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ORIGINAL PAPER

Ischemic preconditioning enhances fatty acid-dependent mitochondrial uncoupling

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Abstract This study tests the hypothesis that ischemic preconditioning (IP) changes fatty acid (FA)-dependent uncoupling between mitochondrial respiration and oxidative phosphorylation. We found that IP does not alter mitochondrial membrane integrity or FA levels, but enhances membrane potential decreases when FA are present, in an ATP-sensitive manner. FA hydroperoxides had equal effects in control and preconditioned mitochondria, and GTP did not abrogate the IP effect, suggesting uncoupling proteins were not involved. Conversely, thiol reductants and atractyloside, which inhibits the adenine nucleotide translocator, eliminated the differences in responses to FA. Together, our results suggest that IP leads to thiol oxidation and activation of the adenine nucleotide translocator, resulting in enhanced FA transport and mild mitochondrial uncoupling.

Keywords Mitochondria · Ischemic preconditioning · Oxidative phosphorylation · Oxygen radicals · Ion transport · Mild uncoupling

Abbreviations

ADAM	9-Anthryldiazomethane
ANT	Adenine nucleotide translocator
ATR	Atractyloside
BSA	Bovine serum albumin
DTT	Dithiothreitol
FA	Fatty acids
CCCP	Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone
IP	Ischemic preconditioning
IR	Ischemia/reperfusion
LA	Linoleic acid
LAOOH	Linoleic acid hydroperoxide
mitoK_{ATP}	Mitochondrial ATP-sensitive potassium channel
NAC	*N*-acetylcystein
ROS	Reactive oxygen species
TBA	Thiobarbituric acid
UCP	Uncoupling protein
$\Delta\Psi$	Mitochondrial transmembrane potential

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Introduction

Ischemic heart damage can be prevented by ischemic preconditioning (IP), a procedure in which brief sub-lethal episodes of ischemia, each separated by periods of reperfusion, paradoxically protect the heart against subsequent lethal ischemia (Murry et al. 1986). IP can reduce the necrosis area, severity of arrhythmias and improve functional recovery after a longer period of ischemia (Liu and Downey 1992; Lasley et al. 1993).

The mechanisms through which IP leads to cardioprotection are still under intense investigation, but clearly involve changes in mitochondria. One mitochondrial function involved in IP is the generation of reactive oxygen species (ROS) at the level of the electron transport chain. IP is dependent on a moderate increase in ROS generation,

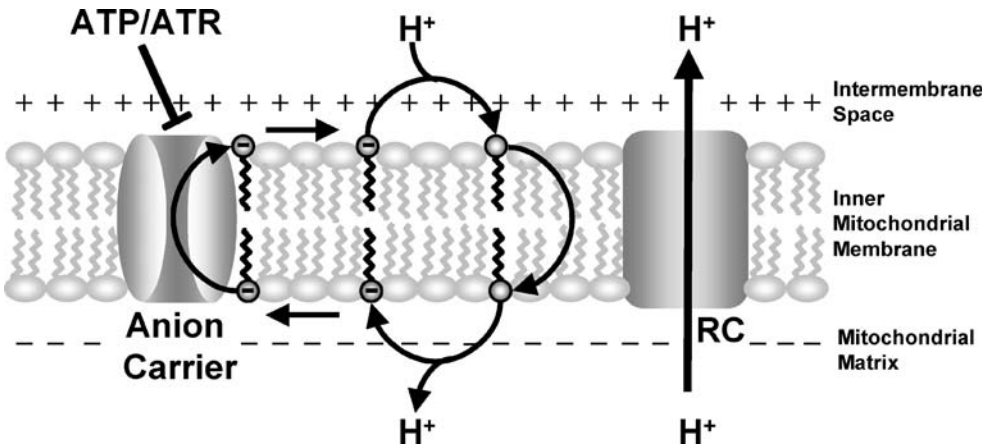
possibly promoted by mild inhibition of respiratory complexes (Vanden Hoek et al. 1998; da Silva et al. 2003). Indeed, the removal of ROS during IP completely abrogates its beneficial effects (Vanden Hoek et al. 1998; Facundo et al. 2006a). Interestingly, increased ROS release observed during IP can protect against oxidative stress after ischemia/reperfusion (IR) (Vanden Hoek et al. 2000), suggesting that mild oxidative conditions occurring during IP activate a signalling pathway that prevents ROS accumulation during IR.

Another mitochondrial event associated with IP is the activity of mitochondrial inner membrane K^+ channels (mitoK_{ATP}). MitoK_{ATP} channel antagonists prevent the beneficial effects of IP, while mitoK_{ATP} agonists lead to cardioprotection in the absence of IP (Garlid et al. 1997; Jaburek et al. 1998; Wang et al. 1999; Vanden Hoek et al. 2000; Fryer et al. 2000; Sato et al. 2000). We demonstrated that early increases in mitochondrial ROS during preconditioning increase mitoK_{ATP} activity (Facundo et al. 2006a, 2007). A consequence of mitoK_{ATP} channel opening is to induce mild uncoupling between mitochondrial respiration and oxidative phosphorylation, moderating secondary ROS release during IR (Ferranti et al. 2003; Facundo et al. 2006a,b).

Although it is a prerequisite for oxidative phosphorylation, an elevated mitochondrial transmembrane potential ($\Delta\Psi$) is associated with enhanced formation of mitochondrial ROS (Korshunov et al. 1997; Skulachev 1998). On the other hand, small decreases in $\Delta\Psi$ (mild uncoupling) very effectively prevent ROS formation without seriously compromising cellular energetics (Korshunov et al. 1997; Starkov 1997; Brand et al. 2004; Brookes 2005). This occurs due to a limitation in the life span of reduced electron transport chain intermediates capable of generating ROS, in addition to a decrease in local oxygen tensions (Korshunov et al. 1997; Skulachev 1998). The prevention of mitochondrial ROS release can avoid cytotoxic effects of ROS generated in excess, including oxidation of proteins, DNA and lipids.

An important endogenous mild uncoupling pathway that prevents ROS release is fatty acid (FA) cycling across the inner mitochondrial membrane (Jezek et al. 1998; Skulachev 1991) (see Scheme 1). In the proton-rich intermembrane space, FA anions are protonated, become uncharged and flip-flop across the inner membrane lipid bilayer. Once in the mitochondrial matrix, the proton is released and the FA anion transported back to the intermembrane space by anion carriers, which include mitochondrial uncoupling proteins (UCPs; Ricquier and Bouillaud 2000; Pecqueur et al. 2001) and the adenine nucleotide translocator (ANT; Andreyev et al. 1988, 1989; Skulachev 1991).

Interestingly, UCP expression promotes tissue protection against IR (Mattiasson et al. 2003; Diano et al. 2003; Hoerter et al. 2004; McLeod et al. 2005). Furthermore, post-ischemic tissue survival in both heart and brain correlates closely with UCP expression (Mattiasson et al. 2003; McLeod et al. 2005) and chemical uncoupling using dinitrophenol mimics IP (Minners et al. 2000). Thus, mitochondrial uncoupling is cardioprotective and may be a mechanism leading to tissue preservation following IP. We directly addressed this hypothesis by measuring mitochondrial inner membrane potentials and quantifying the uncoupling effect of endogenous and exogenous FA. We found that IP enhances FA-dependent mitochondrial uncoupling which occurs due to IR, and investigated the mechanisms through which this activation occurs.



Scheme 1 FA-dependent H^+ transport across the inner mitochondrial membrane. Anion carriers catalyze the uniport of the anionic FA head group from the matrix side to the intermembrane side of the inner mitochondrial membrane. When the charged FA headgroup reaches the intermembrane space side, it is protonated in a manner stimulated by the mitochondrial proton gradient generated by the respiratory chain (*RC*). The protonated FA then flips-flops back through the lipid bilayer, reaching the mitochondrial matrix, where it releases the proton. The now negatively charged FA is again transported to the intermembrane space by an anion carrier. Anion carriers can be ATP-sensitive UCPs or the ANT, which is both ATP-sensitive and inhibited

Methods

Materials

All reagents used were analytical grade or better, and all aqueous solutions were prepared in deionized water. The following reagents were prepared in water: Bovine serum albumin (BSA) (FA-free), atractyloside (ATR), dithiothreitol (DTT), *N*-acetylcystein (NAC), and safranine O. Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (CCCP), linoleic acid (LA), linoleic acid hydroperoxide (LAOOH), oligomycin and rotenone stock solutions were prepared in dimethylsulphoxide. 9-Anthryldiazomethane (ADAM) was prepared in ethyl acetate. NAC, ADP, ATP and GTP solutions were prepared in water and corrected to a pH value between 7.0 and 7.4 with NaOH. Mitochondrial protein concentrations were kept constant in order to ensure equal FA/mitochondrial protein concentrations.

Rat heart perfusions

Heart perfusion was conducted as described previously (da Silva et al. 2003; Belisle and Kowaltowski 2002). Briefly, hearts were rapidly removed from male Sprague–Dawley rats, trimmed over ice, and Langendorff-perfused with 200 mL of oxygenated Krebs–Henseleit buffer containing (in mM) 118 NaCl, 25 $NaHCO_3$, 1.2 KH_2PO_4, 4.7 KCl, 1.2 $MgSO_4$, 1.25 $CaCl_2$, 10 glucose, and 10 HEPES, pH 7.4, at 37°C. Heart beat rates were left unpaced, and perfusion was maintained at a constant pressure of 70 mmHg. During the first 5 min of perfusion, a non-recirculating mode was used to eliminate contaminants. After stabilization, recirculating perfusion was initiated. This perfusion mechanism does not alter general properties of heart ischemia, which continues to present sensitivity to IP or pharmacological cardioprotection. Non-ischemic hearts were perfused continuously for 55 min. Preconditioned hearts were submitted to two 5 min periods of global ischemia separated by 5 min periods of reperfusion. IR hearts were perfused for 25 min. Preconditioned and IR hearts were then submitted to 20 min no-flow ischemia followed by 10 min reperfusion. The IP protocol performed has been proven effective, as shown by da Silva et al. 2003. All studies were approved by the *Comissão de Ética em Cuidado e Uso Animal*, and conducted in accordance to guidelines established by the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health) and the *Colégio Brasileiro de Experimentação Animal*. Hearts were eliminated from the study if the time between heart removal and the beginning of perfusion was over 3 min.

Mitochondrial isolation

Langendorff-perfused hearts were washed in ice-cold buffer containing 300 mM sucrose, 0.5 mM K-EGTA, 0.2% (w/v) non FA-free, grade V BSA, and 10 mM K-HEPES buffer, pH 7.4. The tissue was finely minced with scissors to approximately 1 mm sections and homogenized 3–5 times with a manual glass potter, in the same buffer. The resulting suspension was centrifuged at 600 g for 7 min, and the supernatant was recentrifuged at 12,000 g for 7 min. The mitochondrial pellet was ressuspended in 300 mM sucrose, 10 μM K-EGTA, and 10 mM K-HEPES buffer, pH 7.4 and centrifuged at 12,000 g for 7 min. The pellet was ressuspended in a minimal volume of 300 mM sucrose, 20 μM Na-EDTA, and 10 mM K-HEPES buffer, pH 7.4. Mitochondria isolated in this manner from non-perfused hearts typically present high respiratory control ratios (above 8) when measured using malate plus pyruvate as substrates. Perfusion decreases respiratory control ratios, but mitochondria still remain fully coupled, with respiratory control ratios above 5. As expected, ischemia followed by reperfusion decreases respiratory control ratios, in a manner significantly prevented by IP (2.43±0.25 versus 5.31±0.52, $p=0.001$). This change in respiratory control ratios cannot be attributed only to changes in FA-independent coupling, since state 4 respiratory rates are not significantly different (51.4±11.5 nmols $O_2 \cdot min^{-1} \cdot mg^{-1}$ versus 61.5±9.3 nmols $O_2 \cdot min^{-1} \cdot mg^{-1}$, $p=0.53$), while state 3 respiration was inhibited by IR in a manner prevented by IP (115.4±25.0 nmols $O_2 \cdot min^{-1} \cdot mg^{-1}$ versus 325.1±52.55 nmols $O_2 \cdot min^{-1} \cdot mg^{-1}$, $p=0.006$). To avoid interferences in our experiments, all measurements were conducted in the presence of oligomycin, to promote state 4 conditions (in which respiratory rates are equal) in the absence of added ATP or ADP.

ΔΨ estimation

ΔΨ was estimated by following safranin O (5 μM) fluorescence (Akerman and Wikstrom 1976) at an excitation wavelength of 495 nm and emission wavelength of 586 nm, using 5 nm slits, on a Hitachi F4500 spectrofluorometer. No significant differences in safranine O uptake for a given ΔΨ between mitochondrial groups were noted when ΔΨ was calibrated as described previously (Kowaltowski et al. 2002).

FA extraction and analysis

FAs were extracted by the modified Dole method as described in Puttmann et al. (1993). All glassware was rinsed with 1:1 (v/v) chloroform/methanol. The mitochondrial suspension (100 μL) was mixed with 500 μL 40:10:1 (v/v/v) isopropanol–hexane–2 M phosphoric acid and

20 μL of 0.1 mM heptadecanoic acid as an internal standard. The mixture was vortexed and left to react at room temperature for 10 min, after which 500 μL of hexane and 300 μL of water were added. After being vortexed again, the mixture was centrifuged for 5 min at 1,000 g. 300 μL of the upper organic layer were transferred to a derivatization vial and the solvent was removed under a stream of nitrogen gas. FA were then quantified using the fluorescent probe ADAM (Nimura and Kinoshita 1980). The residue obtained previously was dissolved in 100 μL ethyl acetate and 10 μL of 2.5 mg/mL ADAM were added. The mixture was left to react for 1 hour at 37°C. Analysis was conducted using a Shimadzu Prominence HPLC system composed of two HPLC pumps (LC-20AT), an autoinjector (SIL-20A), a fluorescence detector (RF-10A_{XL}) and a system controller (CBM-20A). Chromatographic separation was carried out using a C_8 column (150×6.0 mm, 5 μm particle size, Tosoh, Japan). The column was isocratically eluted with 80% acetonitrile for 5 min and 90% acetonitrile for 10 min, followed by a linear gradient from 90 to 95% acetonitrile for 10 min and from 95 to 100% acetonitrile for 5 min. The compounds were detected by measuring fluorescence at an excitation wavelength of 365 nm and emission wavelength of 412 nm. The data were acquired using LabSolution software (Shimadzu, Japan).

Malonaldehyde quantification

For the evaluation of lipid peroxidation levels, 1 mg of mitochondrial protein was mixed with 0.025% (w/v) butylated hydroxytoluene, to prevent autoxidation, and 0.35% (w/v) thiobarbituric acid (TBA) diluted in 0.2 M HCl. The suspension was then heated in a water bath at 90°C for 45 min. The TBA-malonaldehyde adduct was extracted with 2-butanol, centrifuged at 1,000 g for 5 min, and quantified by HPLC. The HPLC system (Shimadzu) consisted of 2 LC-10ATVP pumps, an autoinjector (SIL-10AF), a RF-10A_{XL} fluorescence detector and a SCL-10AVP system controller. The column used was a Lichrosorb 10 RP-18 (250×4.6 mm, 5 μm particle size, Phenomenex, Torrence, CA, USA). The mobile phase was 0.025 M KH_2PO_4, pH 7.0, with 50% methanol, and was pumped at an isocratic flow of 1 mL/min. The TBA-malonaldehyde adduct was detected by measuring fluorescence (excitation wavelength=515 nm, emission wavelength=550 nm) and quantified by comparison with a malonaldehyde standard calibration curve prepared previously using the same procedure as for the samples. The data were acquired using Class-VP 6.13 SP2 software (Shimadzu, Japan).

LAOOH synthesis

LAOOH was synthesized by photooxidation using methylene blue as a sensitizer (Miyamoto et al. 2003). Typically, 1 g of LA was dissolved in 50 mL of chloroform containing 0.2 mM of methylene blue and irradiated with a tungsten lamp (500 W) for 5 h. Irradiation was conducted in an ice bath under a continuous flow of oxygen. Methylene blue was removed and LAOOH separated by silica gel column chromatography, following the procedure described by Kühn et al. (1987). Briefly, the products were placed in the column and separated by a discontinuous gradient of *n*-hexane/diethyl ether ranging from 9:1 to 5:5 (v/v). LAOOH concentrations were determined spectrophotometrically at 234 nm (ε=25×10^3 $M^{-1} \cdot cm^{-1}$; Mulliez et al. 1987), assuming that 60% of the hydroperoxides contain conjugated dienes (Terao and Matsushita 1977; Frankel et al. 1979).

Data analysis

Data presented are representative traces (Fig. 1) or averages and standard errors (SEM, Figs. 2, 3, and 4) for at least three experiments. Statistical significance (p<0.05) was calculated using Student t tests, conducted by GraphPad Prism, GraphPad Software™.

Results

This study used isolated mitochondrial preparations to uncover possible changes in coupling occurring during ischemia, reperfusion and IR preceded by IP. Although results obtained with isolated mitochondria may differ from

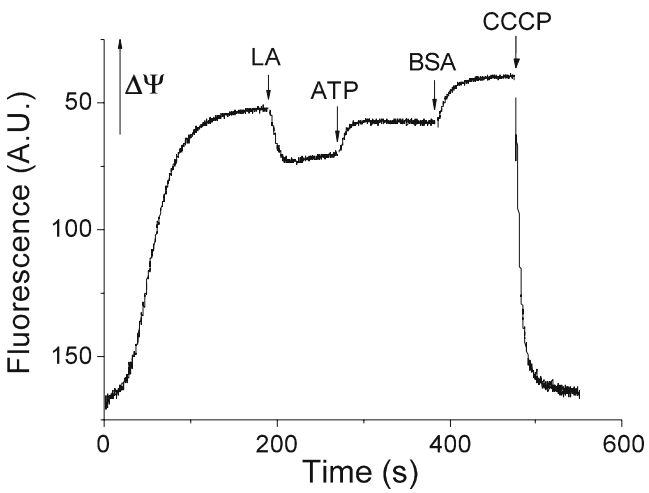


Fig. 1 Mitochondrial ΔΨ is decreased by FA, in an ATP-sensitive manner. Rat heart mitochondria (0.25 mg/mL) were added to 37°C media containing 250 mM sucrose, 100 μM EGTA, 1 mM $MgCl_2$, 1 mM NaH_2PO_4, 2 mM succinate and 10 mM HEPES (pH 7.4, NaOH) supplemented with 1 μM rotenone, 0.5 μg/mg protein oligomycin and 5 μM safranine O. Safranine fluorescence changes promoted by ΔΨ formation over time were followed as described in the "Methods" section. Where indicated, 1 μM LA, 2 mM ATP, 0.1% BSA (w/v) and 1 μM CCCP were added. The results are a representative trace of more than 10 similar repetitions

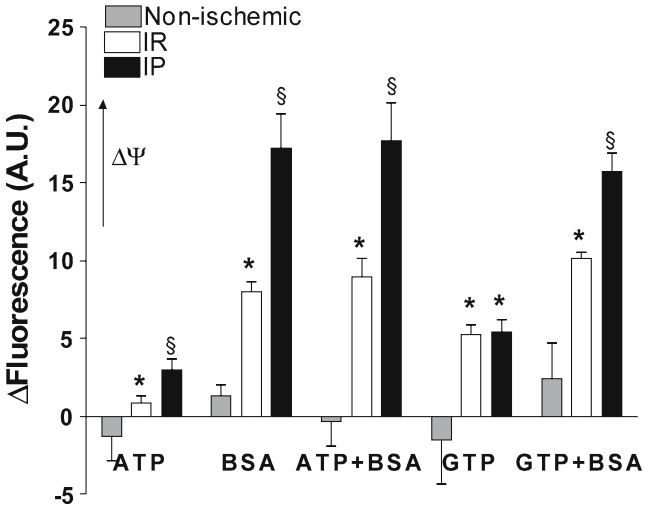


Fig. 2 IP enhances FA-dependent mitochondrial uncoupling. Non-ischemic (*gray bars*), IR (*open bars*) or IP (*closed bars*) mitochondria were incubated under the conditions of Fig. 1, and fluorescence variations induced by the addition of 2 mM ATP, 2 mM GTP and/or 0.1% BSA (w/v) were determined. Results represent averages±SEM of 3–14 repetitions. *Asterisks*, $p<0.05$ versus non-ischemic; *section marks*, $p<0.05$ versus IR

in vivo effects, this experimental setting allows for ready quantifications of bioenergetic parameters and manipulations of experimental conditions not possible in whole heart preparations. All incubations of isolated mitochondria were conducted in K^+-free media (to avoid mitoK_{ATP} effects; Kowaltowski et al. 2001; Garlid and Paucek 2003) in the presence of succinate as a respiratory substrate (to avoid changes in $\Delta\Psi$ caused by complex I inhibition; da Silva et al. 2003). Figure 1 represents a typical trace of mitochondrial $\Delta\Psi$ measurements using safranine O conducted under these conditions. Mitochondria were suspended and allowed to take up the cationic dye safranine until fluorescence levels were stable. As expected, under these conditions the addition of the FA LA lead to an abrupt decrease in $\Delta\Psi$. This decrease was partially reversed by ATP, which inhibits anion carriers such as UCPs and the ANT. BSA, a FA quencher, enhances $\Delta\Psi$ to levels higher than those observed in the absence of LA, since it removes not only added LA, but also free FA present in the mitochondrial preparation. Finally, $\Delta\Psi$ was completely dissipated by the protonophore CCCP.

In Fig. 2, the fluorescence changes observed in experiments such as those shown in Fig. 1 were pooled, and mitochondria from perfused non-ischemic hearts (grey bars) were compared to mitochondria submitted to IR (open bars) and IP followed by IR (IP, full bars). In the absence of added FA, we found that non-ischemic mitochondria presented virtually no change in their membrane potentials when ATP and BSA were added, indicating they had low levels of FA-dependent mitochondrial uncoupling. IR mitochondria, on the other hand, had significant increments in $\Delta\Psi$ when ATP or BSA were added. This effect was not observed when hearts were submitted to ischemia alone (results not shown), indicating that the uncoupling occurred during reperfusion. Interestingly, ATP or BSA enhanced $\Delta\Psi$ even more significantly in IP mitochondria. The presence of ATP and BSA concomitantly did not augment the effect of BSA alone, and still evidenced a more pronounced effect in IP samples. These results indicate two interesting phenomena: (1) ischemia followed by reperfusion increases mitochondrial FA-dependent uncoupling and (2) FA-dependent uncoupling promoted by IR is significantly enhanced by preconditioning, suggesting that IP activates an endogenous FA cycling mechanism catalysed by an ATP-sensitive carrier.

Since the IP protocol used here is strongly cardioprotective (da Silva et al. 2003), we focussed our next studies on the differences between mitochondrial uncoupling in IP samples versus IR. To verify if uncoupling was promoted by changes in endogenous free FA concentrations, we measured levels of saturated and unsaturated FA in IR and IP mitochondrial preparations. Among the main FA types found (C16:0, palmitic acid; C18:0, stearic acid; C18:1, oleic acid; C18:2, LA and C20:4, arachidonic acid), no quantitative difference was observed between IR and IP groups (results not shown, $n=5$). Furthermore, we determined mitochondrial membrane lipid peroxidation levels by quantifying malonaldehyde (a lipid oxidation product) in our preparations (see "Methods"), and found no difference between IR and IP groups (397±38 versus

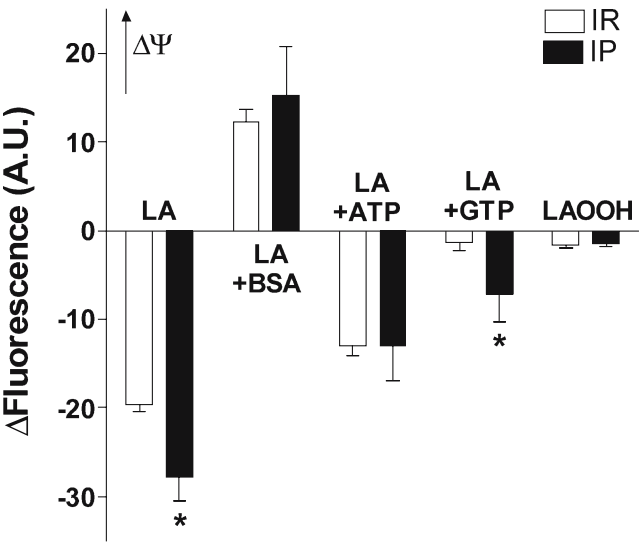


Fig. 3 FA, but not FA hydroperoxides (LAOOH) lead to enhanced uncoupling in preconditioned mitochondria. IR (*open bars*) or IP (*closed bars*) mitochondria were incubated under the conditions of Fig. 1, and fluorescence variations induced by the addition of 1 μM LA, 1 μM LAOOH, 2 mM ATP, 2 mM GTP and/or 0.1% BSA were determined. Results represent averages±SEM of 6–24 repetitions. *Asterisks*, $p<0.05$ versus IR

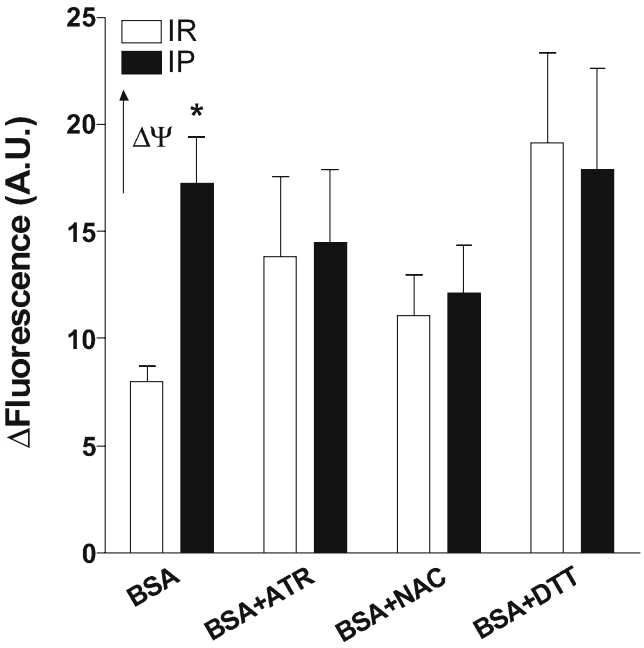


Fig. 4 FA-dependent uncoupling stimulated by IP is prevented by ATR and thiol reductants. IR (*open bars*) or IP (*closed bars*) mitochondria were incubated under the conditions of Fig. 1, and fluorescence variations induced by the addition of 0.1% (w/v) BSA and 10 μM ATR, 4 mM NAC or 300 μM DTT were determined. Results represent averages ± SEM of 6–11 repetitions. *Asterisk*, $p<0.05$ versus IR

442±35 pmols/mg protein, respectively, $n=6$, $p=0.25$). These results suggest that neither changes in lipid oxidation levels nor enhanced levels of free FA are responsible for the results observed in Fig. 2. We also found (da Silva and Kowaltowski, unpublished results) that ΔΨ in IR and IP mitochondria under our conditions was unchanged by the addition of cyclosporin A, indicating the effect observed was not attributable to FA-stimulated mitochondrial permeability transition (Broekemeier and Pfeiffer 1995; Catisti and Vercesi 1999). Thus, enhanced uncoupling promoted by FA in IP mitochondria is probably due to enhanced FA transport activity by an inner membrane anion carrier.

UCPs mediate ischemic protection and present enhanced expression in preconditioned tissues (Mattiasson et al. 2003; McLeod et al. 2005). These proteins would thus be prime candidates for the anion carriers involved in the uncoupling effects observed. Mammalian UCPs are inhibited by ATP and more selectively by GTP (Garlid et al. 2000). Indeed, we found that GTP addition to mitochondrial preparations containing endogenous FA significantly enhanced ΔΨ in IR and IP mitochondria, but not in non-ischemic samples (Fig. 2), suggesting that UCP activity was augmented by ischemia followed by reperfusion. This finding is in line with reports indicating that this tissue expresses UCP2, UCP3 and UCP5 (Ricquier and Bouillaud 2000; Pecqueur et al. 2001; Yu et al. 2000). However, the result of GTP addition was equal in IR and IP mitochondria. Thus, the differences observed between IR and IP samples upon the addition of ATP may not be due to UCP activity.

Further evidence for the lack of participation of UCPs in the IP uncoupling effect was provided by adding exogenous FA (Fig. 3). LA promoted a more pronounced decrease in ΔΨ in IP mitochondria relative to IR, in a manner prevented by BSA, confirming that transport pathways for FA are activated in IP mitochondria. Interestingly, although neither ATP nor GTP could completely reverse the effect of added LA, fluorescence changes promoted by added LA in the presence of ATP were equal in IR and IP mitochondria. On the other hand, IP mitochondria displayed enhanced uncoupling even when GTP was present, indicating that IP activates an ATP-sensitive, GTP-insensitive FA transport pathway. In addition, the hydroperoxide derivative of LA, LAOOH, which is transported with high affinity by UCPs (Jaburek et al. 2004), did not present an enhanced uncoupling effect in IP mitochondria relative to IR, confirming that UCPs are not involved in the form of FA-induced uncoupling modulated by IP.

Another major ATP-sensitive FA transporter in the inner mitochondrial membrane is the ANT (Andreyev et al. 1988, 1989). In order to verify if this protein was activated by IP, we tested the effect of the ANT inhibitor ATR on BSA-induced increases in ΔΨ similar to those observed in Fig. 2. In the presence of ATR without added BSA, ΔΨ levels in IR and IP mitochondria were identical (results not shown). Furthermore, ATR completely removed the differences in response to added BSA observed in IR and IP mitochondria (Fig. 4), leading us to conclude that the ANT is probably responsible for enhanced FA-dependent uncoupling stimulated by IP. We then tested if the quantities of ANT were enhanced in IP mitochondria by titrating the amount of carboxyatractyloside (an irreversible ANT inhibitor) necessary to inhibit ADP transport (Oliveira and Wallace 2006). We found that the response to carboxyatractyloside was equal in IP and IR mitochondria (results not shown), suggesting that ANT activity, but not quantity, was enhanced by IP.

Other mitochondrial uncoupling pathways such as mitoK_{ATP} and UCPs are activated by reversible oxidation of thiol residues (Szewczyk et al. 1999; Zhang et al. 2001; Echtay et al. 2002; Talbot et al. 2004). Furthermore, there is some evidence of ANT-mediated FA transport activation by thiol redox changes (Brustovetsky and Klingenberg 1994). In order to test if ANT activation under our conditions was promoted by oxidation of protein thiol residues, we tested the effect of two thiol-reducing compounds (NAC and DTT) added to isolated IR or IP mitochondria. Unfortunately, compounds such as NAC and DTT present multiple mitochondrial effects, as evidenced by the fact that both compounds slightly decreased ΔΨ when added in isolation

(results not shown). Despite this, we found that these thiol reductants completely eliminated differences between control and IP groups (Fig. 4), suggesting that enhanced ANT activity promoted by IP probably occurs due to protein thiol oxidation.

Discussion

This study demonstrates that mitochondria present increments in FA-mediated uncoupling after undergoing ischemia followed by reperfusion (Fig. 2). Interestingly, IP increases the ability of endogenous FA (Figs. 2 and 4) and added FA (Fig. 3) to promote mild mitochondrial uncoupling during reperfusion. Since neither mitochondrial lipid oxidation nor FA levels were different in IR and IP preparations, we believe that the enhanced $\Delta\Psi$ effect is due to enhanced FA cycling catalysed by anion carriers. Interestingly, although UCPs appeared to be activated by IR, they were not involved in the enhanced uncoupling induced by IP, since GTP did not prevent the effect of endogenous FA (Fig. 2) or added LA (Fig. 3), and LAOOH could not substitute LA (Fig. 3; Jaburek et al. 2004; Garlid et al. 2001). Instead, the ANT seemed to be the main pathway in which FA transport was activated after IP, as confirmed by the observation that ATR abolished the effect of IP (Fig. 4). Since thiol reductants also abrogated the IP effect (Fig. 4), we propose that FA transport is activated when mitochondrial ROS, generated at higher levels during IP (Vanden Hoek et al. 2000; Facundo et al. 2006a), promote the oxidation of protein thiols within the ANT (Brustovetsky and Klingenberg 1994). In agreement with this proposal, ANT-mediated FA transport has previously been shown to be sensitive to the redox state of protein thiol groups (Brustovetsky and Klingenberg 1994). Furthermore, the ANT is an abundant inner membrane protein that can transport FA with high affinity, promoting uncoupling (Andreyev et al. 1988, 1989).

This is the first report, to our knowledge, directly demonstrating that IP can enhance FA-dependent mitochondrial uncoupling. FA transporters such as UCPs can be activated by mitochondrially-generated ROS (Echtay et al. 2002; Brustovetsky and Klingenberg 1994), suggesting that UCP activity could be stimulated by IP. Indeed, Brookes' group has suggested that IP induces mitochondrial uncoupling due to enhanced UCP activity (Nadtochiy et al. 2006). However, in this study all experiments were conducted in the presence of BSA, which quenches FA necessary for UCP activity (Garlid et al. 2000). Other groups have found that UCP expression (but not activity) is enhanced after IP, albeit usually after periods much longer than those that lead to the first window of cardioprotection (McLeod et al. 2005; Nadtochiy et al. 2006). Thus, it is possible that enhanced UCP expression participates in delayed preconditioning. On the other hand, our results do not support the conclusion that UCPs mediate the FA-induced uncoupling we observed after acute IP, pointing instead toward the ANT.

There is ample evidence that mild uncoupling (Skulachev 1998; Brand et al. 2004; Brookes 2005) protects against damage promoted by IR. For example, the expression of UCPs (Hoerter et al. 2004; Bienengraeber et al. 2003; Teshima et al. 2003) or addition of protonophores (Minners et al. 2000; Holmuhamedov et al. 2004) is protective in ischemic tissues. This protection is commonly attributed to the limitation of post-ischemic ROS release promoted by uncoupling (Facundo et al. 2006a; Vanden Hoek et al. 2000). In this sense, our work uncovers a novel consequence of IP: enhanced FA-transport activity by mitochondrial ANT resulting in mild mitochondrial uncoupling. Moreover, it brings further support to the idea that the activation of mitochondrial uncoupling pathways is a central point within the signalling cascade leading to the protective effects of IP. Interestingly, the activation of the ANT under these conditions is probably mediated by thiol oxidation, and results in decreased mitochondrial ROS release (Andreyev et al. 1988, 1989). This illustrates an elegant negative feedback mechanism triggered by IP, controlling redox balance in mitochondria (Facundo et al. 2006a, 2007).

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