Adenosine receptors: regulatory players in the preservation of mitochondrial function induced by ischemic preconditioning of rat liver

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Abstract Although adenosine A₁ receptors (A1R) have been associated to ischemic preconditioning (IPC), direct evidence for their ability to preserve mitochondrial function upon hepatic preconditioning is still missing and could represent a novel strategy to boost the quality of liver transplants. We tested if the A1R antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) prevented IPC in the liver and if the A1R agonist 2-chloro-N⁶-cyclopentyladenosine (CCPA) might afford a pharmacological preconditioning. Livers underwent a 120 min of 70% warm ischemia and 16 h of reperfusion (I/R), and the IPC group underwent a 5-min ischemic episode followed by a 10-min period of reperfusion before I/R. DPCPX or CCPA was administered intraperitoneally 2 h before IPC or I/R. The control of mitochondrial function emerged as the central element affected by IPC and controlled by endogenous A1R activation. Thus, livers from IPC- or CCPA-treated rats displayed an improved oxidative phosphorylation with higher state 3 respiratory rate, higher respiratory control ratio, increased ATP content, and decreased lag phase. IPC and CCPA also prevented the I/R-induced susceptibility to calcium-induced mitochondrial permeability transition, the rate of reactive oxygen species (ROS) generation, and the decreased mitochondrial content of phospho-Ser⁹ GSK-3 β . DPCPX abrogated these effects of IPC. These implicate the control of GSK-3 β activity by Akt-

mediated Ser⁹-GSK-3 β phosphorylation preserving the efficiency of oxidative phosphorylation and ROS-mediated cell death in the ability of A1R activation to mimic IPC in the liver. In conclusion, the parallel between IPC and A1R-mediated preconditioning also paves the way to consider a putative therapeutic use of the later in liver transplants.

Keywords Adenosine A1 receptors · GSK-3 β , glycogen synthase kinase 3 β · Ischemic preconditioning · Liver · Mitochondrial permeability transition · Oxidative phosphorylation

Abbreviations

A1R	Adenosine A ₁ receptor
ALT	Alanine aminotransferase
ANT	Adenine nucleotide translocator
AST	Aspartate aminotransferase
CypD	Cyclophilin D
CCPA	2-Chloro-N ⁶ -cyclopentyladenosine
H ₂ DCF-DA	2',7'-Dichlorodihydrofluorescein diacetate
DPCPX	8-Cyclopentyl-1,3-dipropylxanthine
FCCP	Carbonylcyanide- <i>p</i> -trifluoromethoxyphenylhydrazon
GSK-3 β	Glycogen synthase kinase-3 β
IPC	Ischemic preconditioning
I/R	Ischemia/reperfusion
LDH	Lactate dehydrogenase
$\Delta\Psi$	Mitochondrial membrane potential
MPT	Mitochondrial permeability transition
OXPHOS	Oxidative phosphorylation
PC	Preconditioning
RCR	Respiratory control ratio
RFUs	Relative fluorescence units

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ROS	Reactive oxygen species
TPP ⁺	Tetraphenylphosphonium
VDAC	Voltage-dependent anion channel

Introduction

Ischemia/reperfusion (I/R) injury is a common clinical problem responsible, at least in part, for the morbidity associated with liver surgery under total vascular exclusion or after liver transplantation [1–3]. This has motivated a search for new approaches to reduce ischemic injury, among which ischemic preconditioning (IPC) constitutes a prominent promising strategy. This endogenous adaptive mechanism of protection against a sustained ischemic insult is afforded by exposure to an initial, brief ischemic stimulus followed by a period of reperfusion or by the administration of chemical agents, which is known as pharmacological preconditioning (PC) [2–4]. In livers, PC can reduce the release of transaminases, increase the survival of rodents after liver transplantation, and even improve human hepatic functions after major liver surgery in patients subjected to 30 min of ischemia [5–8]. Furthermore, IPC decreases liver injury after both warm and cold I/R [2, 4, 9, 10].

Several mechanisms have been proposed for protection by IPC, including activation of adenosine receptors [11–14], increased nitric oxide production [15], activation of protein kinase C [16], up-regulation of heat shock proteins, and increased antioxidant capacity [17, 18]. Probably, the process better established to be associated with decrease tolerance to I/R injury is the inability to restore energetic balance following I/R [1, 19, 20]. Accordingly, IPC can prevent I/R-induced mitochondrial dysfunction suggesting that PC protects the integrity of mitochondrial oxidative phosphorylation (OXPHOS) and increases the resistance to the induction of the mitochondrial permeability transition [21–23]. The precise mechanism of PC-induced hepatoprotection is unknown, but it is likely to be a receptor-mediated process, related with preservation of energy metabolism [2, 3, 14, 17, 24]. However, little is known about the coupling of extracellular signals with intracellular adaptive mechanisms involved in preconditioning.

A major extracellular signal involved in preconditioning is adenosine; adenosine is a major homeostatic modulator in eukaryotic tissues since it is produced according to tissue stress or workload and protects from subsequent injury by decreasing metabolic rate [25]. In particular, adenosine A₁ receptors (A1R) have been shown to be responsible for the phenomena of PC in tissues such as the brain, heart, and liver [12–14, 26–37]. However, it is largely unknown how A1R impact on mitochondrial mechanisms recruited to implement preconditioning. Both ischemic and pharmacological PC enhance Akt phosphorylation and its translocation to mitochondria [13, 38, 39]. In mitochondria, Akt can phosphorylate glycogen synthase kinase-3 β (GSK-3 β) on serine 9 causing its inactivation [38,

40]. The mitochondrion is a platform of cell signaling and a decision-maker of cell death, with multiple roles in the development of I/R injury [3, 21, 24, 38]. Cell viability is compromised by energy deficiency due to impaired OXPHOS, increased generation of reactive oxygen species (ROS), mitochondrial calcium overload, and induction of the mitochondrial permeability transition (MPT). In response to cellular stresses, MPT induction abolishes mitochondrial membrane potential and compromises ATP generation. MPT induction can be suppressed by IPC and other interventions that increase phospho-Ser⁹-GSK-3 β [3, 23, 40–43]. GSK-3 β is an important regulator of cell function, controlling gene expression, cell cycle, survival, and apoptosis [44–46]. Upon reperfusion, phospho-Ser⁹-GSK-3 β in mitochondria physically interacts with the adenine nucleotide translocator (ANT) and the voltage-dependent anion channel (VDAC), thus impairing the interaction of the ANT with cyclophilin D (CypD), elevating the threshold for MPT induction [40, 42, 46, 47].

Albeit it is expectable that mitochondrial function should be a key target of A1R-mediated effects, the link between both is still to be established but seems likely in view of the described ability of A1R to preserve mitochondrial membrane potential and the sensitivity to pro-oxidant stressors in the setting of hepatic I/R [14, 35, 36]. The present study was designed to critically evaluate the role of A1R and to identify mitochondrial downstream targets involved in hepatic PC protection activated by A1R. For this purpose, we blocked A1R to test if they were necessary for liver ischemic preconditioning and activated A1R to test if this was sufficient to mimic ischemic preconditioning. In parallel, we evaluated if these opposite manipulations of A1R would trigger mirror alterations of different mechanisms controlling mitochondrial function.

Material and methods

Chemicals and reagents

8-Cyclopentyl-1,3-dipropylxanthine (DPCPX) and 2-chloro-N⁶-cyclopentyladenosine (CCPA) were purchased from Tocris Biosciences, and 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA) was from Sigma. Rabbit monoclonal anti-Bcl2 antibody and rabbit monoclonal anti-cleaved caspase 3 antibody were from Cell Signaling. WesternDot 625 goat anti-rabbit and goat anti-mouse Western blot, TNF- α ELISA Kit, and Ca²⁺-Green were from Invitrogen. All other reagents and chemicals used were the highest grade of purity commercially available.

Animals

Seven-week-old male Wistar rats were obtained from Charles River (France). Upon arrival, animals were allowed 7 days to

acclimatize, being housed in groups of four under controlled light and humidity conditions with free access to food and water. All animals received care according to institutional guidelines, and all procedures were according to EU guidelines (2010/63) after approval by the Animal Care Committee of the University of Coimbra.

Hepatic ischemia/reperfusion

Animals were anesthetized with ketamine (50 mg/kg) and chlorpromazine (50 mg/kg). Partial ischemia (70%) was carried out with occlusion for 120 min of the hepatic artery and portal vein to the left and median liver lobes. Reperfusion (16 h) was initiated by removal of the clamp. In the IPC group, before I/R, brief (5 min) ischemic episodes were applied, then followed by a 10-min period of reperfusion. To probe if A1R were necessary for the expression of IPC, the selective A1R antagonist DPCPX was used at a supramaximal dose of 0.5 mg/kg [48] and was administered intraperitoneally 2 h before IPC. To test if A1R activation was sufficient to induce PC, we used the A1R agonist CCPA of 0.5 mg/kg [28, 29]. CCPA was also administered intraperitoneally 2 h before IR. Both control (Ctl, sham-operated) and I/R (subjected to ischemia/reperfusion without treatment) rats were injected with an equal volume of saline buffer also for 2 h.

All analysis were carried out at single time point, at 16 h after reperfusion, to determine if A1R activation resulted in the activation of the later phase of preconditioning [49, 50].

Plasma biochemical determination

Following reperfusion, animals were killed by decapitation. Blood samples were collected and enzymatic determinations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) performed in the resultant plasma using commercial kits (Hospitex Diagnosis).

Preparation of liver mitochondria

Mitochondria were isolated from animals of the different experimental groups by conventional methods with slight modifications [23, 51]. Protein content was determined by the biuret method, calibrated with bovine serum albumin [52].

Mitochondrial oxygen consumption

Oxygen consumption of isolated mitochondria was polarographically determined with a Clark oxygen electrode [53, 54]. Mitochondria (1 mg) were suspended under constant stirring, at 25 °C, in 1.3 mL of standard respiratory medium (130 mM sucrose, 50 mM KCl, 5 mM MgCl₂, 5 mM KH₂PO₄, 50 μM EDTA, 5 mM HEPES, pH 7.4), supplemented with 3 μM rotenone. State 3 respiration was induced by

adding 200 nmol ADP. The oxygen consumption was also measured in the presence of carbonylcyanide-*p*-trifluoromethoxyphenylhydrazon (FCCP; 1 μM). State 3 and respiratory control ratio (RCR) were calculated according to Chance and Williams [54].

Mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi$) was estimated using an ion-selective electrode to measure the distribution of tetraphenylphosphonium (TPP⁺) [51, 55]. Reactions were carried out at 25 °C, in a temperature-controlled, water-jacketed chamber with magnetic stirring. Mitochondria (1 mg) were suspended in 1 mL of standard respiratory medium and 3 μM rotenone supplemented with 3 μL TPP⁺. A matrix volume of 1.1 μL/mg protein was assumed.

Titration with carboxyatractyloside

Carboxyatractyloside (CAT) is a highly selective inhibitor of cytosolic side-specific ANT that causes stabilization of the “c” conformation of ANT leading to MPT induction and loss of $\Delta\Psi$ [57]. After determining the $\Delta\Psi$, energized mitochondria were titrated with 0.02 nmol of CAT. Cumulative addition of CAT before ADP addition progressively blocks a higher number of ANT units contributing to the observed decreased number of phosphorylation cycles induced by successive addition of ADP [56, 57].

ATP content

To extract adenosine nucleotides, liver samples (20 mg) were pulverized with a mortar and pestle in liquid N₂ and homogenized in 25 μL of ice-cold KOH buffer (1.5 M K₂HPO₄ and 2.5 M KOH), on ice. Samples were vortexed and centrifuged at 14,000×g for 2 min at 4 °C. The supernatants were collected and dissolved in 100 μL of 1 M K₂HPO₄. pH was adjusted to 7.0 and samples were frozen at −80 °C for storage. ATP was quantified with an ATP Bioluminescent Assay Kit (Sigma) using a Victor3 plate reader.

Evaluation of reactive oxygen species

ROS were determined fluorometrically at an excitation wavelength 485 nm and an emission wavelength 538 nm, using a PerkinElmer VICTOR 3 plate-reader fluorometer [58]. Isolated mitochondria (1 mg/mL) were suspended in standard respiratory medium and loaded with 50 μM H₂DCF-DA (in DMSO) for 15 min at 37 °C. The mitochondrial suspension (200 μL) was loaded into a 96-well plate, and the fluorescence was monitored for 30 min to calculate the rate of ROS formation. The results are expressed as relative fluorescence units (RFUs).

Mitochondrial permeability transition

Mitochondrial swelling was estimated by changes of light scattering, monitored spectrophotometrically at 540 nm [59]. Reactions were carried out at 25 °C, and MPT was induced with 20 nmol Ca^{2+} . Experiments were started by the addition of mitochondria (1 mg) to 2 mL of reaction medium (200 mM sucrose, 10 mM Tris-MOPS—pH 7.4, 1 mM KH_2PO_4 , and 10 μM EGTA) supplemented with 3 μM rotenone and 5 mM succinate.

Mitochondrial calcium fluxes

Free calcium was monitored with 100 nM of a calcium-sensitive fluorescent dye, Calcium Green-5 N [60]. The reactions were carried out at 25 °C, in 2 mL of MPT medium supplemented with 3 μM rotenone. Mitochondria (1 mg) were energized with succinate (5 mM) after the addition of 20 nmol Ca^{2+} . Fluorescence was recorded continuously using a PerkinElmer VICTOR 3, with excitation and emission wavelengths of 485 and 535 nm, respectively. Calcium fluxes were expressed as relative RFUs.

Western blotting analysis

Tissue homogenates or mitochondrial pellets were lysed in ice-cold RIPA lysis buffer supplemented with a cocktail of protease and phosphatase inhibitors. Equal amounts of protein were loaded and electrophorized on SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with 5% non-fat milk and incubated with anti-Bcl-2 (1:1000), anti-cleaved caspase-3 (1:1000), anti-phospho-Thr308-Akt (1:100), anti-Akt (1:500), anti-phospho-Ser⁹-GSK-3 β (1:100), or anti-GSK-3 β (1:500), overnight at 4 °C. Immunodetection was performed with WesternDot 625 goat anti-rabbit or goat anti-mouse western blot kits. Membranes were imaged using a Versa Doc 3000, and the densitometry analysis was performed with the ImageJ software.

Statistical analyses

Data are presented as mean $\pm$ SEM and were obtained from five different experiments. Statistical significance was determined using one-way ANOVA followed by Bonferroni post hoc test. A value of $P < 0.05$ was considered as statistically significant. Asterisk indicates the significant difference ($P < 0.05$) versus control, number sign indicates the significant difference versus I/R, ampersand indicates the significant difference versus PC, and dollar sign indicates the significant difference versus DPCPX.

Results

Plasma markers of liver injury

Analyses of serum levels of AST, ALT, and LDH showed that warm I/R induced liver injury (Fig. 1). IPC effectively reduced the plasma levels of these markers of liver injury (Fig. 1). This IPC-induced amelioration was blunted by blocking A1R with DPCPX since AST, ALT, and LDH levels were increased in DPCPX + IPC + I/R group to levels similar to those observed in the I/R group (Fig. 1). Notably, A1R were not only necessary but actually sufficient to mimic IPC since the administration of the agonist CCPA before I/R also reduced AST, ALT, and LDH levels (Fig. 1).

Mitochondrial membrane potential and oxygen consumption

Comparatively to mitochondria isolated from the livers of rats subject to I/R, $\Delta\Psi$ was significantly increased in preconditioned livers (IPC + I/R and CCPA + I/R). $\Delta\Psi$ developed by mitochondria isolated from animals treated with the antagonist DPCPX was similar to that of I/R (Fig. 2a). Notably, DPCPX decreased the capacity to establish $\Delta\Psi$ after repolarization, whereas the A1R agonist CCPA increased $\Delta\Psi$ after repolarization.

The lag phase (time required for ADP phosphorylation) that precedes repolarization was significantly increased by I/R comparatively to control, and this increase was lessened by preconditioning (IPC + I/R or CCPA + I/R) (Fig. 2b). This suggested that preconditioning was able to preserve the efficiency of mitochondrial phosphorylation. In the presence of DPCPX, the effect of preconditioning was blocked, resulting in a lag phase larger than that found upon IPC.

State 3 respiration (Fig. 3a) and the respiratory control ratio (RCR) (Fig. 3b) were significantly decreased in mitochondria isolated from livers subjected to I/R, when compared to the control. preconditioning (IPC + I/R and CCPA + I/R) prevented the deleterious action of I/R on mitochondrial respiration, increasing respiratory state 3 to a value similar to the control group (Fig. 3).

ATP content

To further determine whether preconditioning was able to preserve mitochondrial ATP production upon I/R, endogenous ATP content was quantified in isolated mitochondria (Fig. 4a), energized mitochondria (Fig. 4b), and tissue extracts (Fig. 4c). As shown in Fig. 4, ATP content was decreased upon I/R, but the presence of the A1R antagonist DPCPX further decreased the efficiency of phosphorylation. In contrast, as observed for the parameters of mitochondrial function, both IPC and CCPA administration before ischemia were able to significantly preserve mitochondrial ATP content (Fig. 4).

Fig. 1 Effects of IPC and A1R activation by CCPA on ALT, AST, and LDH plasma levels. Plasma samples were collected and enzymatic determinations were performed using commercial kits. Data are means $\pm$ SEM of experiments performed with six animals/group. *Significant difference ($P < 0.05$) versus Ctl, #significant difference versus I/R, &significant difference versus IPC + I/R, \$significant difference versus IPC + I/R + DPCPX

Generation of reactive oxygen species

A pathological increase of the endogenous production of ROS after I/R decreases the capacity for cell survival. In the presence of antimycin A, an inhibitor of the electron transport chain, mitochondria isolated from livers of animals subject to I/R exhibited increased ROS generation, when compared to the control group (Fig. 5). The generation of ROS was decreased by IPC and CCPA, demonstrating that preconditioning is able to

control the generation of mitochondrial ROS induced by I/R and thus prevent the deleterious cycle of ROS-induced ROS generation (Fig. 5).

Interference with ADP-induced depolarization

The ANT facilitates the one-for-one exchange of extramitochondrial ADP for intramitochondrial ATP during oxidative phosphorylation. Assuming that ADP/ATP transport

Fig. 2 Effects of IPC and A1R activation by CCPA on mitochondrial membrane potential ($\Delta\Psi$) in succinate-energized liver mitochondria. $\Delta\Psi$ was measured with a TPP⁺-selective electrode. Reactions were carried out in 1 mL of reaction medium, supplemented with 3 μ M rotenone and 1 mg of freshly isolated mitochondria. Energization was achieved with 5 mM

succinate and phosphorylation induced by 200 nmol ADP. Data are means $\pm$ SEM of experiments performed with six animals/group. *Significant difference ($P < 0.05$) versus Ctl, #significant difference versus I/R, &significant difference versus IPC + I/R, \$significant difference versus IPC + I/R + DPCPX

Fig. 3 Effects of IPC and A1R activation by CCPA on state 3 respiration and respiratory control ratio (RCR). Oxygen consumption of isolated mitochondria was polarographically determined with a Clark oxygen electrode. Mitochondria (1 mg) were suspended under constant stirring, at 25 °C, in 1.4 mL of standard respiratory medium with 3 μ M rotenone and energized by adding succinate to a final concentration of 5 mM. State 3 respiration was induced by adding 200 nmol ADP (a). The oxygen

consumption was also measured in the presence of oligomycin (0.5 μ g/mg protein) and 1 μ M FCCP to determine RCR (b). Data are means $\pm$ SEM of experiments performed with six animals/group. *Significant difference ($P < 0.05$) versus Ctl, #significant difference versus I/R, &significant difference versus IPC + I/R, \$significant difference versus IPC + I/R + DPCPX

is the rate-limiting step in OXPHOS, depression of the ANT activity would impair the energy-producing capacity of the mitochondria. The observed increase in the lag phase induced by I/R could thus be related with an impaired activity of the ANT. To test this hypothesis, energized mitochondria were titrated with CAT. CAT is a highly selective inhibitor of cytosolic side-specific ANT that causes stabilization of the c

conformation of ANT and blocks the exchange of matrix ATP and cytoplasmic ADP [57]. Matrix ADP is an important modulator of pore opening and acts by decreasing the Ca^{2+} sensitivity of the calcium trigger; therefore, CAT is also an inducer of the MPT [56]. Measuring $\Delta\Psi$ after ADP addition showed that, in mitochondria of rats subjected to I/R, less CAT was needed to abolish repolarization after phosphorylation

Fig. 4 Effects of IPC and A1R activation by CCPA on ATP content in isolated mitochondria (a), energized mitochondria (b), and tissue (c). ATP content was evaluated using a bioluminescent assay kit. Data are means $\pm$ SEM of experiments performed with six animals/group. *Significant difference ($P < 0.05$) versus Ctl, #significant difference versus I/R, &significant difference versus IPC + I/R, \$significant difference versus IPC + I/R + DPCPX

Fig. 5 Effects of IPC and A1R activation by CCPA on ROS generation. ROS were estimated fluorometrically using the probe H₂DFDA. After measuring the basal fluorescence (white), antimycin A was added (black) to block complex III of the mitochondrial chain, thus inducing maximal ROS generation. Data are means $\pm$ SEM of experiments performed with six animals/group. *Significant difference ($P < 0.05$) versus Ctl, #significant difference versus I/R, &significant difference versus IPC + I/R, §significant difference versus IPC + I/R + DPCPX

(Fig. 6), indicating ANT inhibition and thus an impaired phosphorylative system in I/R mitochondria. Preconditioning (either IPC + I/R or CCPA + I/R) prevented this effect of I/R (Fig. 6).

Mitochondrial permeability transition

In isolated mitochondria, the ability to tolerate a calcium challenge is an indicator of the susceptibility to the MPT. Thus, as mitochondria possess a finite capacity to accumulate calcium before undergoing the MPT, we evaluated calcium-induced mitochondrial swelling.

Mitochondria isolated from livers subjected to I/R were more susceptible to undergo mitochondrial swelling induced by calcium, comparatively to control (Fig. 7a). In vitro pretreatment of I/R mitochondria with the MPT inhibitor cyclosporine A prevented mitochondrial swelling induced by calcium (Fig. 7a), demonstrating that the swelling was associated with MPT induction. IPC or the A1R agonist CCPA increased the threshold to MPT induction. Acute treatment with the antagonist DPCPX before preconditioning, increased the susceptibility to the MPT induced by calcium, thus blocking the IPC protective action (Fig. 7a).

Mitochondrial calcium fluxes

Since mitochondria isolated from livers of preconditioned rats displayed a decreased susceptibility to calcium-induced swelling, we next evaluated mitochondrial calcium fluxes. Mitochondria isolated from control animals were able to accumulate the added calcium after energization with succinate and retained it during the entire period of the assay (Fig. 7b). In contrast, mitochondria isolated from animals subjected to

I/R immediately released calcium into the assay media (Fig. 7b). Pretreatment with cyclosporine A prevented calcium release, implying that increased calcium efflux from I/R mitochondria resulted from MPT induction. IPC and treatment with CCPA before I/R prevented both mitochondrial calcium efflux after calcium challenge (Fig. 7b). In contrast, mitochondria from DPCPX-treated rats precociously released the accumulated calcium, indicating the increased susceptibility to a calcium challenge (precociously).

Phosphorylation status of Akt and GSK-3 β

Activation of A1R has been shown to protect liver from I/R injury via pathways involving Akt [13]. Taking into account that activated Akt decreases GSK-3 β activity, which is related with modulation of the MPT, we probed if the protective effect of preconditioning against hepatic I/R injury involves the activation of the Akt/GSK-3 β signaling pathway to prevent mitochondria-mediated I/R injury. Western blot analysis revealed that I/R decreased the ratios of phospho-Thr308-Akt/Akt (Fig. 8a) and mitochondrial phospho-Ser9-GSK-3 β /GSK-3 β (Fig. 8b) compared to control; this indicates decreased Akt and increased GSK-3 β activities upon I/R. IPC blocked this effect of I/R, which was mimicked by CCPA exposure before I/R (Fig. 8). This suggests that the Akt/GSK-3 β pathway might mediate the improvement of mitochondrial function by IPC. Thus, PC maintains the phosphorylated inactive GSK-3 β that regulates the threshold for MPT induction.

Discussion

Accumulated evidence suggests that A1R play a role in the protection from I/R injury in several organs such as heart, brain, and lung [12–14, 26–37] through mechanisms that remain to be defined. The present study suggests that A1R activation prevents I/R injury through the control of OXPHOS efficiency. Notably, preischemic exposure to a selective A1R agonist (CCPA) essentially mimicked preconditioning in the liver through a mitochondrial adaptation similar to ischemic preconditioning. The single used dose of CCPA is expected to engage A1R since CCPA is 32 times selective for A1R versus A3 receptors in rats [61]. The possible involvement of A3 receptors in the effects of CCPA cannot be fully ruled out, although the role of A3 receptors in the preconditioning process is far from clear, with opposite effects in different tissues [62, 63]. Furthermore, CCPA caused effects on different preconditioning-related parameters that mirror these of the selective A1R antagonist, DPCPX, further arguing for our assumption that we have mostly manipulated A1R to control liver preconditioning. The doses of the A1R ligands used were not lethal, but they are expected to cause transient

Fig. 6 Effects of IPC and A1R activation by CCPA on the interference with ADP-induced depolarization. $\Delta\Psi$ was measured with a TPP⁺-selective electrode. Energization was achieved with 5 mM succinate and phosphorylation induced with 200 nmol ADP. Energized mitochondria were titrated with carboxyatractyloside (CAT). Successive additions of CAT aliquots before ADP addition progressively block a

hemodynamic, cardiac, and thermogenic alterations [64], which were not measured, and can indirectly affect the measured impact of I/R on the liver. However, although we did not directly ruled out the possible involvement of other adenosine receptors in the liver or indirectly affecting liver function, we favor the contention that the effects observed upon administration of A1R ligands mostly result from direct effects mediated by liver A1R [30], since preconditioning studies in different organs in vivo and in vitro agreed on a direct effect of A1R in the affected cells [27, 31–33].

Since ATP content declines substantially during I/R, which is associated with hepatocellular injury and higher mortality, prevention of defective energy production plays an important role in the resistance to I/R injury. Associated with alterations in pH, Na⁺, and oxidative phosphorylation, I/R triggers a cascade of events that involve increased ROS generation and loss of Ca²⁺ homeostasis. This culminates in MPT induction and cell death [3, 21, 24, 38]. Therefore, modulation of mitochondria has emerged as a critical survival strategy for the

higher number of ANT units contributing to the observed decreased number of phosphorylation cycles induced by successive ADP additions, as well as to the observed increase in lag phase corresponding to successive phosphorylation cycles. The traces are representative of experiments performed with six independent mitochondrial preparations

prevention of I/R injury. IPC-triggered signal transduction appears to directly preserve several cell functions including intracellular energy state, pH, and redox system [2, 3, 14, 17, 24]. Our data show that PC (both ischemic and pharmacological) was capable to decrease the plasma activities of ALT, AST, and LDH, indicating a clear hepatoprotective effect against warm I/R injury.

PC was associated with an increased performance of mitochondrial function, as shown by the preservation of ATP content. The effects of PC can be differentiated in two phases characterized by different time frames and mechanisms, an early phase (early preconditioning) and the late phase (late preconditioning) [49]. Early preconditioning immediately follows the brief ischemic time, lasts 2–3 h, and involves preservation of both bioenergetics and ionic homeostasis, involving the activation of constitutive molecular systems. Besides DNA transcription and de novo protein synthesis, modulation of mitochondria by late preconditioning is key for the protection against I/R with reduction of mitochondrial calcium

Fig. 7 Effects of IPC and A1R activation by CCPA on the susceptibility to calcium-induced MPT. Mitochondrial swelling (a) was spectrophotometrically monitored at 540 nm. Experiments were started by the addition of mitochondria (1 mg) to 2 mL of reaction medium supplemented with 3 μ M rotenone and 5 mM succinate. MPT was induced with 20 nmol CaCl_2 . Calcium fluxes (b) were fluorometrically monitored using the probe Calcium Green (485 and 535 nm) and

expressed as relative fluorescence units (RFUs). Mitochondria (1 mg) were incubated in 2 mL of standard incubation medium prior to the addition of calcium (20 nmol). Energization was achieved with succinate 5 mM. Cyclosporine A (CsA; 1 μ M) was added to the reaction prior to calcium addition. The traces are representative of experiments performed with six independent mitochondrial preparations

overload, MPT inhibition, and increased OXPHOS efficiency [50]. Our goal was to determine if the protection afforded by the A1R agonist CCPA was as stable and constitutive as ischemic preconditioning, with preservation of mitochondrial homeostasis present 16 h following the beginning of reperfusion. The efficiency of oxidative phosphorylation was impaired in mitochondria isolated from the livers of rats subject to I/R and exposed to DPCPX; in contrast, IPC and administration of CCPA were able to maintain $\Delta\Psi$, state 3 respiration,

RCR, and the lag phase at values similar to control. The increased lag phase may be caused by disruption of mitochondrial ATP/ADP exchange mediated by the ANT. The drop in cytosolic ATP levels caused by impaired mitochondrial ATP/ADP exchange could stimulate metabolic pathways related to cytosolic acidification in the aerobic cell and initiate apoptosis in these cells [65]. The involvement of the ANT in the inhibition of mitochondrial phosphorylation was supported by the fact that less CAT was needed to abolish ADP-induced

Fig. 8 Effects of IPC and A1R activation by CCPA Akt activation (Thr³⁰⁸) and GSK-3 β inhibition (Ser⁹) by phosphorylation. Representative data (lower) and quantitative analyses (upper) are shown. Data are means $\pm$ SEM of experiments performed with six animals/group. *Significant difference ($P < 0.05$) versus Ctl, #significant difference versus I/R

depolarization, indicating that the ANT was rate-limiting phosphorylation in I/R conditions and implying that I/R was enhancing the *c* conformation of the ANT. In energized mitochondria, stabilization of the *c* conformation of the ANT (as it is by CAT) sensitizes the MPT to calcium. Also, oxidative stress activates the MPT by inhibiting ADP binding to the ANT and enhancing CypD-ANT interaction, decreasing the sensitivity of the calcium trigger [66]. Thus, inhibition of ATP/ADP translocation sensitizes mitochondria to MPT. This series of findings provides an integrative scenario of the diverse molecular adaptations caused by I/R that converge to affect mitochondrial efficiency.

Mitochondria isolated from animals subject to I/R were highly susceptible to undergo mitochondrial swelling induced by calcium, but PC (ischemic and pharmacological) was able to restore the capacity of mitochondria to accumulate calcium, without inducing the MPT. The susceptibility to undergo MPT formation was increased by I/R, as demonstrated by the inhibition of liver mitochondrial swelling and calcium efflux in the presence of cyclosporine A.

Modulation of MPT sensitivity by IPC may also be explained by modulation of CypD-ANT binding via Akt/GSK-3 β pathway. It has been proposed that IPC increases the levels of mitochondrial phospho-Ser⁹-GSK-3 β and that phospho-Ser⁹-GSK-3 β physically interacts with the ANT, which inhibits interaction of the ANT with CypD [40, 42, 46, 47]. This may alter mitochondrial function in a way that makes the MPT less likely to form, under oxidant stress and high calcium concentrations, conditions that are associated with I/R. Both IPC and CCPA increased the levels of active Akt and phospho-Ser⁹-GSK-3 β , which may modulate MPT induction.

In conclusion, this study demonstrates that A1R agonist CCPA reproduced the beneficial effects of IPC, an effect abolished by the A1R antagonist DPCPX. This involves preservation of OXPHOS efficiency and decreased susceptibility to MPT induction. Both ischemic PC and pharmacological PC may modulate the MPT in a GSK-3 β -dependent manner. Apart from providing a mechanistic insight into this striking ability of A1R to control IPC, the present study also paves the way to consider A1R agonists as potential strategies to exploit a pharmacological PC in liver transplantation.

Compliance with ethical standards All animals received care according to institutional guidelines, and all procedures were according to EU guidelines (2010/63) after approval by the Animal Care Committee of the University of Coimbra.

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