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Relationship between oxidative stress and mitochondrial function in the post-conditioned heart

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Abstract The pathways activated by post-conditioning may converge on the mitochondria, in particular on the mitochondrial permeability transition pore. We sought to characterize the inhibition status of the mitochondrial permeability transition early after the post-conditioning maneuver and before long reperfusion was established. We observed that post-conditioning maneuvers applied to isolated rat hearts, after a prolonged ischemia and before reperfusion, promoted cardiac mechanical function recovery and maintained mitochondrial integrity. These effects were evaluated by mitochondrial swelling, calcium transport, and NAD^+ content measurements; the improvements were established before restoring a long lasting reperfusion period. Mitochondrial integrity was associated with a diminution in oxidative stress, since carbonylation of proteins was prevented and aconitase activity was preserved in the post-conditioned hearts, implying that ROS might mediate mitochondrial dysfunction and mPTP opening. In addition, we found that cytochrome release was significantly abolished in the post-conditioned heart, in contrast with conventionally reperfused hearts.

Keywords Post-conditioning · Mitochondria · Permeability transition pore · Ischemic-reperfused heart · CSA, cyclosporin A

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Introduction

Paradoxically, although rapid initiation of reperfusion is the most effective treatment to reduce infarct size resulting from myocardial ischemia, it has the potential to introduce an additional lethal injury that is not evident at the end of ischemia; this situation is known as reperfusion injury (Becker et al. 1987; Forman et al. 1990; Hearse and Bolli 1992).

Reperfusion injury is the consequence of numerous mechanisms activated in the intracellular and extracellular environments. Perhaps predicted by its diverse and numerous causes, reperfusion injury is expressed physiologically in equally diverse ways including: endothelial damage, vascular dysfunction, sequels of impaired blood flow, metabolic dysfunction, contractile dysfunction, dysrhythmias, cellular necrosis, and apoptosis. In this respect, calcium overload and reactive oxygen species (ROS) bursting out during reperfusion activate the mitochondrial permeability transition (mPT), a key event in cell death after ischemia-reperfusion. Opening of the non-specific mitochondrial permeability transition pore (mPTP) in the inner mitochondrial membrane results in the collapse of the membrane potential ($\Delta\Psi_m$), uncoupling of the respiratory chain, efflux of cytochrome c and of other pro-apoptotic factors, that may lead to either apoptosis or necrosis (De Moissac et al. 2000; Borutaite et al. 2001; Freude et al. 2000). Relevant to this matter is the demonstration that the mPTP remains closed throughout ischemia but opens at the time of reperfusion (Halestrap 2006; Griffiths et al. 2003). These evidences and data sustaining that mPTP opening is a hallmark event towards irreversible cellular injury, underlie the relevance of mPTP regulation to prevent reperfusion damage.

Several strategies have been developed to contend against reperfusion injury. Recently, Zhao et al. (2003) reported that brief periods of ischemia-reperfusion, performed just before prolonged reperfusion, reduce the infarct size. This mechanical maneuver is known as "post-conditioning". The pathways activated by post-conditioning are the subject of intensive research. Recent reports suggest that they converge on the mitochondria, in particular on the mitochondrial permeability transition pore (Argaud et al. 2005; Bopassa et al. 2006; Gateau-Roesch et al. 2006; Gómez et al. 2008). Particularly, Argaud et al. (2005) have shown that NIM811, an inhibitor of mPTP, produces an effect similar to post-conditioning and that larger calcium loads are required to induce calcium release in mitochondria isolated from post-conditioned hearts subjected to long reperfusion. Recently, Penna et al. (2007) suggested that, during the brief periods of post-conditioning, the heart releases and intermittently accumulates downstream mediators that provide cardioprotection. In this sense, we sought to determine if, after the brief post-conditioning cycles of ischemia and reperfusion, the mitochondrial permeability transition is in its closed state, independently of mechanisms activated during reperfusion. To accomplish this goal we measured indicators of mitochondrial functionality and their relationship to oxidative damage, at the beginning and after long reperfusion.

We present evidence that post-conditioning maneuvers maintain the mPTP in its closed state, very early at reperfusion, and that a close correlation exists between cardioprotection and the redox status of mitochondria.

Material and methods

This investigation was performed in accordance with the Guide for the Care and Use of Laboratory Animals, published by the United States National Institutes of Health (US-NIH).

Male Wistar rats (400–450 g) were anesthetized with sodium pentobarbital (60 mg/kg) and anti-coagulated with sodium heparin (1000 U/kg). Five minutes after the heparin injection, a midsternal thoracotomy was performed and the heart was rapidly excised and placed in ice-cold Krebs-Henseleit buffer solution, consisting of 118 mM NaCl, 4.75 mM KCl, 1.18 mM KH_2PO_4, 1.18 mM $MgSO_47H_2O$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 5.5 mM glucose, and 100 μM sodium octanoate, pH 7.4. The heart was quickly fixed onto a Langendorff heart perfusion system and perfused retrogradely via the aorta at a constant flux of 12 ml/min with Krebs-Henseleit solution that was continuously bubbled with 95% O_2 and 5% CO_2, at 37°C. Mechanical work was measured at a left ventricular end-diastolic pressure of 10 mm Hg, using a latex balloon inserted into the left ventricle and connected to a pressure transducer. All variables were recorded using a computer acquisition data system designed by the Instrumentation and Technical Development Department of the National Institute of Cardiology (Mexico, D.F., México).

Experimental protocols

Krebs-Henseleit buffer was perfused for 20 min to stabilize the hearts. The ischemic and reperfused hearts were subjected to global ischemia for 30 min, by turning off the pumping system, and then to reperfusion for additional 5 (I/R_5) or 60 (I/R_{60}) minutes. The control hearts were continuously perfused as long as the I/R_{60} hearts. The post-conditioning maneuver consisted of five cycles of 30 s ischemia and 30 s of reperfusion; then, the hearts were subjected to 60 min of reperfusion (I/R + PC). Another group of hearts was only subjected to ischemia and to the post-conditioning cycles, without reperfusion (PC). In addition, some hearts were perfused during 20 min with 0.2 μM Cyclosporin A (CSA), a well known inhibitor of the mPTP, and then subjected to ischemia and reperfusion. A summary of all the protocols used in this work is shown in Fig. 1.

Hearts that developed arrhythmias before the ischemia were discarded and replaced. Thus, all analyses represent hearts that did not show electrical dysfunction before the ischemia.

Preparation of mitochondria

At the end of the ischemia/reperfusion protocol, the hearts were removed from the Langendorff system and placed in cold isolation buffer containing 250 mM sucrose, 10 mM TRIS-HCl, and 1 mM EDTA, pH 7.3. The hearts were minced

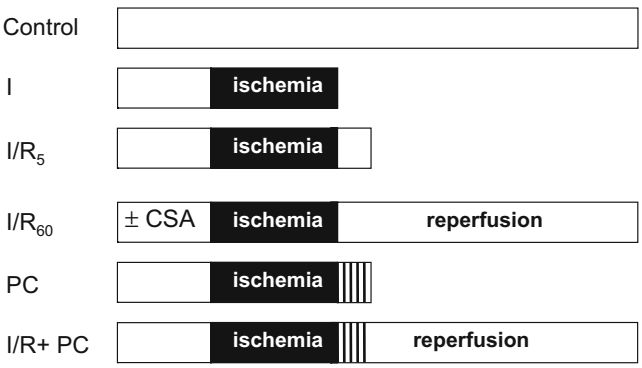


Fig. 1 Schematic representation of the experimental protocols. Control represents hearts perfused during 120 min; $I/R_{60,}$ represents hearts subjected to 30 min of ischemia and 60 min of reperfusion; I/R + PC, represents post-conditioned hearts exposed to 30 min of ischemia and 60 min of reperfusion; PC, represents hearts with only 30 min of ischemia and the post-conditioning cycles; and I/R_5, represents hearts exposed to 30 min of ischemia and only 5 min of reperfusion

and incubated for 10 min in isolation buffer containing 1 mg/ml subtilisin A, centrifuged at 3,000x*g* for 5 min and washed with the same buffer before being homogenized. Mitochondria were obtained by differential centrifugation as previously described (Chávez et al. 1985). Protein was measured by the Lowry method (Lowry et al. 1951).

Opening of the mPTP

Mitochondrial integrity was evaluated by measuring mitochondrial swelling, calcium accumulation, NAD^+ content and oxygen consumption. Mitochondrial swelling was followed by changes in optical density at 540 nm in a basic medium, containing 125 mM KCl, 10 mM HEPES, 3 mM Pi, 10 mM succinate, 160 μM ADP and 5 μg/ml rotenone, pH 7.3. Where indicated 0.5 μM CCCP and 50 μM $CaCl_2$ were added. Mitochondrial calcium movements were evaluated spectrophotometrically in a double beam spectrophotometer at 675–685 nm, by using the metallochromic dye Arsenazo III (Kendrick 1976).

Mitochondrial NAD^+ was measured after trichloroacetic acid extraction, as described previously by Di Lisa et al. (2001) with minor modifications. Briefly, 0.5 ml of 21% (v/v) $HClO_4$ were added to 10 mg of mitochondrial protein per milliliter of suspension and incubated during 30 min in an ice cold bath. The suspension was centrifuged at 8,000x*g* and the supernatants neutralized. NAD^+ was determined fluorometrically at λ_{ex} 340 nm and λ_{em}= 460 nm, by measuring NAD^+ − dependent lactate dehydrogenase activity in a medium containing 3 μg of lactate dehydrogenase from rabbit muscle, 400 mM hydrazine, 500 mM glycine and 10 mM L-lactate, pH 9.0, at 25°C.

Mitochondrial oxygen consumption was measured using a Clark-type oxygen electrode (Yellow Springs Instruments, OH, USA). The experiments were carried out in 1.5 ml of basic medium. State 4 respiration was evaluated in the presence of 10 mM succinate plus 1 $\mu g.ml^{-1}$ rotenone, or 10 mM sodium glutamate and 5 mM sodium malate. State 3 respiration was measured after addition of 200 μM ADP. The respiratory control index (RC) was calculated as the ratio between state 3 and state 4 rates. Uncoupled respiration was measured by adding 1 μM carbonyl cyanide m-chlorophenylhydrazone.

Protein oxidation and aconitase activity in post-conditioned hearts

In order to evaluate oxidative stress in the post-conditioned hearts, we measured protein oxidation in homogenates with the OxyBlot™ protein oxidation detection kit (Chemicon® International, Inc.) and aconitase activity in mitochondria. Briefly, heart tissue was homogenized in phosphate buffered saline (PBS), pH 7.0, containing 1 mM PMSF and 50 mM DTT, to prevent oxidation of proteins after cell lysis. Then, two aliquots of each sample (20 μg) were transferred to eppendorf tubes and denatured by adding a final concentration of 6% SDS. One aliquot was derivatized to 2,4 dinitrophenylhydrazone (DNP) with 2,4-dinitrophenylhydrazine (DNPH), while the aliquot used as the negative control was incubated with the same volume of derivatization-control solution. Then, samples were neutralized and separated by SDS-PAGE, transferred to PVDF membranes and incubated with rabbit anti-DNP antibodies (1:150) in 1% BSA/PBS-T. HRP-conjugated secondary antibodies (1:300) and a chemiluminiscent reagent were used for signal detection.

Aconitase activity was evaluated by measuring cis-aconitate formation at 240 nm, as described by Hausladen and Fridovich (1994).

Cytochrome c release from mitochondria

Cytochrome c content in mitochondria was evaluated by western blot, using a primary monoclonal antibody against cytochrome c (1:1000). Phosphatase alkaline-conjugated secondary antibodies were used, followed by enhanced chemiluminescence system detection. To assess protein loading, the membranes were "stripped" in a buffer containing 62.5 mM Tris/HCl, 100 mM β-mercaptoethanol, 2% SDS, pH 6.7, for 20 min at 50°C; then, the membranes were incubated against anti-ANT polyclonal antibodies (1:500).

Data analysis

Data are presented as means ± SD for each experimental protocol. Significance ($P \leq 0.005$) was determined for discrete variables by analysis of variance (ANOVA), using the data analysis and technical program Microcal™ Origin™ from Microcal Software, Inc. (1999).

Statement of responsibility

The authors had full access to the data and take responsibility for their integrity. All authors have read and agreed to the manuscript as written.

Results

Cardiac function

Cardiac mechanical work of hearts subjected to 30 min of ischemia and 60 min of reperfusion decreased dramatically from the first minutes of reperfusion, in remarkable contrast with the hearts subjected to the post-conditioning maneuvers (Fig. 2). There was no significant difference between

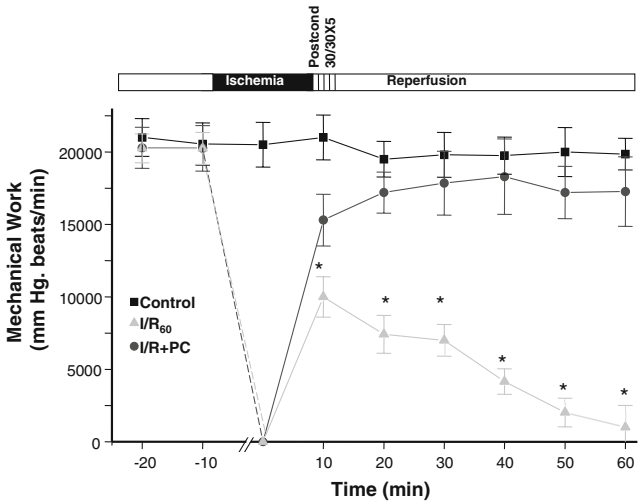


Fig. 2 Post-conditioning protects myocardial performance against reperfusion injury. After a 20-minute equilibration time, the hearts were subjected to global ischemia for 30 min, indicated by the dotted line. Then, the perfusion pump system was restarted and the hearts were reperfused during 60 min. Post-conditioned hearts were subjected to five cycles of 30 s of ischemia and 30 s of reperfusion, before starting prolonged ischemia and reperfusion. (■) Control; (▲) I/R and (●) I/R + PC. Data represent the mean of 14 experiments ± S.D. * P≤0.05 vs. Control

mechanical work of I/R + PC hearts and control hearts after reperfusion from minute 20 to minute 60.

Mitochondrial integrity and mPTP opening

At the end of the reperfusion, the hearts were removed from the perfusion system and mitochondria were obtained as described. The results in Table 1 show that mitochondrial function, evaluated by state 3 respiration, RC and uncoupled respiration, either using succinate or malate/glutamate as substrates, was only depressed in I/R_{60} hearts, but was maintained in I/R + PC hearts compared with the control values.

We measured mitochondrial swelling, calcium transport and NAD^+ content; all these parameters were used to evaluate mitochondrial integrity and are indicative of mPTP opening. An increase in inner membrane permeability has been frequently proposed as a mechanism to explain irreversible cardiac injury in ischemia-reperfusion. In this regard, it was important to demonstrate that mitochondria from post-conditioned hearts were resistant to the opening of this mega-channel. Only I/R_{60} mitochondria showed extensive and spontaneous swelling; mitochondria from I/R_5 hearts had a delayed swelling response, whereas mitochondria from control, I/R + PC, and PC hearts maintained the same volumes until the uncoupler CCCP was added (Fig. 3A). Extensive swelling in I/R_{60} mitochondria was fully prevented by treatment with CSA, a classic mPTP inhibitor.

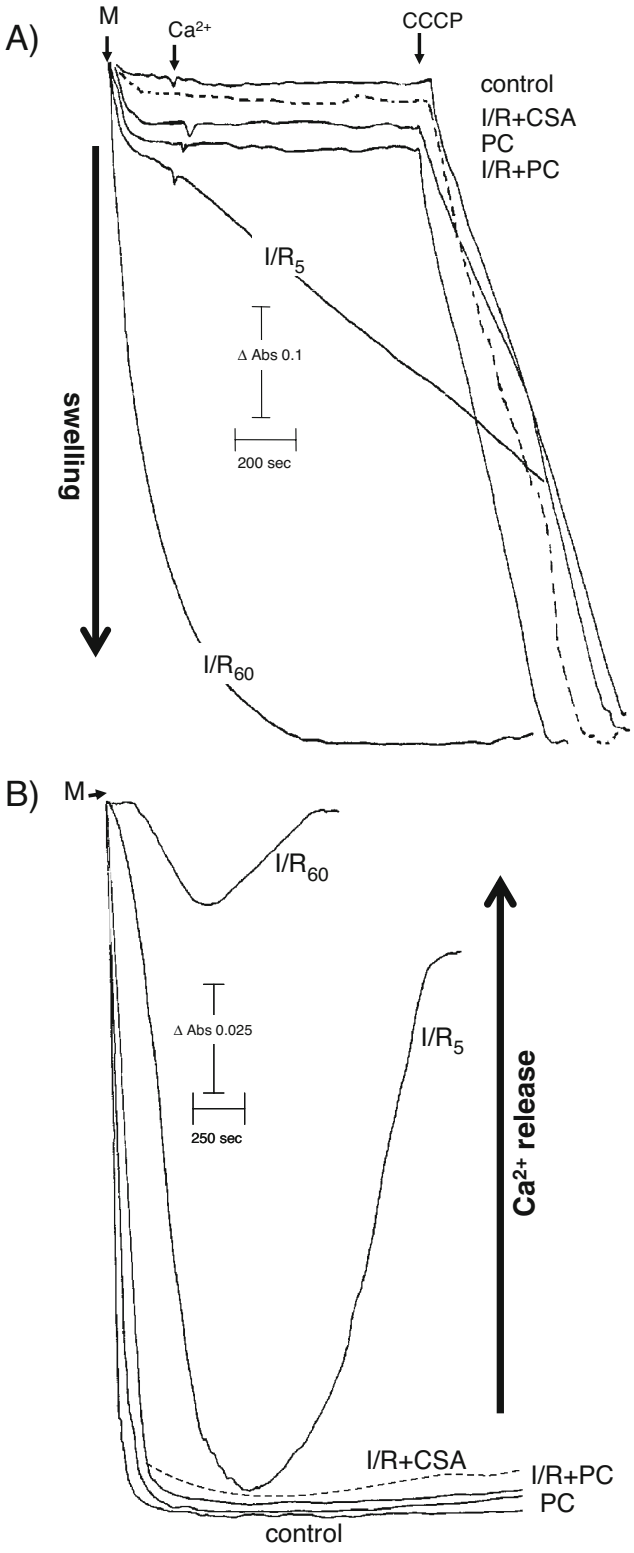
mPTP is also characterized by the non-specific efflux of calcium and other metabolites from the mitochondrial matrix. Calcium was accumulated in control mitochondria, contrasting with mitochondria isolated from I/R_{60} hearts, which, as a consequence of mPTP opening, were unable to retain calcium; again, mitochondria exposed only to 5 min of reperfusion (I/R_5) showed a partial activation of the mPTP. This condition was also fully prevented by CSA treatment. Conversely, no calcium efflux was observed in either I/R +PC mitochondria or in mitochondria obtained from hearts at the end of the post-conditioning cycles of ischemia and reperfusion (PC), indicating that the pore remained closed (Fig. 3B).

A recent demonstration indicated that mitochondrial NAD^+ content in the intact heart is severely depressed during reperfusion. This process is largely decreased by CSA, therefore reflecting the transition pore opening *in situ* (Di Lisa et al. 2001). Our results indicate that NAD^+ content diminished as a function of reperfusion time (Fig. 4). At only 5 min after reperfusion (I/R_5), 6.4± 1.8 nmol NAD^+/mg protein were detected and after 60 min of reperfusion (I/R_{60}) this value diminished to 1.4±0.5 nmol NAD^+/mg protein. Remarkably, at the end of the post-conditioning maneuver (PC), NAD^+ content was maintained comparable to control values (9.7± 1.3 nmol NAD^+/

Table 1 Oxygen consumption in mitochondria from post-conditioned hearts

	State 4 malate/glutamate (ngAO/min/mg)	State 3 malate/glutamate (ngAO/min/mg)	RC	Uncoupled respiration (ngAO/min/mg)	State 4 succinate + rotenone(ngAO/min/mg)	State 3 succinate + rotenone(ngAO/min/mg)	RC
Control	53.0	169.5	3.2	84.3	15.2	81.3	5.3
I/R_{60}	39.6	39.6Plus succinate:51.8	1	41.6	41.6	41.6	1
I/R + PC	56.5	224.7	3.9	108.4	20.3	88.9	4.3

The experiments were carried out at 25°C in 1.7 mL of respiration medium containing 125 mM KCl, 10 mM Hepes and 3 mM phosphate, pH 7.3. Incubations were started by adding 1.5 mg of mitochondrial protein. State 4 respiration was evaluated with 10 mM succinate plus 1 μg/mL rotenone and with 10 mM sodium glutamate and 5 mM sodium malate as substrates. State 3 respiration was stimulated by the addition of 200 μM ADP. RC was calculated as the ratio between state 3 and state 4 rates. Uncoupled respiration was determined after addition of 1 μM CCCP. Values are the means of two different preparations in duplicate for each group.



◀ **Fig. 3** Mitochondrial permeability transition pore opening in mitochondria isolated from post-conditioned hearts. **A**) Mitochondrial swelling. 2 mg of mitochondrial protein were added to the medium described in Material and Methods. Where indicated 50 μM $CaCl_2$ and 0.5 μM CCCP were added. **B**) Mitochondrial calcium transport. Calcium uptake and release were determined by Arsenazo III absorbance changes. 50 μM $CaCl_2$ was present in the medium before mitochondrial addition. Dotted lines indicate mitochondria from hearts perfused with 0.2 μM Cyclosporin A (CSA). Tracings are representative of at least three different experiments

mg protein vs. 10.45±1.5 nmol NAD^+/mg protein) and persisted along the prolonged reperfusion (9.0±0.9) (I/R + PC). Noteworthy was the fact that NAD^+ content in hearts subjected to 60 min of reperfusion, but previously treated with CSA (10.34±0.66 nmol NAD^+/mg protein), remained similar to that of control heart value (10.45±1.5 nmol NAD^+/mg protein). These findings indicate that post-conditioning maneuvers activate mechanisms that maintain conditions that inhibit mPTP opening at early reperfusion.

Protein oxidation in homogenates and aconitase activity in post-conditioned hearts

Oxidative modification of proteins by oxygen free radicals results in the introduction of carbonyl groups into protein side-chains. Carbonyl groups were derivatized to DNP-hydrazone and detected with primary antibodies, specific to the DNP moiety of the proteins. Oxidative modification of proteins was evident at early reperfusion (I/R_5) and it was exacerbated in I/R_{60}. A slight increase in protein oxidation was observed early after the post-conditioning maneuver (PC), but carbonylation was totally abolished at the end of reperfusion (I/R + PC) (Fig. 5A).

Also, mitochondrial aconitase activity, a Fe-S cluster enzyme that is inactivated by superoxide and peroxynitrite was measured, as its inactivation is a reliable marker for mitochondrial superoxide production. The activity in mitochondria from ischemic (106±50.5 nmol/min/mg), I/R_5 (127±34.8 nmol/min/mg) and I/R_{60} hearts (157±18.7 nmol/min/mg) hearts was significantly lower than in mitochondria obtained from control (255±24.6 nmol/min/mg) and post-conditioned hearts (197±47.9 nmol/min/mg). Interestingly, aconitase activity in hearts only subjected to post-conditioning without reperfusion, was slightly higher than the activity found after long reperfusion in post-conditioned hearts (233±54.4 nmol/min/mg vs 197±47.9 nmol/min/mg) (Fig. 5B).

Cytochrome c release in post-ischemic hearts

To determine if apoptosis triggering was also prevented by post-conditioning, we measured cytochrome c release under the referred conditions. Cytochrome c content of mitochondria obtained from hearts subjected to 30 min of ischemia and to 60 min of reperfusion were analyzed by western blot. Mitochondrial cytochrome c obtained from reperfused hearts (I/R_{60}) showed a clear diminution as compared with mitochondria obtained from control and I/R + PC hearts

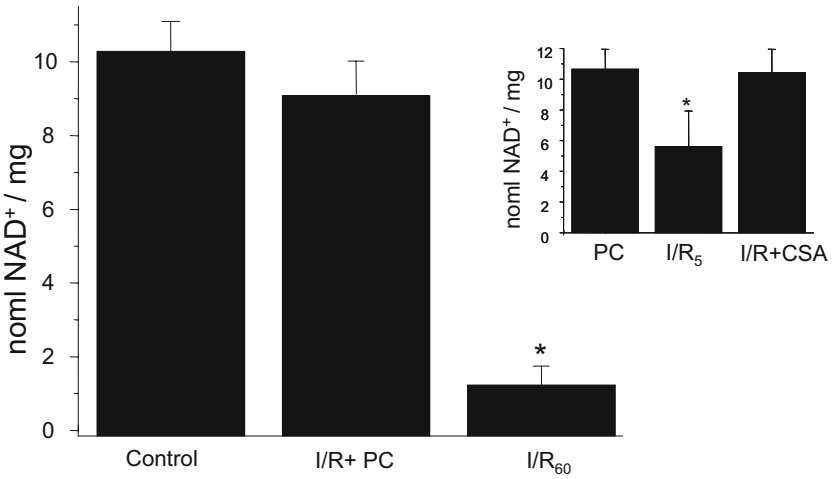


Fig. 4 Mitochondrial NAD^+ content in post-conditioned hearts at early reperfusion and after prolonged reperfusion. The post-conditioned hearts were removed from the Langendorff system to obtain mitochondrial fractions after early and prolonged reperfusion. The insert represents NAD^+ content in PC, IR_5 and I/R_{60} hearts perfused with CSA. Data represent the mean ± S.D of three different experiments. * $P \leq 0.05$ vs. Control

(Fig. 6). It should be noted that in our experiments cytochrome c content in ischemic mitochondria was comparable to cytochrome c levels from control mitochondria, although it has been reported that cytochrome c is released during ischemia by a direct effect of oxygen deprivation in reperfused rat livers (Morin et al. 2004).

Discussion

Recently Zhao et al. (2003) reported that brief cycles of occlusion and reperfusion of the coronary artery, applied before reperfusion and after a relatively prolonged ischemia, dramatically diminished the infarct size. Emerging studies suggest that suppression of mPTP opening may underlie the cardioprotection elicited by ischemic post-conditioning. For example, in mitochondria isolated from post-conditioned rabbit hearts, the calcium concentration required to induce the opening of the mPTP was higher than in mitochondria isolated from untreated hearts (Argaud et al. 2005). Relevant to the idea that post-conditioning is related to mPTP regulation, is the demonstration that pharmacological inhibition of the mitochondrial K_{ATP} channels inhibits the post-conditioning effect on the infarct

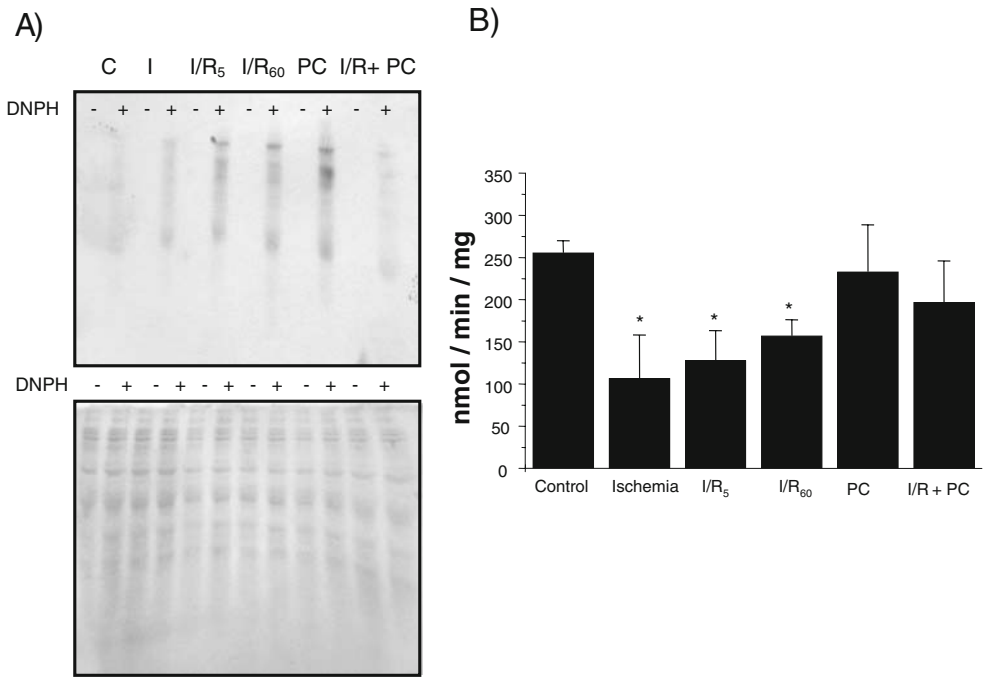


Fig. 5 Oxidative stress in homogenates from post-conditioned hearts and aconitase activity in mitochondria isolated from post-conditioned hearts. **A**) Autoradiography and Coumassie staining of cardiac homogenates derivatized with DPNH (+) and negative controls for each sample (-). **B**) Aconitase activity in mitochondria expressed as the mean ± SD of three different experiments. * $P \leq 0.05$ vs. Control

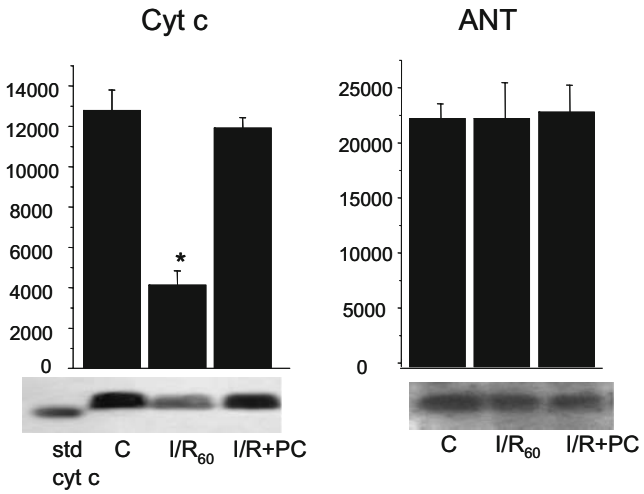


Fig. 6 Cytochrome c content in post-conditioned hearts. Mitochondria obtained from the post-conditioned hearts were evaluated for cytochrome c content by western blot. Total protein (50 μg) was loaded into each lane of the SDS-PAGE gels and transferred to PVDF membranes. The blots were incubated with a monoclonal anti-cytochrome c antibody (1:1000) in TRIS-buffered saline (TBS), pH 7.0, supplemented with 0.1% Tween 20. The membranes were incubated with an alkaline phosphatase-conjugated secondary antibody before enhanced chemiluminescent detection. ANT content was also determined as the loading marker. The blots are representative of three separate experiments using different hearts for each experimental protocol. *P≤0.05 vs. Control

size (Penna et al. 2007). In this sense, it has been suggested that the cardioprotective effect of the activation of the mitochondrial K_{ATP} channels promotes transient mitochondrial depolarization and, thereby, a reduction in mitochondrial calcium overload, avoiding mitochondrial irreversible damage (Holmuhamedov et al. 1999).

In addition, a recent report shows that hearts deficient in cyclophilin-D, a key component of the mPTP, are resistant to the cardioprotective action of post-conditioning (Lim et al. 2007). Although the actual mechanism by which post-conditioning inhibits the mPTP has not been described, it has been postulated that mPTP inhibition may occur through the activation of protein kinases, such as Akt, GSK-3β (Juhaszova et al. 2004), or PKC-ε (Costa et al. 2006).

An important question in mPTP experiments with isolated mitochondria is if the isolation procedure selected mitochondrial populations with different susceptibility to reperfusion damage, and if so, whether calcium-induced mPTP opening is a consequence and not a cause of myocardial damage. In this respect, NAD^+ diminution in the intact heart during reperfusion has been related to mPTP gating *in situ* (Di Lisa et al. 2001). This research group reported that the NAD^+ stored in the mitochondrial matrix becomes available for cytosolic and nuclear processes only after its release through the permeability transition pore, as the process is largely prevented by CSA. Accordingly, we have showed that mPTP opening and NAD^+ depletion are abolished in hearts subjected to post-conditioning. It is worthwhile to mention that in our experiments, the NAD^+ content in control mitochondria isolated from perfused hearts, was comparable to NAD^+ values from normoxic hearts perfused with CSA or MeVal-4-CS before mitochondria isolation (Di Lisa 2001), discarding the possibility that NAD^+ depletion occurred as a consequence of the mitochondrial isolation procedure.

In a previous work we found that, in mitochondria obtained from reperfused hearts, cytochrome c release leads to a significant activation of caspase-3 activity after 30 min of reperfusion, in close correlation with mPTP opening (Correa et al. 2007). Similar results using mPTP inhibitors, such as CSA and NIM811 (Argaud et al 2005), reinforced a primary role of the mPTP for triggering the intrinsic apoptotic pathway, during reperfusion damage.

Cytochrome c release remains as a hallmark for the involvement of mitochondria in apoptosis. A suggested mechanism to explain cytochrome c release from mitochondria is that calcium, along with other stimuli, induces the opening of the mitochondrial permeability transition pore (mPTP), promoting extensive swelling, outer membrane rupture and delivery of pro-apoptotic mitochondrial modulators. Several apoptotic pathways could act concertedly in response to reperfusion/reoxygenation-mediated events. For example, some groups have demonstrated the participation of JNK and p38-mitogen-activated protein kinase signaling pathways in the attenuation of cardiomyocyte apoptosis during post-conditioning (Sun et al. 2006).

An increase in ROS generation would lead to irreversible damage to mitochondrial membrane lipids and proteins resulting in mitochondrial dysfunction and ultimately in cell death (Kowalstowski et al. 1999). Some research groups suggest that post-conditioning protection is achieved, at the time when blood supply is restored, by inhibiting oxidant generation or oxidant-mediated injury (Kin et al. 2004). Penna et al (2007) proposed that intermittent targeting of bradykidin B_2 receptors and mitochondrial K_{ATP} channels during early reperfusion, triggers post-conditioning protection via ROS signaling and that the post-conditioning protection is only achieved if ROS production is timely and not reduced by free radical scavengers. In this respect, our experimental data showed that carbonylation of proteins was prevented in post-conditioned heart homogenates (I/R + PC), but such prevention was not evident at the end of the post-conditioning maneuver (PC). However, aconitase activity was early preserved in PC mitochondria, strengthening the notion that the threshold between the signaling and the deleterious effect of ROS is highly regulated. It is known that ROS, NO and peroxynitrite exert beneficial or deleterious effects depending on their amount, compartmentalization and timing of generation (Pagliaro 2003; Becker 2004).

In summary, mitochondrial integrity could be maintained in the post-conditioned heart as a result of a reduction of the intracellular calcium overload and a diminution of ROS-generation and ROS-mediated lipid and protein peroxidation. In fact, it has been reported that ROS and high intramitochondrial Ca^{2+} may act together to trigger MPT opening (Crompton 1999; Doran & Halestrap 2000; Duchen 2000); however, further investigation is needed to gain an insight into whether ROS generation is an integral part of the signaling machinery of mPTP rather than a consequence of mPTP opening.

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