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Original article

Cardiac specific ATP-sensitive K^+ channel (K_{ATP}) overexpression results in embryonic lethality

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ABSTRACT

Transgenic mice overexpressing SUR1 and gain of function Kir6.2[ΔN30, K185Q] K_{ATP} channel subunits, under cardiac α-myosin heavy chain (αMHC) promoter control, demonstrate arrhythmia susceptibility and premature death. Pregnant mice, crossed to carry double transgenic progeny, which harbor high levels of both overexpressed subunits, exhibit the most extreme phenotype and do not deliver any double transgenic pups. To explore the fetal lethality and embryonic phenotype that result from K_{ATP} overexpression, wild type (WT) and K_{ATP} overexpressing embryonic cardiomyocytes were isolated, cultured and voltage-clamped using whole cell and excised patch clamp techniques. Whole mount embryonic imaging, Hematoxylin and Eosin (H&E) and α smooth muscle actin (αSMA) immunostaining were used to assess anatomy, histology and cardiac development in K_{ATP} overexpressing and WT embryos. Double transgenic embryos developed in utero heart failure and 100% embryonic lethality by 11.5 days post conception (dpc). K_{ATP} currents were detectable in both WT and K_{ATP}-overexpressing embryonic cardiomyocytes, starting at early stages of cardiac development (9.5 dpc). In contrast to adult cardiomyocytes, WT and K_{ATP}-overexpressing embryonic cardiomyocytes exhibit basal and spontaneous K_{ATP} current, implying that these channels may be open and active under physiological conditions. At 9.5 dpc, live double transgenic embryos demonstrated normal looping pattern, although all cardiac structures were collapsed, probably representing failed, non-contractile chambers. In conclusion, K_{ATP} channels are present and active in embryonic myocytes, and overexpression causes in utero heart failure and results in embryonic lethality. These results suggest that the K_{ATP} channel may have an important physiological role during early cardiac development.

1. Introduction

In the adult heart sarcolemma, K_{ATP} channels are present at higher density than any other K^+ channels although, under physiological conditions, these channels are essentially closed, and opening of as few as 1% of K_{ATP} channels is expected to shorten cardiac action potential by about 50% [1]. Opening of the K_{ATP} channel protects the heart during ischemia and reperfusion injury [2,3] and loss of channel activity is associated with reduced cardiac stress handling, heart failure and cardiomyopathy [4–6]. Structurally, K_{ATP} channels are hetero-octameric complexes of two separate proteins: the sulfonylurea receptor (SURx) subunits and pore-forming (Kir6.x) subunits [7]. Kir6.1 (*KCNJ8*) and Kir6.2 (*KCNJ11*) are members of the inwardly rectifying potassium channel family while the regulatory subunits, SUR1 (*ABCC8*) and SUR2 (*ABCC9*), belong to the ATP-binding cassette (ABC) superfamily of membrane proteins. Different combinations of Kir6.x and SURx subunits generate tissue-specific channels [7]. In rodents, ventricular K_{ATP} is composed predominantly of Kir6.2 and SUR2A [1,8]. Kir6.2 and SUR1 predominate in mouse atria [1], although SUR1 is also present in ventricular tissue [9,10].

Transgenic mice overexpressing SUR1 or gain-of-function Kir6.2 mutant (Kir6.2[ΔN30, K185Q]) under cardiac α MHC promoter control have been generated [11,12]. Kir6.2[ΔN30, K185Q] single transgenic (STG) mice exhibit ~40 fold reduction in ATP sensitivity combined with overexpression of the subunit of up to 400 fold [11], depending on line. SUR1 STG overexpress the SUR1 subunit by up to

Abbreviations: K_{ATP}, ATP-sensitive K^+ channel; αMHC, α myosin heavy chain; DTG, double transgenic; WT, wild type; H&E, Hematoxylin and Eosin; αSMA, α smooth muscle actin; dpc, days post conception; ABC, ATP-binding cassette; STG, single transgenic; PBS, phosphate buffered saline; Vhold, holding potential; GFP, green fluorescent protein; S.E.M, standard error of the mean; NCX, Na–Ca exchanger; UD, Undetermined; BPM, beats per minute.

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100 fold [12], again depending on line. None of these STG animals exhibits an overt cardiac phenotype, other than PR prolongation in SUR1 overexpressors [11,12]. However, cross-breeding of the two STG strains yielded SUR1-Kir6.2[ΔN30, K185Q] DTG mice that demonstrate marked arrhythmia susceptibility and premature death, even with relatively low expression of one or the other of the transgenes. Pregnant mice, crossed to carry double transgenic (DTG) progeny that harbor high levels of both overexpressed subunits, exhibit the most extreme phenotype and do not deliver any DTG pups [13].

It has previously been demonstrated that K_{ATP} channels are expressed and active, from early stages and throughout cardiac development in both mice and rats, although the physiological role and expression pattern in these early stages is not well characterized [14–18]. Here we characterized the embryonic lethality of K_{ATP} overexpressing DTG mice, showing that it is associated with in utero heart failure and arrest of normal cardiac development. We show that K_{ATP} channels are readily detected in WT embryos from early cardiac development stages. Together, these results indicate that K_{ATP} activity is required for normal development of the embryonic heart, and that marked over-activity of the channel can be lethal.

2. Materials and methods

2.1. Generation of transgenic mice

Heterozygous STG mice overexpressing SUR1 (Line 720) or gain of function Kir6.2[ΔN30, K185Q] (Line 4) were generated as described previously [11,12]. All procedures complied with the standards for the care and use of animal subjects as stated in the Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, revised 1996). All studies were approved by the Animal Studies Committee at Washington University.

2.2. Embryonic ultrasound imaging

Ultrasound examination of embryos was performed using a Vevo 770 Ultrasound System (VisualSonics Inc., Toronto, Ontario, Canada). Mice were lightly anesthetized with 1.5% isoflurane in 100% oxygen by nose cone and were warmed using a heated pad and a heat lamp. Physiologic parameters of adult pregnant female mice, including heart rate, respiratory rate and core body temperature, were continuously monitored by a built-in monitoring system. Maternal heart rates ranged between 480 and 640 BPM. Rectal temperature was monitored, and heating was adjusted to maintain rectal temperature between 36 °C and 38 °C. Once anesthetized, the abdomen was shaved and further cleaned with a chemical hair remover to minimize ultrasound attenuation. Transuterine embryonic imaging was conducted in pregnant mice after surgical exteriorization of the uterus. The uterus was laid out over the maternal abdomen on a sterile gauze pad presoaked in phosphate buffered saline (PBS) at 37 °C. Pre-warmed ultrasound gel was applied to the region of interest, to provide a coupling medium for the transducer. Embryos were imaged individually. Standardized imaging planes were obtained [19] and imaging was performed using 55 MHz ultrasound. Doppler flow waveforms were recorded using 40 MHz ultrasound. As reported previously, the embryonic heart was well visualized at 9.5–10.5 dpc [19]. At the end of the study, mice were euthanized by cervical dislocation while still anesthetized, and the embryos were collected for further ex-vivo studies.

2.3. Isolation and culture of embryonic cardiomyocytes

For timed mating, male and female mice were paired overnight. In the morning, the female was examined for a vaginal plug. The morning on which a mating plug was observed, was designated as 0.5 dpc. Pregnancy follow up included documentation of weight gain of the pregnant females [20]. For embryonic cell culture, pregnant females were anesthetized with Avertin administered intraperitoneally at a dose of 0.25 mg/g body weight and subsequently euthanized by cervical dislocation. The embryos were extracted from the uterus and transferred to prewarmed dissection buffer containing (in mM): 116 NaCl, 5 KCl, 0.8 $MgSO_4$, 1 NaH_2PO_4, 20 HEPES, and 5.5 glucose (pH 7.3). The embryonic heart was excised, cut into 2–4 pieces and placed in fresh dissection buffer with 0.5 mg/ml collagenase type 2 (Worthington) and 1.0 mg/ml Pancreatin (Sigma). After incubation for 15 min at 37 °C, the tissue was mechanically dispersed, plated on polylysated glass coverslips, and incubated in DMEM supplemented with 10% fetal bovine serum at 37 °C and 5% CO_2 [21].

2.4. Cellular electrophysiology

Following incubation for 24 h, glass coverslips with adherent cells were transferred into a recording chamber. Macroscopic currents in embryonic cardiomyocytes were recorded with standard whole cell voltage-clamp recording techniques performed at room temperature. Patch-clamp electrodes (1–3 MΩ when filled with electrode filling solution) were fabricated from soda lime glass microhematocrit tubes (Kimble & Chase #2502). Cell capacitance and series resistance were estimated with a 5- to 10-mV hyperpolarizing square pulse from a holding potential (Vhold) of −70 mV following establishment of the whole cell recording configuration. In all whole cell experiments, current was elicited with a slow voltage ramp protocol from −120 to 40 mV over 4 s (Vhold = −70 mV during interpulse periods). Bath solution consisted of calcium-free Tyrode solution (137 mM NaCl, 5.4 mM KCl, 0.16 mM NaH_2PO_4, 10 mM glucose, 0.5 mM $MgCl_2$, 5 mM HEPES, 3 mM $NaHCO_3$, pH 7.3–7.4). K_{ATP} activation was assessed by addition of 100 μM pinacidil, followed by inhibition with the same solution plus 10 μM glibenclamide. Pipette solution consisted of 4 mM K_2HPO_4, 120 mM K-aspartate, 1 mM $MgCl_2$, 10 mM HEPES, 20 mM KCl, 5 mM EGTA, 0.5 mM $CaCl_2$, and 1 mM ATP (pH 7.3). Excised inside-out patch data were obtained at a membrane potential of −50 mV. Standard bath (intracellular) and pipette (extracellular) solution used in the excised patch clamp experiments contained 150 mM KCl, 5 mM HEPES, and 10 mM EGTA (pH 7.3), with additions as noted. Data were filtered at 5 kHz. pCLAMP 8.2 software and a DigiData 1322 converter were used to generate command pulses and collect data.

2.5. Confocal imaging

The gain of function Kir6.2[ΔN30, K185Q] (Line 4) construct is tagged at the C-terminus with green fluorescent (GFP) protein [11]. Confocal images of WT and line 4 whole embryos and dissected hearts were acquired using a Zeiss LSM 5 Pascal confocal system mounted on Zeiss Axiovert 200 inverted microscope (Carl Zeiss Microimaging Inc, Thornwood, NY). To fit the specimen into the scanning area, images of embryos and dissected hearts were collected using a 2.5×/0.075 Plan-Neo lens (Zeiss) with a 1× scan zoom and 10×/0.3 Plan Neofluor lens with 0.8 scan zoom, respectively at 2048×2048 resolution. The fluorescence was excited using a 488 nm Ar laser line and collected through an LP505 filter. The images of GFP expression patterns in WT and line 4 specimens were collected under the same imaging conditions.

2.6. Histological and whole mount preparations

For histological analysis, embryos were fixed in either Bouin's solution or 4% parformaldehyde. Embryos were processed for routine paraffin embedding, sectioned at 5 μm and stained with H&E. For immunostaining, sections were exposed to αSMA antibodies (goat anti mouse Alexa 488 secondary antibody). Whole mount images