Mice with cardiac overexpression of PPARγ have impaired repolarization and spontaneous fatal ventricular arrhythmias

SUPPLEMENTAL MATERIAL

Supplemental Online Methods

Animal Care and Breeding

The PPARγ transgenic mouse has cardiac-specific overexpression of PPARγ driven by the myosin heavy chain (MHC) promoter as previously described [1]. Animal protocols were approved by the Columbia University Institutional Animal Care and Use Committee and were carried out in accordance with the NIH guidelines for the care and use of laboratory animals. Pioglitazone-chow (30 mg/kg) was purchased from Research Diets[2].

Telemetry and ECG analysis

Telemetry devices (Data Sciences International, model EA-F20) were implanted in 10 week-old mice using inhaled isoflurane anesthesia. The two subcutaneous leads were positioned to approximate limb lead II of a human ECG. The mice recovered for one week after surgery before initiating 24-hour recordings. ECG intervals were measured manually, blinded to genotype, using Ponemah 3 software from recordings with minimal artifact at heart rates of 520-550 bpm to avoid the issues of correcting QT for rate. Intervals were averaged from 4 consecutive beats. PVC and arrhythmias counts were tallied manually by review of daily telemetry recordings. Daily heart rates were averaged for 3 animals in each group.

Echocardiography

Transthoracic echocardiography was performed using a high-resolution imaging system with a 30-MHz imaging transducer (Vevo 770; VisualSonics, Toronto, ON, Canada). The mice were anesthetized with isoflurane throughout the procedure. Care was taken to minimize sedation by monitoring the heart rate and respiratory rate of the mice. Images were obtained using short-axis views at the level of papillary muscles, and each parameter was measured using M-mode view. Images were recorded in a digital format and were then analyzed off-line. Percent fractional shortening (%FS) was calculated as follows: %FS = (LVDd-LVDs)/LVDd X 100), where LVDd is left ventricular diastolic dimension and LVDs is left ventricular systolic dimension.

Isolation of Cardiomyocytes and Cellular Electrophysiology

Cardiomyocytes were isolated using methods previously described [3]. Briefly, the mouse heart was removed and the aorta was cannulated. After perfusing Ca^{2+}-free buffer for two min, a mixture of collagenase and protease was then perfused through the coronary arteries for 5-7 min (Blendzyme 4 or Liberase TH, 0.3 mg/mL, Roche) at a $[Ca^{2+}]$ =12.5 μM. The LV tissue was teased apart with fine forceps and briefly pipetted to release individual cells. After enzymatic dispersion, $[Ca^{2+}]$ in the buffer containing 3.5 mg/ml BSA was elevated in 4 steps up to 0.8 mM. Cells were transferred into experimental temperature controlled chamber (Delta T Culture Dish, Bioptechs Inc). Only non-contracting rod shaped cells with clear striations were used in this study. Experiments were performed on freshly isolated cardiomyocytes from left ventricle of PPARγ mice (10 animals) and their littermate controls (12 animals).

Membrane currents were measured by the whole-cell patch-clamp method [4] using a MultiClamp 700B amplifier (Axon Instruments, Union City, CA). For action potential duration (APD) measurements (Fig. 3), perforated, whole-cell patch-clamp was utilized, using amphotericin B (300 μg/ml; Sigma A9528) at 35ºC. Micropipettes were pulled from borosilicate glass capillaries (BF150-110-7.5, Sutter Instruments, Novato CA) on a programmable horizontal puller (S-97; Sutter Instruments, Novato CA). The pipettes had inner tip diameters of about 1 to 1.5 μm. When filled with internal solutions, they had resistances of 1.5 to 2.5 MΩ. Data were filtered at 4 KHz with a four-pole low-pass Bessel filter and sampled at 10 KHz. All experiments were performed using pCLAMP 10.2 software (Axon Instruments, Union City CA). Boltzman's fits were performed as previously described [5].

To record action potentials and K^+ currents, the pipette solution contained 130 mM K^+ gluconate, 10 mM NaCl, 10 mM EGTA , 1 mM $MgCl_2$, 2 mM Mg-ATP, 2.0 mM $CaCl_2$ and 10 mM HEPES, adjusted to pH 7.2 with KOH. Cells were superfused at room temperature with HEPES-buffered Tyrode's containing 137 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, and 10 mM HEPES (pH was adjusted to 7.4 with NaOH). Voltage clamp correction for a liquid junction potential of -13.6 mV was made by configuring the recording files in CLAMPEX of pCLAMP 10.2. Series resistances were usually less than 2 MΩ after 60% compensation. All voltages in current clamp recording were also corrected for the junction potential.

The decay phases of the outward K^+ currents evoked during 4.0 s depolarizing voltage steps were fitted by a double exponential function of the form:

$$Y(t) = A_1\exp(-t/\tau_1) + A_2\exp(-t/\tau_2) + B,$$

where t is time, τ_1 and τ_2 are the decay time constants, A_1 and A_2 are the amplitudes of the inactivating current components ($I_{to,f}$ and $I_{K,slow}$), and B is the amplitude of the non-inactivating current component, I_{ss}

To study L-type Ca^{2+} current, we used a bath solution containing 140 mM TEA-Cl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES (pH was adjusted to 7.4 with CsOH). In these experiments, pipettes were filled with solution contained 135 mM CsCl, 10 mM EGTA, 1 mM Mg Cl_2, 2 mM Mg-ATP, 2.0 mM $CaCl_2$ and 10 mM HEPES, adjusted to pH 7.2 with CsOH. Series resistance was usually less than 2 MΩ after 60% compensation. Leak currents and capacitance transients were subtracted by a P/4 protocol. Voltage clamp correction for a liquid junction potential of -3.6 mV was made by configuring the recording files in CLAMPEX of pCLAMP 10.2. To evaluate the steady state activation of Ca^{2+} currents, the cell membrane potential was held at -70 mV and stepped for 450 ms to -60 to +50 mV in 10 mV increments. The interval between pulses was 10 s. To study the steady state inactivation of Ca^{2+} currents, the cell membrane potential was held at -70 mV and stepped to -70 to +30 mV for 650 ms in 10 mV increments and then stepped to the test potential +10 mV for 650 ms. The interval between pulses was 10 sec. Na^+ currents were measured as described previously [6].

Action potential parameters, K^+, Na^+ and Ca^{2+} currents were measured and analyzed using pCLAMP 10.2, Excel and Origin 7.5 (OriginLab, Northampton, MA) software. $V_{1/2}$ and k were calculated from Boltzmann function fitting for each cell. Statistical significance of observed differences were evaluated using TTEST ($p<0.05$). Regression analysis was done using GraphPad Software.

Real-time PCR

Samples of ventricular tissue from PPARγ mice and WT littermates, at 10-12 weeks of age, were used for harvesting RNA for RT-PCR. Cardiac tissue was homogenized with a Mini-BeadBeater

(Glen Mills, Inc). RNA was then purified using a Qiagen RNeasy kit (item 74104). cDNA was synthesized using the Applied Biosystems high capacity RNA to cDNA kit (#4387406) and diluted to 10 ng/μL for use as a template (20 ng template was used for each 20 μL reaction). Real-time PCR was performed using an Applied Biosystems StepOne Plus Real-Time PCR system with StepOne Software v2.0 and inventoried primers from Applied Biosystems. PCR was performed for 40 cycles with automated detection of crossing threshold. PCR reactions were performed with duplicate wells with actin as a control reaction and no-template lanes for negative controls.

Immunoblots

Cardiac lysates were made by homogenizing ventricular tissue in buffer containing 1% (v/v) Triton X-100, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10 mM EDTA, 10 mM EGTA, phosphatase inhibitor cocktail, and protease inhibitors (complete mini-tablet, calpain I and II inhibitors, Roche). Lysate were used for PAGE and then transferred to nitrocellulose membranes for immunoblots. The following antibodies were used: anti-Cx43 (Invitrogen), anti-K_V1.5 and anti-K_V2.1 (Alomone), anti-K_V4.2 and anti-K_V1.4 (Thermo-Scientific Pierce), and anti-tubulin (Santa Cruz Biotechnology). Chemiluminescence signal was obtained using a Kodak Image Station 400R Pro digital camera with Kodak Molecular Imaging Software v4.5.1. Signal intensity was quantified using ImageJ software (NIH). Blots for tubulin were performed to normalize loading of lanes, using the same membrane.

Immunohistochemistry

Heart tissue was fixed with 4% paraformaldehyde, embedded in paraffin wax, and then sectioned. Sections were deparaffinized and underwent antigen retrieval treatment (autoclaved with pH 9.0 Tris-buffer at 121˚C for 15 min). For DAB staining, sections were treated with 0.3% H_2O_2 to block endogenous peroxidase. Sections were incubated with rabbit polyclonal antibodies against Cx43 (1:200 dilution, Invitrogen Corp.) or non-immune rabbit polyclonal IgG at the same concentration, at 4˚C overnight. For DAB staining, after exposure to anti-rabbit swine antibody conjugated to biotin (1:500, DakoCytomation Denmark A/S, Glostrup, Denmark) for 1 hr in room temperature, the sections were treated with peroxidase-labeled ABC (VECTASATIN ABC Kit, Vector Laboratories, Inc., Burlingame, CA) and developed with DAB solution (ImmPACT DAB Peroxidase Substrate, Vector Laboratories, Inc.). After the reaction, the sections were counterstained with hematoxylin and observed by a light microscope. For immunofluorescent staining, after reaction with anti-rabbit donkey antibody conjugated to Alexa Fluor 488 (1:500, Invitrogen Corp.), the sections were counterstained with DAPI and observed by a fluorescent microscope. Tissues from two mice in each group (PPARγ overexpression and WT littermates) were used. Slides were photographed with a digital camera. Signal intensity was quantified using ImageJ software (NIH).

Supplement References:

1. Son NH, Park TS, Yamashita H, Yokoyama M, Huggins LA, Okajima K, Homma S, Szabolcs MJ, Huang LS, Goldberg IJ. Cardiomyocyte expression of PPARgamma leads to cardiac dysfunction in mice. *J Clin Invest.* 2007;117:2791-2801.

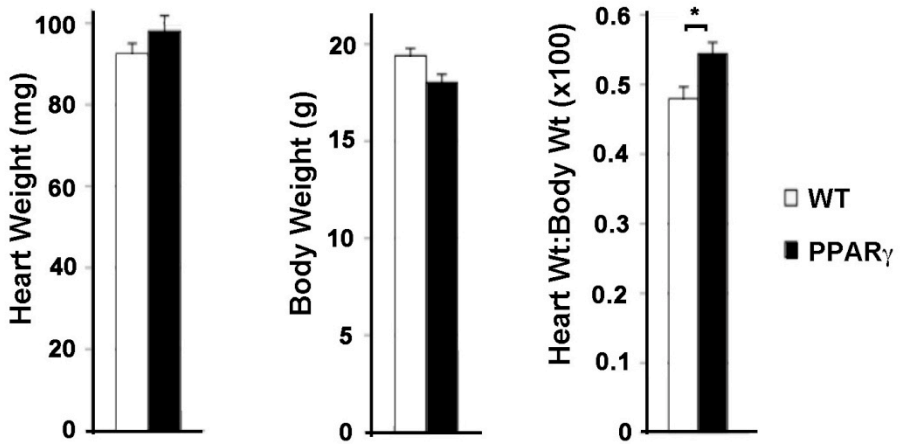
2. Raphael KL, Strait KA, Stricklett PK, Baird BC, Piontek K, Germino GG, Kohan DE. Effect of pioglitazone on survival and renal function in a mouse model of polycystic kidney disease. *Am J Nephrol.* 2009;30:468-473.

3. O'Connell TD, Rodrigo MC, Simpson PC. Isolation and culture of adult mouse cardiac myocytes. *Methods Mol Biol.* 2007;357:271-296.

4. Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch.* 1981;391:85-100.

5. Yang L, Katchman A, Morrow JP, Doshi D, Marx SO. Cardiac L-type calcium channel (Cav1.2) associates with gamma subunits. *FASEB J.* 2011;25:928-936.

6. Knollmann BC, Knollmann-Ritschel BE, Weissman NJ, Jones LR, Morad M. Remodelling of ionic currents in hypertrophied and failing hearts of transgenic mice overexpressing calsequestrin. *J Physiol.* 2000;525:483-498.



Supplemental Figure 1. Heart weight, body weight and heart weight:body weight ratio for WT and PPARγ mice. Mean + SEM. n=8, * indicates $p<0.05$ by U-test.

	WT		PPARγ	
	mean, pF	SEM	mean, pF	SEM
Whole-cell membrane capacitance	145.9	5.2	144.4	5.4
	mean, mV	SEM	mean, mV	SEM
Resting membrane potential	-80.0	0.7	-79.2	0.4
	mean, mV	SEM	mean, mV	SEM
Action potential amplitude	113.7	1.5	112.3	1.3

Supplemental Table 1. Additional patch-clamp data. WT: n=43; PPARγ: n=31. None of the comparisons were statistically significant by t-test