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Preconditioning prevents loss in mitochondrial function and release of cytochrome *c* during prolonged cardiac ischemia/reperfusion ☆

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Abstract

Loss in mitochondrial function and induction of mitochondrial-mediated apoptosis occur as a result of cardiac ischemia/reperfusion. Brief and repeated cycles of ischemia/reperfusion, termed ischemic preconditioning, prevent or minimize contractile dysfunction and apoptosis associated with prolonged episodes of cardiac ischemia and reperfusion. The effects of preconditioning on various indices of ischemia/reperfusion-induced alterations in mitochondrial function and structure were therefore explored. Utilizing an in vivo rat model data is provided indicating that preconditioning completely prevents cardiac ischemia/reperfusion-induced: (1) loss in the activity of the redox sensitive Krebs cycle enzyme α-ketoglutarate dehydrogenase; (2) declines in NADH-linked ADP-dependent mitochondrial respiration; (3) insertion of the pro-apoptotic Bcl-2 protein Bax into the mitochondrial membrane; and (4) release of cytochrome *c* into the cytosol. The results of the current study indicate that preconditioning prevents specific alterations in mitochondrial structure and function that are known to impact cellular viability and provide insight into the collective benefits of preconditioning.

Keywords: Heart; Ischemia/reperfusion; Preconditioning; Calcium; Free radicals; Mitochondria; α-Ketoglutarate dehydrogenase; Bax; Cytochrome *c*; Apoptosis

Brief and repeated cycles of ischemia/reperfusion, termed ischemic preconditioning, prevent or minimize contractile dysfunction and myocardial necrosis and apoptosis associated with prolonged episodes of cardiac ischemia and reperfusion [1–4]. While mechanism(s) by which these protective effects are mediated have not been fully delineated, preconditioning diminishes reperfusion-induced mitochondrial Ca^{2+} overload, [5,6] free radical production, [7,8] and declines in respiratory activity [7,9–11] events that can result in the disruption of cellular processes and in the induction of cell death [12–14]. Processes initiated at and/or disseminated from the mitochondria are likely central factors in ischemia/reperfusion injury and prevention of their occurrence may underlie the benefits of preconditioning [7]. Mitochondria exhibit dramatic increases in Ca^{2+} concentrations, [15–17] are a major source of oxygen-derived free radicals, [7,18–23] and exhibit declines in the rate of respiration and oxidative phosphorylation during cardiac ischemia/reperfusion [7,19,23–32]. In addition, necrotic and apoptotic cell death are dependent on changes in mitochondrial function and/or integrity and can be initiated by disruption of Ca^{2+}, energy, and/or redox homeostasis [12–14].

Given that preconditioning is associated with a reduction in ischemia/reperfusion-induced Ca^{2+} overload [5,6] and free radical production, [7,8] we sought evidence for protection from various indices of mitochondrial dysfunction. We have previously shown that cardiac ischemia/reperfusion resulted in inactivation of the redox

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sensitive Krebs cycle enzyme α-ketoglutarate dehydrogenase (KGDH)[1] [28,30,33–36]. Inactivation of KGDH was largely responsible for observed declines in NADH-linked ADP-dependent respiration [28,30]. In addition, ischemia/reperfusion resulted in release of cytochrome *c* from the mitochondria, [37–39] consistent with the induction of mitochondrial-mediated apoptosis [40–42]. Utilizing an in vivo rat model of coronary occlusion and reperfusion, the effects of preconditioning on ischemia/reperfusion-induced alterations in mitochondrial respiration, KGDH activity, and cytochrome *c* distribution were determined. The results of the current study indicate that preconditioning prevents specific alterations in mitochondrial structure and function that have previously been associated with increases in Ca^{2+} concentration and free radical production.

Materials and methods

In vivo model of coronary occlusion/reperfusion

Male Fisher-344 rats (6 months) were subjected to left anterior descending coronary occlusion and reperfusion for varying periods of time as previously described [39]. Four experimental protocols were investigated: (1) control perfusion: (60 min instrumented perfusion); (2) control perfusion + preconditioning: (40 min instrumented perfusion followed by 2 cycles of 5.0 min LAD occlusion and 5.0 min reperfusion); (3) ischemia/reperfusion: (30 min LAD occlusion followed by 60 min reperfusion); and (4) preconditioning + ischemia/reperfusion: (2 cycles of 5.0 min LAD occlusion and 5.0 min reperfusion followed by 30 min LAD occlusion and 60 min reperfusion).

Preparation of cytosolic and mitochondrial extracts

Following each experimental protocol, hearts were excised and flushed with ice-cold homogenization buffer (210 mM mannitol, 70 mM sucrose, 5.0 mM Mops, and 1.0;mM EDTA, pH 7.4). The left ventricular free wall was then isolated and cytosolic extract and mitochondria were prepared as previously described [39].

Detection of cytochrome c by Western blot analysis

Cytosolic extracts were diluted to 0.75 mg of protein/ml of homogenization buffer followed by a 1:1 dilution with 2× SDS sample buffer (126 mM Tris–HCl, 20% glycerol, 4.0% SDS, 2.0 mM EDTA, 0.005% bromophenol blue, and 100 mM DTT, pH 6.8). Proteins were resolved on 4–20% SDS–PAGE gels (Bio-Rad) then transferred to nitrocellulose membrane (0.22 μm) at 100 V for 30 min (Bio-Rad, Criterion System). Membranes were then incubated for 1 h at room temperature in 1× PBS, 0.05% Tween 20, and 5% non-fat milk with polyclonal anti-cytochrome *c* antibodies (prepared to the C-terminus amino acids 88–105 of rat cytochrome *c*, BioSynthesis, Inc., Lewisville, TX). Primary antibody binding was detected utilizing an alkaline phosphatase based chemiluminescence system (Tropix).

Bax extraction and detection by Western blot analysis

The strength of association of Bax with mitochondria was probed using high pH/high salt extraction as previously described with modifications [39,43–45]. Mitochondria (5.0 mg protein/ml homogenization) were pelleted and resuspended at 5.0 mg protein/ml in 100 mM Na_2CO_3, pH 11.5. Samples were then incubated for 30 min on ice followed by centrifugation at 100,000*g* for 30 min yielding the supernatant (loosely associated) and pellet (tightly associated) fractions. A volume of 100 mM Na_2CO_3, pH 11.5, corresponding to the supernatant volume was added to the pellet. Pellets were then brought into solution by sonication (20 min, water bath sonnicator) followed by a 1:1 dilution in 2× SDS sample buffer. Samples were heated at 100 °C for 10 min and protein was then resolved on 4–20% SDS–PAGE gels (Bio-Rad) and transferred to PVDF (0.45 μm) at 100 V for 45 min. Membranes were then incubated overnight at room temperature with anti-Bax N-20 antibodies (Santa Cruz Biotechnology) at a dilution of 1/500 in 1× PBS, 0.5% Tween 20, and 5% non-fat milk. Primary antibody binding was detected utilizing a horseradish peroxidase based chemiluminescence system (Super Signal West, Pierce).

Evaluation of mitochondrial O_2 consumption

Rates of NADH-linked ADP-dependent (state 3) and ADP-independent (state 4) respiration were measured using a Clark-type oxygen electrode (Instech, Plymouth Meeting, PA). Briefly, mitochondria were diluted to a concentration of 0.5 mg/ml in assay buffer (210 mM mannitol, 70 mM sucrose, 5.0 mM Mops, and 5.0 mM KH_2PO_4, at pH 7.4) and placed in a sealed oxygen chamber. Respiration was initiated by addition of glutamate (15 mM). After 2.0 min, state 3 respiration was induced by addition of ADP (0.5 mM). Upon depletion of ADP, the rate of state 4 respiration was evaluated.

Measurement of KGDH and citrate synthase activities

As previously described, [35] mitochondria were diluted to 0.05 mg/ml in 25.0 mM KH_2PO_4, 0.1% Triton X-100, 0.5 mM EDTA at pH 7.25. KGDH activity was assayed as the rate of NAD^+ reduction upon addition of 5.0 mM $MgCl_2$, 2.5 mM α-ketoglutarate, 0.1 mM CoASH, 0.2 mM thiamine pyrophosphate, and 1.0 mM NAD^+. Citrate synthase activity was monitored by detection of DTNB reactive CoASH (412 nm, $\varepsilon = 13{,}600$) upon addition of 0.1 mM DTNB, 0.3 mM acetyl CoA, and 0.5 mM oxaloacetate. All enzyme assays were performed at room temperature.

[1] *Abbreviation used:* KGDH, α-ketoglutarate dehydrogenase.