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Cardiac mitochondrial connexin 43 regulates apoptosis

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Abstract

Connexin 43 (Cx43) is thought to be present largely in the plasma membrane and its function solely to provide low resistance electrical connection between myocytes. A recent report suggested the presence of Cx43 in the mitochondria as well. We confirmed the presence of Cx43 in the mitochondria isolated from adult rat ventricles with the Cx43 immunoreactivity fractionating to the outer mitochondrial membrane. Mitochondrial Cx43 is mostly phosphorylated only detected by a phospho-specific antibody. Using a Ca^{2+}-sensitive electrode and Western blot, we showed that the gap junction inhibitors 18-β-glycyrrhetinic acid (β-GA), oleamide, and heptanol all induced concomitant release of Ca^{2+} and cytochrome *C* in isolated mitochondria whereas the inactive analog 18-β-glycyrrhizic acid failed to do so. In low density neonatal myocyte culture with no appreciable cell–cell contacts, β-GA induced apoptosis as assessed by TUNEL staining. Our results suggest a novel role of Cx43 as a regulator of mitochondrial physiology and myocyte apoptosis.

Keywords: Mitochondria; Connexins; Apoptosis; β-Glycyrrhetinic acid; Gap junction inhibitors; Cytochrome *C*; TUNEL; Cardiac myocytes

Cellular apoptosis plays a critical role in cardiovascular diseases [1,2]. While many regulators of this programmed cell death pathway have been identified the list is clearly incomplete. Connexin (Cx) 43, the major gap junction forming protein in the adult cardiac ventricles, plays a pivotal role in mediating tissue injury and post-ischemic cardiac dysfunction, however, the mechanism by which Cx43 modulates cellular injury is not known.

Most of the function ascribed to Cx43 in cardiac pathophysiology is within the context of its role in forming the gap junction [3]. However, recent literature reports atypical Cx43 functions in roles outside of the traditional gap junction [4,5]. We recently discovered that Cx43, independent of its ability to form functional gap junctions, regulates susceptibility of cells to several cell injury paradigms suggesting a novel function of this protein in modulating cell death [6]. Cx43 is abundantly expressed in the adult cardiac ventricles, yet little study exists addressing the role of cardiac Cx43 in modulating myocyte death. Myocyte apoptosis is now recognized to mediate cell death in a variety of acute and chronic heart diseases and understanding how Cx regulates apoptosis may lead to a new novel approach to preventing myocyte death.

A recent report suggested that Cx43 is present in the mitochondria and may play a role in mediating the cardioprotective effect of ischemic preconditioning [7]. In the present study, we confirmed the presence of Cx43 immunoreactivity in the outer membrane of the mitochondria isolated from adult cardiac ventricles and provide evidence that mitochondrial Cx43 as a novel regulator of mitochondrial function where inhibition results in the release of cytochrome *C* and myocyte apoptosis.

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Methods

Isolation of rat ventricular mitochondria and fractionation. All procedures were conducted in accordance with institutional animal care regulations. Minced rat ventricular tissue chunks were suspended in a mannitol–sucrose (M/S) buffer (in mM): 255 mannitol, 10 sucrose, 0.5 EGTA, 1 glutathione, 10 Hepes, pH 7.4, and cells lysed by 10 strokes of a Dounce homogenizer. The first low speed centrifugation at $2000g \times 10$ min separated the unlysed cells and the nuclei. The supernatant from the first spin (S1) was re-spun at $6000g \times 10$ min and the pellet (P2) was collected as the fraction enriched in mitochondria. For the mitochondria functional assay, the P2 pellet was resuspended in the M/S buffer without EGTA and oxygen applied by blow-by until used. For protein isolation, the P2 pellet was resuspended in RIPA buffer (1% Nonidet P40, 10 mM Tris pH 7.6, 50 mM NaCl, 30 mM NaPPi, 50 mM NaF, 1% Triton X-100, and 0.1% sodium dodecyl-sulfate) and sonicated (50% power for $2\,s \times 10$ pulses, Cole Palmer Model 130 Ultrasonic Processor). All procedures were done at 4 °C or on ice.

Subcellular fractionation of the organelles by idioxanol gradient (19–27%) centrifugation was carried out according to manufacturer's instructions (Axis–Shield, Oslow, Norway). For the sub-mitochondrial fractionation, the P2 pellet suspended in M/S buffer supplemented with 1 mM DTT and 0.2 mM PMSF was sonicated, as noted above, followed by 3 freeze-thaw cycles (modified from Beutner et al. [8]). The mitochondrial fragments were loaded on a sucrose gradient (from 60 to 30%) in 10 mM Hepes, pH 7.4, supplemented with a protease inhibitor cocktail (Complete, Roche Applied Science, Indianapolis, IN, USA), and centrifuged at 100,000 *g* for 3 h at 4 °C (Beckman Optima TLX centrifuge with a TL55 swinging bucket rotor).

Western blotting. Proteins subjected to SDS–PAGE and transferred to a nitrocellulose membrane was blocked in 3% milk-TBST and probed with the following primary antibodies: *ANT* (1:500, Santa Cruz Biotech, Santa Cruz, CA, USA; sc-11433); *Calreticulin* (1:500, Affinity Bioreagents, Golden, CO, USA; #PA1–903); *Cx43* (1:500, Chemicon International, Temecula, CA, USA; MAB3067), *GAPDH* (1:100,000, Advance Immunochemical, Long Beach, CA, USA; #RGM2); *N-cadherin* (1:1000, Zymed Lab, South San Francisco, CA, USA; #18–0224), *VDAC* (1:1000, Oncogene, Boston, MA, USA; #PC548), all in 3% milk-PBST, and reacted with the horseradish peroxidase-conjugated secondary antibody (1:2000) in 1% milk-TBST. After reaction with the Western Lightning chemiluminescence reagent (NEN Life Science Products, Boston, MA, USA), the images were captured on the EpiChemi Darkroom System (UVP Inc., Upland, CA). The commercially available anti-Cx43 antibody raised against a peptide–epitope (pan-Cx43) used in this study is well-characterized specifically recognizing both the non-phosphorylated and phosphorylated Cx43 protein with little non-specific reactivity [9]. The Cx-1B1 anti-Cx43 antibody (#13–8300, Zymed Laboratory) only recognizes Cx43 when the serine at residue 368 is unphosphorylated (P0-Cx43) [10]. Dephosphorylation was accomplished by incubating mitochondrial lysate with calf intestinal alkaline phosphatase (10 U) at 37 °C for 20 h and separated by a 12% SDS–PAGE to optimize separation of multiple bands in the 40–50 kDa range.

Immunohistochemistry. Cultured cardiomyocytes were fixed with 4% paraformaldehyde in 0.1 M PB for 15 min. After permeablization with 0.2% Tween 20 in phosphate buffered saline (PBST), and blocking (10% normal goat serum in PBST) both for 15 min at room temperature, the samples were incubated with the following antibodies: Cx43 antibody (1:200), VDAC (1:200), and α-actinin (1:500, Sigma–Aldrich, St.Louis, MO, USA) all in PBST with 2% normal goat serum for 2 h at room temperature. After washing, the cells were reacted with the appropriate secondary antibody conjugated to Alexa 487 or 594 (1:500, Molecular Probes, Portland, OR, USA), and visualized under a Zeiss LSM 510 NLO confocal microscope (Oberkochen, Germany) and psuedo-colored in Photoshop.

Measurement of mitochondrial calcium uptake. Mitochondrial suspension in MS buffer without EGTA was placed in a stirred container and the free-[Ca^{2+}] within the solution adjusted to approximately 10 μM and thereafter continuously monitored by a Ca^{2+}-sensitive electrode (MI-600, Microelectrodes Inc., Bedford, NH, USA) connected to an Accumet 25 pH meter (Fisher Scientific, Pittsburg, PA, USA). The voltage output was calibrated using solutions of known free-[Ca^{2+}] (WP Instruments, Sarasota, FL, USA) and demonstrated a superior log-linearity over a wide range of [Ca^{2+}].

Myocyte culture and apoptosis cell count assay. Rat neonatal ventricular myocytes were isolated from day 2–4 neonatal pups by enzymatic digestion following a procedure recommended by the manufacturer (Worthington, Lakewood, NJ, USA). After pre-panning the remaining myocyte-enriched suspension was plated at a low density (50,000 cells/35 mm well). The growth of background cells was inhibited by addition of cytosine arabinoside (1 μM) 24 h after plating and thereafter, the culture was maintained in DMEM supplemented with 10% newborn calf serum, 10% equine serum, and 1% penicillin/streptomycin.

For the apoptotic cell count assay, myocytes were treated with β-GA (0–100 μM) or its inactive analogue (GZ, 100 μM) for 24 h in the serum containing medium. The presence of the serum reduced the total number of apoptotic cells making the visualization and counting of apoptotic cells easier. Fixed (4% PFA) myocytes were subjected to the terminal deoxyribonucleotide transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay as per manufacturer's instructions (Promega, Madison, WI, USA). The cells counts were taken from no less than 12 random fields, where the total number of myocytes counted was typically 1000 cells. Data (mean ± SEM) is expressed as % TUNEL-positive.

Chemicals. All chemicals were purchased from Sigma–Aldrich except for carbonlycyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP) (Biomol, Plymouth Meeting, PA, USA) and MitoTracker (Invitrogen, Carlsbad, CA, USA).

Results

Anti-Cx43 immunoreactivity in the cardiac mitochondria

Immunoreactivity to anti-Cx43 antibody was investigated in cultured rat neonatal ventricular myocytes. The culture prepared by a standard method consisted of myocytes and non-myocyte support cells; however, the myocytes were easily identified because of their characteristic stellate morphology and the immunoreactivity to anti-α-actinin antibody. Immunostaining with anti-Cx43 antibody (Fig. 1A, red) demonstrated numerous punctate widely distributed immunoreactive spots in addition to the expected linear streaks at the cell borders. The punctate immunoreactive spots resembled the distribution of mitochondria in these cells which was confirmed by MitoTracker staining (Fig. 1A, green) and by anti-VDAC antibody immunoreactivity (data not shown). However, inspection of the images indicated that the two markers only partially overlapped.

Localization of Cx43 in low density cultured neonatal rat ventricular myocytes maybe abnormal, therefore, we attempted to demonstrate the presence of anti-Cx43 immunoreactivity in the adult ventricular mitochondria by biochemical organelle and protein fractionation methods. A crude fractionation of cellular organelles was accomplished by a simple 2-step centrifugation and the isolated fractions probed by immunoblotting (Fig. 1B). The following immunoreactive signals were used as markers: anti-VDAC (mitochondrial marker), anti-GAPDH (cytosolic marker), and N-cadherin (plasma membrane marker). The P2 pellet