c2-KO mice, but not SMC Panx1-KO mice, showed a reduction in MAP following injection of 90 mg/kg spironolactone, indicating that acute decreases in MAP is Panx1-dependent, even at high doses (N≥4 mice per group). (F) Injection of 10 mg/kg finerenone did not result in a decrease in MAP in all mouse genotypes (N≥5 mice per group, BPH/2 N=3). (G) Injection of 40 mg/kg trovafloxacin reduced MAP in all mouse genotypes except the SMC Panx1-KO mouse (N≥3 mice per group). One-way ANOVA; * p<0.05; ** p<0.01; *** p<0.001. Mean ± s.e.m. (H) Injection of 40 mg/kg spironolactone reduced MAP in mice with Panx1 globally deleted (Panx1−/−, N=5), but not control Panx1+/+ (N=4) mice. Students t-test; ** p>0.01. (I) Continuous recording of blood pressure (5 min averages) reveals a significant drop in MAP following i.p. injection of 40 mg/kg trovafloxacin in C57Bl6, SMC Nr3c2-KO, and hypertensive BPH/2 mice, but not in SMC Panx1-KO mice. Data points presented as averaged MAP taken every 5 min (mean ± s.e.m.). Dashed blue line indicates time of injection.
Supplemental Figure IV: Concentration dependent inhibition of TO-PRO-3 dye uptake and ATP release by apoptotic cells. (A) Dose-dependent inhibition of TO-PRO-3 uptake by CBX, spironolactone, canrenone, and eplerenone in apoptotic Jurkat T cells (mean ± s.e.m.; n=3). One-way ANOVA, with Bonferroni’s test, showed significant reduction in TO-PRO-3 uptake by all compounds ($p<0.05$ for $20 \mu$mol/L eplerenone, otherwise $p<0.001$). (B) Dose-dependent inhibition of ATP release by spironolactone, canrenone, and eplerenone from apoptotic Jurkat T cells (mean ± s.e.m.; n=3). Statistical analysis using one-way ANOVA, followed by Bonferroni’s multiple comparison test, found significant reduction ($p>0.001$) in ATP release by spironolactone ($\geq 5 \mu$mol/L), canrenone ($\geq 5 \mu$mol/L), or eplerenone ($\geq 20 \mu$mol/L), compared to vehicle controls (black).