Glibenclamide reverses cardiovascular abnormalities of Cantu Syndrome driven by K_{ATP} channel overactivity

Conor McClenaghan, Yan Huang, Zihan Yan, Theresa Harter, Carmen M. Halabi, Rod Chalk, Attila Kovacs, Gijs van Haaften, Maria S. Remedi, and Colin G. Nichols

SUPPLEMENTARY METHODS

Generation of mouse models

As previously described (1), CRISPR-Cas9 genome editing was used to introduce a single nucleotide mutation into *ABCC9* (c.1427C>T), generating the equivalent of human CS-associated SUR2[A478V] mutation. Heterozygous SUR2[A478V] ($SUR2^{wt/AV}$) mice were in-crossed to generate mutant and WT littermate mice used for experiments.

To generate mice expressing the SUR2[A478V] mutation and dominant negative (DN) Kir6.1-AAA in an inducible, tissue-specific, manner we crossed Tg[CX1-eGFP-Kir6.1-AAA] (6.1-AAA) mice (2) with transgenic mice (SM-Cre) that express inducible Cre recombinase ($CreER^{T2}$) driven by the SMMHC promotor (3) (Figure 1A). These double-transgenic mice were then crossed with $SUR2^{wt/A478V}$ mice to generate double-transgenic CS mice ($SM\text{-}DN^{wt/AV}$), and double-transgenic mice lacking the SUR2[A478V] mutation ($SM\text{-}DN^{wt/wt}$), as well as wild type, CS ($SUR2^{wt/AV}$), and additional uninducible single transgenic (STG) control animals. Eight week old mice of all genotypes were treated with serial tamoxifen injections (1 injection of 50 µg/g body weight per day for 5 days), which induced dominant-negative Kir6.1-AAA expression in $SM\text{-}DN^{wt/AV}$ and $SM\text{-}DN^{wt/wt}$ animals, and experiments were performed 4 weeks after induction.

Experimental groups included male and female mice for all comparisons, except for SMMHC-Cre dominant-negative mice which were male only, as the transgene is carried on the Y chromosome.

Patch clamp electrophysiology of VSM cells

VSM cells were acutely isolated from descending aorta as described (1). K_{ATP} currents were recorded in whole-cell mode using an Axopatch 200B amplifier and Digidata 1322A (Molecular Devices). Data were sampled at 3 kHz and low-pass filtered at 1 kHz. Cells were voltage-clamped at -70 mV and currents were initially recorded in a high Na^+ bath solution containing (in mM): 136 NaCl, 6 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose (pH 7.4) before exchange with a high-K^+ bath solution (140 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose, pH 7.4). Pinacidil and glibenclamide were then administered as indicated. The pipette solution contained (in mM) 110 potassium aspartate, 30 KCl, 10 NaCl, 1 $MgCl_2$, 10 HEPES, 0.5 $CaCl_2$, 4 K_2HPO_4, and 5 EGTA (pH 7.2). K_{ATP} current density was calculated by dividing whole cell currents in pinacidil by cell capacitance.

Blood pressure measurements

Adult mice (3 months old) were anesthetized using 1.5% isoflurane and body temperature was maintained with a heating pad throughout recordings. BPs were recorded following catheterization of the left carotid artery with a Millar pressure transducer advanced to the ascending aorta. Data were acquired using the Powerlab data acquisition system (ADInstruments), and MAP was analyzed using LabChart 7 (ADInstruments).

Heart weight measurements

Adult (3 months old) mice were euthanized using 2.5% Avertin (Tribromoethanol). Hearts were removed, rinsed in 10 % KCl solution and blotted to remove excess liquid and weighed. Heart weight was normalized to tibia length (HW/TL; mg/mm).

Subcutaneous glibenclamide pellet implantation in mice

8 week old mice were anesthetized with 2.5 % Avertin. Fur on the dorsal neck was trimmed and 5 mg/kg carprofen was administered subcutaneously as a pre-operative analgesic. For an approximate glibenclamide dose of 1 mg/kg/day, 2.5 mg (90 day) slow-release pellets (Innovative Research of America) were implanted subcutaneously under the loose skin of the scruff of the neck using a trochar. High dose (approximately 19 mg/kg/day), 25mg (60 day) pellets (Innovative Research of America) were surgically implanted. In control mice, placebo pellets (5mg; 21 day release; Innovative Research of America), were implanted subcutaneously using a trochar. 4 weeks following pellet implantation BPs and heart size were measured.

Echocardiography

Echocardiography was performed using a Vevo 2100 Imaging System (VisualSonics) equipped with a 30-MHz linear-array transducer according to previously published methods (4, 5). Cardiac images were obtained by a handheld technique using 100 mg/kg i.p. tribromoethanol anesthetic; aortic images were obtained under 1.5% inhaled isoflurane. Quantitative image analysis was performed using a speckle-tracking algorithm to obtain volumetric data. Cardiac index was calculated by normalizing cardiac output (stroke volume x heart rate) by tibia length. Systemic vascular resistance was calculated from mice (> 20 g) by dividing mean arterial pressure (determined from catheter measurements from anesthetized mice) by cardiac output (in turn calculated from stroke volume measurements from echocardiographic data multiplied by heart rate measurements from blood pressure recordings). Mice administered with both moderate- and high-dose pellets were grouped together as glibenclamide-treated mice for SVR and cardiac index measurements.

Histology

Hearts were fixed in 10% buffered formalin (24 h), and embedded in paraffin. Sections (3 μm) were cut and Gomori stained as previously reported(6). Left ventricle free-wall sections were imaged from placebo and high-dose glibenclamide treated WT and $SUR2^{wt/AV}$ mice (n of 3 for each group). No differences in collagen staining between any group were observed.

Carotid artery compliance measurement

After euthanasia, the left common carotid artery was excised and placed in physiologic saline solution (PSS) composed of 130 mM NaCl, 4.7 mM KCl, 1.6 mM $CaCl_2$, 1.18 mM $MgSO_4$-$7H_2O$, 1.17 mM KH_2PO_4, 14.8 mM $NaHCO_3$, 5.5 mM dextrose, and 0.026 mM EDTA (pH 7.4). The vessel was cleaned from surrounding fat, mounted on a pressure arteriograph (Danish Myotechnology, Aarhus, Denmark) and maintained in PSS at 37°C. An inverted microscope connected to a charged-coupled device camera and a computerized system was used to visualize the artery and continuously record its diameter. As intravascular pressure was increased from 0 to 175 mm Hg by 25 mmHg increments, the vessel outer diameter was recorded at each step (12 seconds per step). The average of three measurements at each pressure was reported.

Blood Glucose measurement in mice

Blood glucose (BG) was measured from tail-bleed samples using a digital blood glucose meter (Contour). BG was measured in fed mice (food available *ad libitum*) prior to pellet implantation and then periodically over 18 days post-implantation. To assess fasted blood glucose and for glucose-tolerance tests (GTT), food was removed overnight (for ~ 10h) and BG measured the

following morning. GTT was performed by measuring blood glucose from tail bleed samples immediately prior to administration of 1.5 g/kg D-glucose (in PBS), injected I.P.

LC-MS/MS analysis of plasma glibenclamide concentrations

60µl of mouse plasma was spiked with d11 glibenclamideand diluted to 1ml with 2% acetonitrile, 0.1 % formic acid prior to solid phase extraction. Eluted samples were evaporated, re-supended in ammonium formate buffer and then subjected LC-MS/MS analysis using an ion trap mass spectrometer following the method described by Lahmann et al. (7). Six experimental replicates were performed and concentrations were determined with reference to a glibenclamide standard curve.

Preparation of glibenclamide standards: For the glibenclamide calibration standards, a stock solution of glibenclamide (Santa Cruz Biotechnology) was dissolved in methanol (1mg/ml). Mouse plasma was spiked with the stock glibenclamide solution, and using serial dilutions the following standards were prepared: no drug, 1 ng/ml, 5 ng/ml, 10 ng/ml, 20 ng/ml, 30 ng/ml, 40 ng/ml, 50 ng/ml, 100 ng/ml 150 ng/ml. Quality control (QC) samples were also prepared at 50 ng/ml . Deuterated glibenclamide (d11-glibenclamide, Santa Cruz Biotechnology) was used as the internal standard, and was added at a concentration of 333 ng/ml into all the calibration, QC and study samples.

Study sample preparation and extraction: Study plasma samples (8 from low-dose and 8 from high-dose pellet-implanted mice) were thawed and centrifuged at 14,000 x g for 10 minutes prior to use, and 60µl was added to 20µl of internal standard (at 1µg/ml) and acidified with 80µl of 4% orthophosphoric acid (from 85% w/w stock). The samples were then diluted by adding 800 µl of buffer A (2% ACN, 0.1% FA), processed by reverse phase solid phase extraction (C18-SPE, Biotage Isolute C18). Samples were washed with a further 1 ml buffer A followed by 1 ml 10 %

ACN and then eluted using two volumes of 150 µl buffer B (80% ACN, 0.1% FA) The eluates were then transferred to labelled 1.5 ml Eppendorf tubes and evaporated using a Rotovac vacuum centrifuge at 35oC for 1-2 hours. The residue was re-suspended in 12µl of 100% methanol and 48µl of 0.5 mM ammonium formate using an ultrasonic bath for 10 minutes. Finally, the samples were centrifuged for 10 minutes and the supernatant was transferred to the liquid chromatography tandem-mass spectrometry (LC-MS/MS) instrument.

Liquid chromatography tandem-mass spectrometry: LC-MSMS was performed using a Dionex U3000 nano HPLC coupled to a Bruker Esquire HTC ion trap mass spectrometer. Chromatographic separation was performed using a 50 mm x 2.1 mm, 2.6µm Accucore™ C18 RP column and pre-column (ThermoFisher Scientific). A gradient of 20-35 % B was developed over 1 minute, then 35-90 % B over 5 minutes, followed by isocratic elution at 90 % B for 3 minutes and equilibration at 20 % B for 2 minutes at 200 µl/min flow rate. The mass spectrometer was operated in positive ion mode with a scan range 300-600 m/z and scan speed 26,000 m/z/sec. The source parameters were: nebuliser gas 10 psi, drying gas 5 l/min, drying gas temperature 300oC, capillary voltage 4000V. MRM parameters were programmed for transition 494.4 m/z to 369.0 m/z (glibenclamide) and transition 505.1 m/z to 369.0 m/z (d11 glibenclamide) with isolation widths 3 Da and 4 Da respectively. Sample injection volume was 2 µl. Six replicates of all samples were performed each preceded by a blank injection. Data analysis was performed using QuantAnalysis 2.0 software (Bruker Daltonik).

1. Huang Y, McClenaghan C, Harter TM, Hinman K, Halabi CM, Matkovich SJ, et al. Cardiovascular consequences of KATP overactivity in Cantu syndrome. *JCI insight.* 2018;3(15).
2. Malester B, Tong X, Ghiu I, Kontogeorgis A, Gutstein DE, Xu J, et al. Transgenic expression of a dominant negative K(ATP) channel subunit in the mouse endothelium: effects on coronary flow and endothelin-1 secretion. *FASEB J.* 2007;21(9):2162-72.

3. Wirth A, Benyo Z, Lukasova M, Leutgeb B, Wettschureck N, Gorbey S, et al. G12-G13-LARG-mediated signaling in vascular smooth muscle is required for salt-induced hypertension. *Nat Med.* 2008;14(1):64-8.
4. Schugar RC, Moll AR, Andre d'Avignon D, Weinheimer CJ, Kovacs A, and Crawford PA. Cardiomyocyte-specific deficiency of ketone body metabolism promotes accelerated pathological remodeling. *Mol Metab.* 2014;3(7):754-69.
5. Cheng SL, Behrmann A, Shao JS, Ramachandran B, Krchma K, Bello Arredondo Y, et al. Targeted reduction of vascular Msx1 and Msx2 mitigates arteriosclerotic calcification and aortic stiffness in LDLR-deficient mice fed diabetogenic diets. *Diabetes.* 2014;63(12):4326-37.
6. Gomori G. A rapid one-step trichrome stain. *Am J Clin Pathol.* 1950;20(7):661-4.
7. Lahmann C, Kramer HB, and Ashcroft FM. Systemic Administration of Glibenclamide Fails to Achieve Therapeutic Levels in the Brain and Cerebrospinal Fluid of Rodents. *PLoS One.* 2015;10(7):e0134476.