Supplemental Files

Materials and methods

Patients selection and tissues.

Patients with BAH were recruited at the Division of Internal Medicine and Hypertension, University of Torino, Italy, as previously described. Briefly, patients were screened using the serum aldosterone: plasma renin activity (PRA) ratio (ARR) and PA was confirmed using the intravenous saline loading test. All patients underwent adrenal CT scanning and AVS. The diagnosis of BAH was performed when the aldosterone:cortisol ratio from one adrenal was < 4 times that of the other adrenal gland.

In the index case carrying the p.Y152C KCNJ5 mutation, the diagnosis of APA was performed on the basis of an adrenal CT finding of left adrenal nodule. AVS was performed, but right adrenal vein cannulation was not successful. First degree relatives of the index case were not available for genotyping.

Normal human adult adrenals were obtained from patients undergoing laparoscopic nephrectomy for localized renal carcinomas (in all cases histological examination excluded the involvement of the adrenal in the tumor lesion).

KCNJ5 sequencing.

KCNJ5 cDNA from APA and adjacent adrenal tissue was PCR amplified using intron spanning primers as described elsewhere.

SNP genotyping.

SNP genotyping was performed on genomic DNA extracted from 100 BAH patients according to the manufacturer’s instructions (Applied Biosystem, Foster City, CA). Briefly each reaction was performed in a total volume of 25 µL containing 12.5 µL of TaqMan Universal PCR Master Mix, 0.625 µL of SNP Genotyping Assay Mix, 5µL of gDNA (20-25 ng) and 6.875 µL of nuclease-free water.

Cell culture and transfection.
HAC15 human adrenocortical carcinoma cells were cultured in Dulbecco’s Modified Eagle/F12 medium (Invitrogen, Carlsband, CA) supplemented with 10% Cosmic Calf Serum (HyClone, Logan, UT), 1% insulin/transferrin/selenium Premix (BD Biosciences, Sparks, MD) and antibiotics. HAC15 cells were electroporated using the Amaxa electroporator (program X005, Amaxa Biosystems, Cologne, Germany) in 100 µL of nucleofector solution R. After electroporation, cells were plated in 6-well plates with 5 mL of growth medium/well and allowed to recover for 24 h, then starved overnight in 0.1% low serum media (0.1% Cosmic calf serum and antibiotics).

In incubations in the presence of nifedipine, nucleofected cells were plated directly in 5-mL complete medium supplemented with nifedipine (10µM; Sigma-Aldrich) added from 50 mg/mL of stock solutions in dimethyl sulfoxide. This resulted in a final concentration of 0.007% dimethyl sulfoxide that was maintained for all of the cell incubations.

**Immunohistochemistry**

Sections of 10 μm thickness from formalin-fixed, paraffin-embedded adrenal tissue from the patient harboring the Y152C germline KCNJ5 mutation was quenched for endogenous peroxidase using H2O2 and then incubated with antibodies against human KCNJ5 (Sigma HPA071353, 1:500 diluted) or human CYP11B2 (a generous gift from Dr. Celso Gomez Sanchez, 1:100 diluted) for 1 h. After a short rinse, the EnVision reagent (Dako, Carpinteria, CA) coupled with peroxidase-labeled polymer was incubated as secondary antibody or 30 min. The proteins were visualized using 3,3′-diaminobenzidine tetrahydrochloride, counterstained with hematoxylin, and mounted.

**RNA extraction and gene expression assay.**

Adrenal tissue was homogenized in Lysing Matrix D tubes (MP Biomedicals, Santa Ana, CA) with 700 µL of RLT buffer (Qiagen, Hilden, Germany) with 10% β-mercaptoethanol. Total RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s recommendations. For cDNA generation, 2 µg total RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) following the manufacturer’s instructions. qPCR was performed in
triplicate using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) using TaqMan
gene expression assays (Applied Biosystems, Foster City, CA) for KCNJ5, nuclear receptor subfamily 4, group A,
member 2 (NR4A2) and human aldosterone synthase (CYP11B2). Gene expression levels were analyzed
using the $2^{-\Delta\Delta C_T}$ relative quantification method, using GAPDH as endogenous control.

Microarray analysis
RNA samples used for microarray experiments were assayed for purity and quality using an Experion
Automated Electrophoresis System (Bio Rad, Life Sciences Group, Hercules, CA).
RNA samples were hybridized to an Illumina bead chip containing more than 48,000 probes representing
over 25,000 human genes (Illumina, San Diego, CA). The arrays were scanned at high resolution on the
iScan system (Illumina) at the GHSU core facility. Results were analyzed using GeneSpring GX (version 11.5)
software (Silicon Genetics, Redwood City, CA). Fold changes for genes differentially regulated in the KCNJ5
mutation bearing adrenal tissue versus the 4 control adrenal samples were calculated, and used to
generate a heat map based on log$_2$ signal intensity.

Electrophysiological characterization of the KCNJ5$^{Y152C}$ mutant
Human KCNJ3 and KCNJ5 cDNAs were purchased from Invitrogen/Geneart. The mutation c.455A>G of
KCNJ5 (resulting in the mutant protein KCNJ5$^{Y152C}$) was generated by site-directed mutagenesis. For
electrophysiological measurements, KCNJ3 cDNA was subcloned into pIRES2-AcGFP (Clontech), whereas
KCNJ5 and KCNJ5$^{Y152C}$ were subcloned into pIRES2-DsRed (Clontech) expression vectors. For functional
studies, 50% confluent HEK293 cells were cotransfected with wildtype KCNJ3 and wildtype or mutated
KCNJ5 using Lipofectamine (0.5 µg of each plasmid per 35 mm dish). For patch-clamp experiments, only
cells with both green (KCNJ3 expressing) and red (wildtype KCNJ5 or KCNJ5$^{Y152C}$ expressing) fluorescence
were used. Patch-clamp recordings were performed using an EPC-10 amplifier without leak subtraction
(Heka, Germany). The following solutions were used (all concentrations in mM): control solution: pH 7.4;
10 HEPES; 140 NaCl; 5 KCl; 1.8 MgCl$_2$; 1.8 CaCl$_2$. High K$^+$ solution: pH 7.4; 10 HEPES; 95 NaCl; 50 KCl; 1.8
MgCl$_2$; 1.8 CaCl$_2$. Na$^+$-free solution: pH 7.4; 10 HEPES; 5 KCl; 1.8 MgCl$_2$; 1.8 CaCl$_2$; 140 N-methyl-D-
glucamine chloride (NMDG\(^+\)). Pipette solution: pH 7.4; 5 HEPES; 140 KCl; 4 MgCl\(_2\); 1 CaCl\(_2\); 1 EGTA. Ba\(^{2+}\)-
sensitive currents were calculated by subtracting the currents in the absence and presence of Ba\(^{2+}\) (5 mM).

4 Ca\(^{2+}\) measurements

For cytoplasmic Ca\(^{2+}\) measurements KCNJ3, KCNJ5, KCNJ5\(^{Y152C}\) and KCNJ5\(^{G151E}\) cDNAs were subcloned into

the bicistronic expression vector pIRES-CD8\(^4\). Cytoplasmic Ca\(^{2+}\) measurements were performed by loading

the cells for 1 h with 5 µM of Fura-2 AM in the presence of Power Load permeabilizing reagent (Molecular

Probes, Darmstadt, Germany). To measure the effect of extracellular Ca\(^{2+}\) on intracellular Ca\(^{2+}\) activities,

cells were superfused with solutions containing 0.1, 0.7, 1.8 or 5 mM of Ca\(^{2+}\). The Fura-2 ratio of emissions

after excitations with 340 nm and 380 nm was used as a measure of intracellular Ca\(^{2+}\) concentrations. For

comparison, the known mutant KCNJ5\(^{G151E}\) was used\(^5\). All the experiments were performed at room

temperature.

14 Statistical analyses.

IBM SPSS Statistics 19 (SPSS INC, Chicago, IL) was used for statistical analyses. Data are expressed as mean ±

S.D. or S.E. and individual experiments were repeated at least three times. Differences between variables

were evaluated using unpaired \(t\) test or Mann-Whitney test. A probability of less than 0.05 was considered

statistically significant.

19 References


  Veglio F, Boscaro M, Rainey WE, Ito S, Mulatero P 2012 Effect of adrenocorticotropic hormone

  stimulation during adrenal vein sampling in primary aldosteronism. Hypertension 59(4):840-846


  Stowasser M 2010 Impact of different diagnostic criteria during adrenal vein sampling on

  reproducibility of subtype diagnosis in patients with primary aldosteronism. Hypertension 55:667-673


Supplemental figure Legends

Supplemental Figure 1
Sections of adrenal tissue harboring the Y152C mutation stained with hematoxylin and eosin (H&E) (A,D) and immunostained using antibodies against human aldosterone synthase (CYP11B2) (B,E) and human Kir 3.4 (KCNJ5) (C,F).

Supplemental Figure 2 Heatmap comparing steroidogenic enzymes in adrenal tissue harboring the Y152C mutation in the KCNJ5 gene versus normal adrenal tissues obtained from patients of renal cancer or from cadaver donors. Heatmap colors are based on log₂ signal intensity. Absolute fold change (FC) is also provided. NR4A2 = nuclear receptor subfamily 4, group A, member 2; CYP11B2 = cytochrome P450, family 11, subfamily B, polypeptide 2 (aldosterone synthase); POR = P450 (cytochrome) oxidoreductase; NR4A3 = nuclear receptor subfamily 4, group A, member 3; HSD3B2 = 3 beta-hydroxysteroid dehydrogenase type II; CYP21A2 = steroid 21-hydroxylase; CYP11B1 = steroid 11-beta-hydroxylase; CYB5 = cytochrome b5; SULT2A1 = sulfotransferase family, cytosolic, 2A, member 1; AKR1C3 = aldo-keto reductase family 1, member C3; CYP17 = steroid 17-alpha-hydroxylase/17,20 lyase; StAR = steroidogenic acute regulatory protein; CYP11A1 = cholesterol monoxygenase (side-chain cleaving).
Supplemental Figure 3  Ba2+-sensitive currents as a measure of the KCNJ5/KCNJ3-induced current in HEK293 cells. (A) Ba2+-sensitive currents at low extracellular K+. (B) Ba2+-sensitive currents at high extracellular K+. Values are mean values ± SEM

Supplemental Figure 4  ATP-induced release of Ca\(^{2+}\) from intracellular stores under Ca\(^{2+}\)-free conditions. HEK293 cell were co-transfected with KCNJ3/KCNJ5\(^{Y152C}\) (Y152C) or KCNJ3/KCNJ5\(^{WT}\) (WT) or non-transfected cells (sham). After removal of bath Ca\(^{2+}\) (free), ATP (100 µM) was added to stimulate purinergic receptors and induce release of Ca\(^{2+}\) from IP\(_3\)-sensitive intracellular stores. The amounts of released Ca\(^{2+}\) were similar in all three types of cells. Values are mean values ± SEM, number of cells in brackets.
**Normal adrenals (n=4)**

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**Y152C adrenal**

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A) Ba\textsuperscript{2+}-sensitive currents
Extracellular K\textsuperscript{+} 5 mmol/l

B) Ba\textsuperscript{2+}-sensitive currents
Extracellular K\textsuperscript{+} 50 mmol/l