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M-LDH physically associated with sarcolemmal K_{ATP} channels mediates cytoprotection in heart embryonic H9C2 cells

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ABSTRACT

Muscle form of lactate dehydrogenase (M-LDH) physically associate with K_{ATP} channel subunits, Kir6.2 and SUR2A, and is an integral part of the ATP-sensitive K^+ (K_{ATP}) channel protein complex in the heart. Here, we have shown that concomitant introduction of viral constructs containing truncated and mutated forms of M-LDH (ΔM-LDH) and 193gly-M-LDH respectively, generate a phenotype of rat heart embryonic H9C2 cells that do not contain functional M-LDH as a part of the K_{ATP} channel protein complex. The K^+ current was increased in wild type cells, but not in cells expressing ΔM-LDH/193gly-M-LDH, when they were exposed to chemical hypoxia induced by 2,4 dinitrophenol (DNP; 10 mM). At the same time, the outcome of chemical hypoxia was much worse in ΔM-LDH/193gly-M-LDH phenotype than in the control one, and that was associated with increased loss of intracellular ATP in cells infected with ΔM-LDH/193gly-M-LDH. On the other hand, cells expressing Kir6.2AFA, a Kir6.2 mutant that abolishes K_{ATP} channel conductance without affecting intracellular ATP levels, survived chemical hypoxia much better than cells expressing ΔM-LDH/193gly-M-LDH. Based on the obtained results, we conclude that M-LDH physically associated with Kir6.2/SUR2A regulates the activity of sarcolemmal K_{ATP} channels as well as an intracellular ATP production during metabolic stress, both of which are important for cell survival.

1. Introduction

ATP-sensitive K^+ (K_{ATP}) channels are gated by intracellular ATP and are viewed as a link between cellular metabolism and membrane excitability. In the heart, the activation of these channels protects the cells against metabolic stress, such as hypoxia and ischaemia. It is generally accepted that cardiac sarcolemmal K_{ATP} channels are composed of Kir6.2, an inward rectifier, and SUR2A, an ABC protein (Inagaki et al., 1996). It has been recently suggested that *in vivo* these two subunits physically interact with enzymes regulating ATP production and glycolysis. One of these enzymes is a muscle form of lactate dehydrogenase (M-LDH), a minor form of LDH present in the heart (M-LDH; Carrasco et al., 2001; Crawford et al., 2002a,b; Jovanović et al., 2005; Jovanović and Jovanović, 2005; Dhar-Chowdhury et al., 2005). The physical interaction between M-LDH and Kir6.2 and SUR2A subunits has been demonstrated by co-immunoprecipitation at the recombinant and native levels, immunofluorescence and FRET analysis (Crawford et al., 2002b). However, the functional significance of M-LDH physically associated with sarcolemmal K_{ATP} channels is yet to be understood.

H9C2 cells are embryonic rat heart myocytes that have been used with success to study sarcolemmal K_{ATP} channels (Ranki et al., 2002; Crawford et al., 2003). Here, we have taken advantage of this experimental model to determine the role that species of M-LDH physically associated with Kir6.2/SUR2A play in the regulation of sarcolemmal K_{ATP} channels and cellular resistance to metabolic stress. We report that not only that M-LDH-mediated regulation of sarcolemmal K_{ATP} channels activity is crucial for cell survival during metabolic stress, but that ATP produced by sarcolemmal K_{ATP} channel protein complex mediates cytoprotection independently from the channel activity.

2. Methods

2.1. H9C2 cells and viral constructs

Rat embryonic heart H9C2 cells (ECACC, Salisbury, UK) were cultured in a tissue flask (at 5% CO_2) containing Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 2 mM glutamine. For electrophysiological experiments, the cells were plated on a 35 mm × 10 mm culture dish containing 25-mm glass cover-slips. The cells were cultured in incubators (Galaxy, oxygen control model, RS Biotech, Irvine, UK). For the experiments H9C2 cells were infected with adenoviral constructs containing either green fluorescent protein (GFP, gift from C. Sunderland,

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University of Dundee; cells infected with GFP have served as control cells in this study), gly193-M-LDH (a catalytically inactive mutant of M-LDH, (Crawford et al., 2002b) together with truncated M-LDH (ΔM-LDH) or Kir6.2AFA (a mutant form of Kir6.2 where the pore GFG was mutated into AFA leading to largely reduced K^+ conductance, Van Bever et al., 2004). When intracellular ATP levels were measured cells were infected with adenovirus containing luciferase gene. All adenoviruses were generated using the AdEasy XL Adenoviral Vector System (Stratagene) as described by the manufacturer. All of the genes were subcloned by PCR using primers containing restrict enzyme sites. Truncation of the first 19 N- and the last 48 C-terminal amino acids of mouse M-LDH was achieved using nested PCR of M-LDH gene and the following primers, sense 5′-GGCAGATCTATGCAGAACAAGATTACAGTTGT-3′, antisense, 5′-GCCTCGAGTTAATTGATTCCATAGAGACCCT-3′. gly193-M-LDH was already generated in our laboratory (Crawford et al., 2002b) and we have subcloned this gene using the following primers: sense, 5′-GGCAGATCTATGGCAACCCTCAAGGACCA-3′, antisense, 5′-GCCTCGAGTTAGAACTGCAGCTCCTTCT-3′ using gly193-M-LDH as a template. To generate Kir6.2AFA, Kir6.2 gene was subcloned using sense, 5′-GCAGGATCCACCATGCTGTCCCGAAAGGGC-3′, antisense, 5′-GCATCTAG ATCAGGACAAGGAATCTGGAG-3′ and the QuickChange Site-directed mutagenesis kit (Stratagene) was used to generate Kir6.2AFA according to the manufacturer's instructions; the mutagenic primers had the following sequences, sense, 5′-TCCAGGTGACCATTGCATTCGCAGGGCGCATGGTGACA-3′, antisense, 5′-TGTCACCATGCGCCCTGCGAATGCAATGGTCACCTGGA-3′. Luciferase gene was subcloned using the following primers: sense, 5′-GCCTCGAGGCCACCATGGAAGACGCCAAA-3′, antisense, 5′-GCGTAAGCTTACACGGCGATCTTTCCGCC-3′ using pGL3-Enhancer vector (Promega) as a template. PCR was performed by using the highest fidelity PfuUltra™ DNA polymerase (Stratagene) under the following condition: the PCR was run with a hot start for 2 min at 95 °C, followed by 25 cycles of 0.5 min at 95 °C, 0.5 min at 56 °C, and 1 min at 72 °C; and a final extension 10 min at 72 °C. The PCR products were cloned between the Bgl II and Xho I sites of the pShuttle-CMV vector. All of the positive clones containing DNA inserts were verified by DNA sequencing. After construction, the shuttle vectors were linearized with Pme I and transformed into BJ5183-AD-1 competent cells to perform homologous recombination in *Escherichia coli* with these shuttle vectors and a large adenovirus-containing plasmid following electroporation. Recombinants were identified from single colonies, linearised, and then transfected into HEK293 cells to produce infective adenovirus virions. Adenoviral particles were obtained by cell extraction after 7–10 days of transfection, and the primary virus was further amplified by infection of AD-293 cultures. The virus titer was determined using QuickTiter Adenovirus Titer Immunoassay Kit (Cell Biolabs, Inc.) according to manufacturer's instructions. Typical virus titers were in the 10^9–10^{10} pfu/ml range. To infect H9C2 cells, a solution of recombinant adenovirus was mixed with culture medium, and cells were exposed to the virus with a multiplicity of 10 viral particles/cell for 48 h. More specifically, we have added 25 μl of 10^8 pfu/ml of viral solution into each well containing $\sim 2.5 \times 10^5$ cells.

2.2. Real time RT-PCR

To determine mRNA levels of native and mutated/truncated forms of M-LDH we have used real time RT-PCR (as described in Du et al., 2006; Jovanović et al., 2008). Total RNA was extracted from H9C2 cells using TRIZOL reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. Extracted RNA was further purified by RNeasy Plus Mini Kit (Qiagen, Crawley, UK) according to the manufacturer's instruction. The specific primers used to detect the total mRNA levels for muscle lactate dehydrogenase (M-LDH) were as follows: sense, 5′-GCAGAACAAG ATTACAGTTGT-3′, antisense, 5′-CTTGATTCCATAGAG ACCCT-3′ (the size was 795 bp of the PCR product). The primers used to detect native M-LDH were: sense, 5′-GTCCTAGCACTTCACTGTCCAG-3′ antisense, 5′-CACACTAACCAGGTCAC CACTAC-3′ (the size of the PCR product was 173 bp). The specificity of primers was tested by melting curve analysis and for their ability to produce no signal in negative controls by dimer formation (Du et al., 2006). The RT reaction was carried out with ImProm-II Reverse Transcriptase (Promega, Madison, WI). A final volume of 20 μl of RT reaction containing 4 μl of 5 × buffer, 3 mM $MgCl_2$, 20 U of RNasin® Ribonuclease inhibitor, 1 U of ImProm-II reverse transcriptase, 0.5 mM each of dATP, dCTP, dGTP, and dTTP, 0.5 μg of oligo(dT), and 1 μg of RNA was incubated at 42 °C for 1 h and then inactivated at 70 °C for 15 min the produced cDNA were used as template for the quantitative real-time PCR. A SYBR Green I system was utilized in the reaction. The 25 μl reaction mixture contained: 12.5 μl iQ™ SYBR® Green Supermix (2×), 7.5 nM each primers, 9 μl of ddH2O, and 2 μl of cDNA. The thermal cycling conditions were as follows: an initial denaturation at 95 °C for 3 min, followed by 38 cycles of 10 s of denaturing at 95 °C, 15 s of annealing at 56 °C, and 50 s of extension at 72 °C. The real-time PCR was performed in the same wells of a 96-well plate in the iCycler iQ™ Multicolor Real-Time Detection System (Bio-Rad, Hercules, CA). The data were collected following each cycle and displayed graphically (iCycler iQ™ Real-time Detection System Software, version 3.0A, BioRad, Hercules, CA). Threshold cycle (CT) values were determined automatically by software. The melting curve data were collected to check the PCR specificity. The cDNA sample was duplicated, the corresponding no-RT mRNA sample was included as a negative control. The calculation of relative mRNA expression was performed as described (Pfaffl, 2001). The relative expression ratio (R) of gene encoding M-LDH is calculated using equation $R = (E_K)\Delta CP_K(C-I)/(E_R)\Delta CP_R(C-I)$ where E_K is the real time PCR efficiency of M-LDH gene transcript, E_R is the real time PCR efficiency of a reference gene (glyceraldehyde 3-phosphate dehydrogenase; GAPDH; primers and protocol used was as described in Jovanović et al., 2009), ΔCP_K is the crossing point deviation of GFP-ΔM-LDH/gly193-M-LDH of M-LDH gene transcript while ΔCP_R is the crossing point deviation of GFP-ΔM-LDH/gly193-M-LDH of a reference (GAPDH) gene transcript.

2.3. Patch clamp electrophysiology

To monitor whole cell K^+ current the giagaohm seal patch-clamp technique was applied in the whole cell configuration. For whole-cell electrophysiology applied on H9C2 cells were superfused with Tyrode solution (in mM: 136.5 NaCl; 5.4 KCl; 1.8 $CaCl_2$; 0.53 $MgCl_2$; 5.5 glucose; 5.5 HEPES-NaOH; pH 7.4). All pipettes (resistance 3–5 MΩ), were filled with (in mM): KCl 140, $MgCl_2$ 1, EGTA-KOH 5, HEPES-KOH 5 (pH 7.3). Depending whether open or closed K_{ATP} channels were required, 3 μM (for ATP-free pipette solution; this small amount of ATP was added to prevent the channel run-down) or 3 mM (to keep K_{ATP} channels closed) of ATP was added. When the effect of LDH substrates on whole cell K^+ current was assessed NADH plus pyruvate (20 mM each) was added into the pipette solution. The effect of 2,4-dinitrophenol (DNP; 10 mM) on K^+ current in H9C2 cells was measured using perforated patch clamp electrophysiology with essentially the same pipette solution as above just ATP was omitted and amphotericin B (Sigma, 240 μg/ml; Lippiat, 2008) added. For all cells monitored, the membrane potential was normally held at −40 mV and the currents evoked by a series of 400 ms depolarising and hyperpolarising current steps (−100 mV to +80 mV in 20 mV steps) recorded directly to hard disk using an Axopatch-200B amplifier, Digidata-1321 interface and pClamp8 software (Axon Instruments, Inc., Forster City, CA). The capacitance compensation was adjusted to null the additional whole-cell