Calcium preconditioning inhibits mitochondrial permeability transition and apoptosis

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Calcium preconditioning inhibits mitochondrial permeability transition and apoptosis. Am J Physiol Heart Circ Physiol 280: H899–H908, 2001.—We tested the hypothesis whether calcium preconditioning (CPC) reduces reoxygenation injury by inhibiting mitochondrial permeability transition (MPT). Cultured myocytes were preconditioned by a brief exposure to 1.5 mM calcium (CPC) and subjected to 3 h of anoxia followed by 2 h of reoxygenation (A-R). Myocytes were also treated with 0.2 μM cyclosporin A (CsA), an inhibitor of MPT, before A-R. A significant increase of viable cells and reduced lactate dehydrogenase release was observed both in CPC- and CsA-treated myocytes compared with the A-R group. Cytochrome c release was predominantly observed in the cytoplasm of myocytes in the A-R group in contrast with CPC- or CsA-treated groups, where it was restricted only to mitochondria. Similarly, the cell death by apoptosis was also markedly attenuated in these groups. Electron-dense Ca2+ deposits in mitochondria were also less frequent. Atractyloside (20 μM), an adenine nucleotide translocase inhibitor, caused changes similar to those in the A-R group, suggesting a role of MPT in A-R injury. Protection by inhibition of MPT by CsA and CPC suggests that MPT plays an important role in reoxygenation/reperfusion injury. The data further suggest that preconditioning inhibits MPT by inhibiting Ca2+ accumulation by mitochondria.

References

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chemia-induced cell death and CPC could ameliorate cell injury by prevention of MPT in cultured neonatal rat myocytes.

MATERIALS AND METHODS

All animals were treated in accordance with the Guidelines for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication No. 85-23, Revised 1985). Cytochrome c (from the bovine heart), atracyloside (ATR), bromodeoxyuridine (BrdU), and goat anti-mouse IgG (Fab fragment) peroxidase conjugate were purchased from Sigma Chemical (St. Louis, MO); cyclosporin A (CsA) was purchased from Biomol Research Laboratories (Plymouth Meeting, PA).

Preparation of Myocyte-Rich Culture

Primary cultures of the neonatal rat myocytes were prepared as described previously (38). To selectively enrich myocytes, dissociated cells were preplated for an hour to allow nonmyocytes to attach to the bottom of the culture dish. The resultant suspension of myocytes was transferred onto collagen-coated 60-mm or 100-mm culture dishes. BrdU (100 μM) was added during the first 24 to 36 h to prevent proliferation of nonmyocytes. Cultured cells were further confirmed using immunofluorescence staining with a monoclonal antibody against sarcomeric α-actinin (14).

Experimental Protocols

The experiments were performed on myocyte-rich cultures on the third day. Cultured myocytes were divided into five groups (Fig. 1), and the medium was replaced by Tyrode solution (pH 7.4 at 37°C), which contained (in mM) 125 NaCl, 2.6 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 1.0 CaCl₂, and 25 HEPES with or without glucose.

Group 1: control. Myocytes were incubated in aerobic Tyrode solution with glucose (25 mM) during the entire experimental period.

Group 2: anoxia-reoxygenation. Myocytes were incubated with anaerobic glucose-free Tyrode solution for 3 h of anoxia followed by 2 h of reoxygenation (A-R). Myocytes in Tyrode solution were transferred into the anoxic chamber (Forma 1025 anaerobic system). To induce complete anoxia, Tyrode solution was deoxygenated by bubbling with purified nitrogen for 1 h before the experiments. Reoxygenation was carried out by returning the myocytes to the incubator.

Group 3: CsA + A-R. Myocytes were preincubated with CsA (0.2 μM/l) for 20 min before anaerobic incubation and reoxygenation. CsA is a strong inhibitor of MPT (12) and thus reduces cell necrosis.

Group 4: CPC.

GROUP 4A. Myocytes were exposed two times each for 4 min with Tyrode solution containing 1.5 mM/l Ca²⁺ followed by 6 min of normal Ca²⁺-containing Tyrode solution (1.0 mM/l) before the A-R protocol.

GROUP 4B: CPC + ATR. To determine whether inhibition of MPT is the mechanism of CPC, ATR (20 μM/l), an inhibitor of ANT, which opens the pore, was added during the preconditioning protocol.

Cell Viability and ATP Assay

Determination of myocyte injury was carried out at the end of the A-R. The extent of A-R-induced injury was quantitated based on the number of dead cells, ATP content, and lactate dehydrogenase (LDH) release as well as morphological examination.

Cell viability was calculated by dividing the number of trypan blue-negative cells from the total number of cells examined and then multiplied by 100%. Ultrastructural assessment of myocytes was carried out by transmission electron microscopy. Myocytes cultured on the coverslips were...
immersed in 2.5% buffered glutaraldehyde for 4 h, rinsed in 0.1 mol/l sodium cacodylate buffer (pH 7.3), and postfixed in 1% buffered osmium tetroxide. The cells were embedded in epon resin and cut into 600-nm-thick sections with a Sorvall MTB2 ultramicrotome. The sections were stained with uranyl acetate and lead citrate and examined with a Hitachi H-600 electron microscope at 75 kV.

ATP was determined as previously described (23). Isolated myocytes were homogenized with a sonic dismembrator in 6% trichloroacetic acid. The homogenates were centrifuged at 25,000 g for 10 min. The extracts were neutralized with potassium carbamate. ATP was analyzed at 340 nm in a Beckman spectrophotometer by using an ATP detection kit (Sigma). The results were expressed in nanomoles per milligram protein. LDH release from myocytes was measured by using a LDH detection kit (Sigma) and expressed as milliunits per milligram protein.

Detection of Apoptotic Cells

To visualize apoptotic nuclei in cardiac myocytes in situ, the ApoTag in situ apoptosis detection kit (Onco) was used. The cultured myocytes were fixed in 4% paraformaldehyde (pH 7.4) and subjected to terminal transferase-mediated dUTP-biotin nick end-labeling (TUNEL) assay. In brief, myocytes were incubated for 1 h at 37°C in TdT buffer (pH 7.2) containing (in mM) 140 sodium cacodylate, 1 cobalt chloride, 30 Tris-HCl, 50 units of terminal deoxyxynucleotide transferase, and 1 nM of FITC-conjugated dUTP. After the TdT reaction, myocytes were washed three times in phosphate-buffered saline (PBS) and mounted on glass slides. As negative control, sections were incubated in the absence of TdT and expressed as milliunits per milligram protein.

Immunocytochemistry. The distribution of cytochrome c in intact myocyte was assayed as described by Roberg et al. (30). Cultured myocytes on coverslips were fixed in 2% formaldehyde and blocked with 10% normal goat serum. Mouse monoclonal anti-cytochrome c antibody was used at a dilution of 1:100. After washing coverslips with PBS-0.1% Tween 20, cells were incubated with secondary antibody consisting of FITC-conjugated anti-mouse IgG antibody. For mitochondrial staining, unincubated cells were incubated with 500 μM Mito Tracker Orange CMTMRos (Molecular Probes) for 30 min at 37°C. After washing and fixation in 2% formaldehyde, cells were stained further with anti-cytochrome c antibody as described above.

Statistical Analysis

All the experiments were carried out from at least three independent myocyte cultures with replicates of two for each condition. The data were expressed as means ± SE. Group comparisons were analyzed by a two-way analysis of variance. Statistical significance between groups was determined by Student’s t-test. Spearman’s analysis was used for the correlation between the parameters. A value of $P < 0.05$ was considered significant.

RESULTS

CPC Prevents A-R Injury

The purification of cardiac myocytes was assessed with the mouse anti-α-actinin and FITC-labeled mouse anti-IgG antibody. With the use of the present method, we obtained 90% myocytes in the cultured cells. The effect of A-R on the cell viability was examined by using a trypan blue exclusion assay (Fig. 2). In the control neonatal myocytes, 86.5 ± 2.7% cells excluded trypan blue and were considered normal, whereas in the A-R group only 38.5 ± 3.1% excluded trypan blue. CPC significantly increased the number of viable cells (54.3 ± 3.3% vs. 38.5 ± 3.1%, $P < 0.05$) compared with A-R alone. In the transmission electron microscope, the cell membrane was intact, and well-defined rows of mitochondria were observed between the compact myofibrils or scattered loosely throughout the cytoplasm. Nuclear chromatin material was uniformly dispersed (Fig. 3A). The myocytes subjected to A-R were characterized by calcified mitochondria, clumped chromatin material, wavy myofibrils, and granularity of cytoplasm with distorted subcellular organelles (Fig. 3B). The cellular structures were extremely well preserved in CPC cells subjected to the A-R and were similar to structures in the control cells. Mitochondria were usually elongated. Nuclear chromatin material was uniformly dispersed (Fig. 3C).

LDH release was significantly increased (130.3 ± 11.0 mU/mg protein) in the A-R group compared with that of control group (45.5 ± 6.5 mU/mg protein) (Fig.

Quantitation of Cytochrome c

Isolation of mitochondrial and cytosolic fractions. Cells were harvested by centrifugation at 600 g for 10 min at 4°C. The pellet was washed with PBS, and cells were scraped into HEPES buffer (pH 7.5) containing (in mM/l) 10 HEPES, 200 mannitol, and 70 sucrose, which contained protease and phosphatase inhibitors. After chilling on ice for 3 min, the cells were disrupted by 40 strokes of a sonic dismembrator (Fisher model 60). The samples were centrifuged (500 g) to pellet nuclei, unbroken cells, and plasma membrane debris (nuclear fraction). The supernatants were recentrifuged (10,000 g) to separate the mitochondrial fraction from the cytosolic fraction. The mitochondrial fraction was resuspended in HEPES buffer containing 1% (vol/vol) Triton X-100. The supernatants were recentrifuged once more at 200,000 g for 30 min, resulting a supernatant (cytosolic fraction).

Western blot analysis. Cytochrome c release from the mitochondria into cytosol was determined at the end of the experiment by using a modified method of Cook et al. (5). Aliquots of mitochondria and cytosol were boiled, and 10 μg of protein from both fractions was added on 13% SDS-polyacrylamide gels. After electrophoresis, the samples were transferred to Trans-Blot transfer medium-supported nitrocellulose membranes (Bio-Rad). The blots were then blocked with 5% nonfat milk and incubated with the first antibody and second antibody at room temperature. The first antibody was mouse monoclonal antibody to denatured cytochrome c (1:500; Pharmingen). The secondary antibody was horseradish peroxidase-conjugated goat anti-mouse antibody (1:2000; Sigma). The bands were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech), and blots were exposed to Hyperfilm MP for 30 s–2 min. Laser scanning densitometry was used for the semiquantitative determination of the proteins.
In the CPC group, the LDH release was similar to that of control group (66.9 ± 11.9 mU/mg protein). ATP in the myocytes subjected to A-R was significantly decreased when compared with the cells in the control group (7.6 ± 2.5 vs. 27.5 ± 1.8 nM/mg protein in control, *P*, 0.05) (Fig. 4B). The ATP content was well preserved in the CPC group (17.3 ± 1.4 nM/mg protein).

Cell shrinkage and nuclear fragmentation, which are the typical morphological features of apoptosis, were commonly observed. In our experimental conditions, the number of TUNEL-positive nuclei in the control was <10% (Fig. 5A). However, in the A-R group, the number of TUNEL-positive cells was markedly increased (39.2 ± 3.0%) (Fig. 5B). In the CPC group (Fig. 5C), the number of TUNEL-positive cells was significantly decreased (20.2 ± 2.1%) compared with that in the A-R group.

CPC Inhibits MPT

A-R has adverse effects on mitochondrial structure and function. We used a specific inhibitor of MPT to observe whether the induction of MPT is critical for
A-R-induced injury. We then tested whether a specific agonist for the mitochondrial pore induces cell injury similar to A-R. Finally, the distribution of cytochrome c was determined.

The treatment with CsA (0.2 μM/l) inhibited the killing of cells and increased the cell survival (56.3 ± 2.8 vs. 38.5 ± 3.1%, P < 0.05, CsA group vs. A-R group) similar to that of the CPC group (Fig. 2). The release of LDH from myocytes was significantly reduced (56.9 ± 9.0 mU/mg protein) compared with the A-R group (130.3 ± 11.0 mU/mg protein) (Fig. 4A). In addition, CsA also significantly inhibited the A-R-stimulated apoptosis (19.5 ± 2.0 vs. 39.2 ± 3.0%) (Fig. 5) and reduced the morphological changes (Fig. 3).

However, the effect of CPC was inhibited by administration of ATR (20 μmol/l) during CPC. The cell survival rate was significantly decreased in the treated group compared with the CPC group (39.3 ± 3.2 vs. 54.3 ± 3.3%) (Fig. 2). The LDH release from myocytes was increased (100.9 ± 9.4 vs. 66.9 ± 11.9 mU/mg protein, ATR group vs. CPC group), and ATP was exhausted (Fig. 4B). Furthermore, the percentage of apoptotic cardiac myocytes was increased (27.5 ± 2.2 vs. 20.2 ± 2.1%) (Fig. 5), and myocytes underwent severe structural changes (Fig. 3D).

The mitochondrial impairments may lead to the induction of apoptosis through the release of cytochrome c. As expected, cytochrome c release was increased in the cytosol when myocytes were subjected to A-R compared with the control myocytes or CPC group (Fig. 6). Pretreatment of myocytes with CsA significantly blocked the release of cytochrome c from cells subjected to A-R (P < 0.05) and was similar to the CPC group. In

![Fig. 4. Effect of anoxia and reoxygenation and other treatments on lactate dehydrogenase (LDH) release (A) and cell ATP (B). +P < 0.05 vs. control; +P < 0.05 vs. A-R; #P < 0.05 vs. CPC + A-R.](image)

![Fig. 5. Effect of anoxia and reoxygenation and other treatments on apoptosis as determined by terminal transferase-mediated dUTP-biotin nick end-labeling (TUNEL) staining. Nuclei were stained red by propidium iodide to identify cells. A: control, a limited number of TUNEL-positive nuclei were seen; B: during anoxia and reoxygenation, TUNEL-positive cells (green) were increased; C: preconditioned myocytes showed a limited number of TUNEL-positive nuclei; D: quantitative estimate of TUNEL-positive cells. Results are expressed as means ± SE for 3 independent experiments. +P < 0.05 compared with control; +P < 0.05 compared with A-R alone; P < 0.05, compared with CPC + A-R.](image)
contrast, ATR aggravated the release of cytochrome c from mitochondria (Fig. 6). The concentration of cytochrome c in mitochondria displayed a negative linear correlation with the percentage of apoptosis in the myocytes (Fig. 7). To confirm translocation of cytochrome c to the cytosol in individual apoptotic cells, immunocytochemistry was performed (Fig. 8). In control cells with normal nuclear morphology, cytochrome c immunostaining was observed in oval to elongated or punctate bodies, which coincided the distribution of mitochondria that were stained with Mito Tracker Orange CMTMRos. In contrast, the myocytes that underwent A-R exhibited a diffuse cytochrome c immunostaining (Fig. 8).

**DISCUSSION**

Molecules released after a brief ischemic or Ca\(^{2+}\) stress (so-called preconditioning phenomenon) are known to induce endogenous protection against prolonged ischemia. Among these molecules, Ca\(^{2+}\) is an important molecule for both cell death and life. A slight increase in \([\text{Ca}^{2+}]_i\), as observed in preconditioning (21, 22), is beneficial and an important trigger for the CPC. Intracellular Ca\(^{2+}\) can activate several important enzymes, which can initiate different signaling pathways for the cardiac protection. Some of these enzymes include protein kinase C (36, 37, 41), inducible nitric oxide synthase (2), and mitogen-activated protein kinases (19, 20). On the other hand, the excessive Ca\(^{2+}\) accumulation can lead to cell death under different pathological conditions (10, 18).

This study utilized Ca\(^{2+}\) as a stress molecule to activate intracellular machinery for the attenuation of anoxic injury. Calcium-mediated pathways have provided protection against ischemia using neonatal myocytes (this study) and intact hearts (22, 29) similar to that provided by IPC. It has been suggested that free radicals are important intracellular signaling components in producing IPC in cardiocytes (9, 31, 40). Although increased free radicals production during hypoxic preconditioning appears to originate from the mitochondrial electron transport system (9), more experiments are needed to confirm the mechanism by which CPC affects intracellular free radical production.

One of the mechanisms involved in the protection is the preservation of mitochondrial structure and function. Once the mitochondria accumulate Ca\(^{2+}\), the ATP synthesis ceases, and the myocytes undergo irreversible cell injury after lethal ischemia (15). This is preceded by the opening of a pore in the inner mitochondrial membrane, and it occurs soon after reoxygenation, which is accompanied by Ca\(^{2+}\) overload (8). MPT induces cytochrome c release and, consequently, apoptosis (26), and this is in agreement with the data of this study. Apoptosis was significantly
Fig. 8. Immunocytochemical localization of cytochrome c in myocytes. A: staining of control myocytes with 500 nm/l Mito Tracker Orange CMTMRos shows the location of mitochondria (arrowhead) (red); B: anti-cytochrome c antibody (labeled FITC) (green) shows location of cytochrome c in discrete oval to elongated bodies, which represent mitochondria (see A). C: superimposition of A and B images shows mitochondria and cytochrome c localization (arrowhead). D–F: anoxic and reoxygenated myocytes, which are stained similar to A–C, respectively. Mitochondria (arrow) are swollen and fuzzy in D, and cytochrome c is released from mitochondria into the cytoplasm (E), and superimposition of D and E images (F) shows no definite pattern of cytochrome c and mitochondria. G–I: preconditioned myocytes showing well preserved mitochondria (G) and cytochrome c (H) is seen in oval bodies representing mitochondria (arrowhead). Superimposition of G and H images (I) shows the mitochondria containing cytochrome c (orange).
reduced in the preconditioned myocytes. MPT was inhibited by CsA, an inhibitor of MPT, and was promoted by ATR, an ANT inhibitor (11). Thus it is clear that CPC maintains the mitochondrial Ca\(^{2+}\) homeostasis and prevents MPT. MPT was accompanied by cytochrome \(c\) release from mitochondria, which is a typical feature of apoptosis. Cytochrome \(c\) was seen distributed throughout the cytoplasm in the myocytes after A-R and in myocytes treated with ATR. Cytochrome \(c\) release can also result from changes in mitochondrial membrane permeability after loss of membrane potential during apoptosis (42). This may result in the swelling of the mitochondria, rupturing the outer membrane and releasing cytochrome \(c\) into cytosol (3). It has been demonstrated that the induction of MPT causes release of cytochrome \(c\) from mitochondria, which is required for the genesis of apoptosis, caspase 3 activation, and nuclear laddering (39). Our study further confirmed this observation that release of cytochrome \(c\) from mitochondria preceded the apoptosis in cultured myocytes during A-R.

The mechanism by which preconditioning inhibits MPT is not yet totally clear. It appears that initial excessive Ca\(^{2+}\) accumulation by mitochondria after reoxygenation promotes the opening of pores in the inner membranes of mitochondria, which subsequently allows unlimited entry of Ca\(^{2+}\) during reoxygenation. Crompton and Costi (6) have shown that Ca\(^{2+}\) accelerates the opening of pores in isolated mitochondria and that it was dependent on the level of cellular ATP. We have previously shown that preconditioning preserves ATP contents in the ischemic myocardium (36) and maintains the structural integrity of mitochondria (35). Therefore, it is likely that preconditioning prevents mitochondrial pore opening. Similarly, activation of mitochondrial K\(_{\text{ATP}}\) channels during preconditioning leads to reduction in Ca\(^{2+}\) accumulation by mitochondria, as observed by electron microscopy (35). The study by Holmuhamedov et al. (13), in which isolated preloaded mitochondria released their Ca\(^{2+}\) contents on opening of the mitochondrial K\(_{\text{ATP}}\) channel, is also in agreement with our conclusions that the degree of Ca\(^{2+}\) accumulation by mitochondria may be a determinant of MPT. Further direct measurements of mitochondrial Ca\(^{2+}\) and its role in the opening of mitochondrial pores during reoxygenation are needed to determine its role in cell death and apoptosis.

In summary, MPT is a leading determinant of myocyte cell death, and preconditioning suppresses the pore opening and apoptosis.

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