Ischaemic and pharmacological preconditionings protect liver via adenosine and redox status following hepatic ischaemia/reperfusion in rats

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ABSTRACT

Although IPC (ischaemic preconditioning) is considered as a protective strategy in HI/R (hepatic ischaemia/reperfusion), the mechanisms for this effect have not been fully elucidated. In the present study we investigate whether PPC (pharmacological preconditioning) by transient activation of A1R (adenosine A1 receptor) protects against long-term HI/R and whether the protective effects of IPC depend on A1R activation and whether both preconditionings affect remote organs. Wistar rats underwent IPC and long-term HI/R. Another set of animals were pharmacologically preconditioned with the A1R-agonist CCPA [2-chloro-N6-cyclopentyladenosine; 0.1 mg/kg of body weight, i.p. (intraperitoneally)] 24 h before HI/R. In other groups, rats received an A1R-antagonist, DPCPX (1,3-dipropyl-8-cyclopentylxanthine; 0.1 mg/kg of body weight, i.p.) 24 h before HI/R. Hepatic damage was evaluated by transaminase [AST (aspartate transaminase), ALT (alanine transaminase)] release; inflammation was assessed by hepatic MPO (myeloperoxidase) and serum TNFα (tumour necrosis factor α) and NO; oxidative stress was estimated by MDA (malondialdehyde) and 4-HDA (4-hydroxyalkenals), SOD (superoxide dismutase) activity, GSH and ADA (adenosine deaminase) as adenosine metabolism. Both preconditionings protected liver and lung against HI/R as indicated by the reduction in transaminases, MPO, MDA + 4-HDA, NO, TNFα and ADA activity as compared with HI/R (P < 0.05). However, pre-treatment with DPCPX abolished the protective effects of IPC and PPC. Preconditionings induced a significant increase in hepatic MnSOD (manganese SOD) activity and NO generation compared with the sham group, and this activity was abolished by DPCPX pre-treatment. A1R activation induced hepatic delayed preconditioning and blockade of A1R abolished hepatic IPC. IPC, as well as PPC, were able to prevent lung damage. These protective effects are associated with a reduction in oxidative stress, inflammation and endogenous antioxidant preservation.

Key words: adenosine, 2-chloro-N6-cyclopentyladenosine (CCPA), inflammation, ischaemia/reperfusion, oxidative stress, preconditioning.

Abbreviations: ADA, adenosine deaminase; ALT, alanine transaminase; A1R, adenosine A1 receptor; AST, aspartate transaminase; CCPA, 2-chloro-N6-cyclopentyladenosine; Cu,Zn-SOD, copper/zinc superoxide dismutase; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; 4-HDA, 4-hydroxyalkenals; HI/R, hepatic ischaemia/reperfusion; iNOS, inducible NO synthase; i.p., intraperitoneal; IPC, ischaemic preconditioning; IPost, ischaemic postconditioning; LPO, lipid peroxidation; MDA, malondialdehyde; MnSOD, manganese superoxide dismutase; MPO, myeloperoxidase; NF-κB, nuclear factor κB; PKC, protein kinase C; PPC, pharmacological preconditioning; ROS, reactive oxygen species; SOD, superoxide dismutase; TNFα, tumour necrosis factor α; XO, xanthine oxidase.

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INTRODUCTION

HI/R (hepatic ischaemia/reperfusion) is the main cause of hepatic damage in many settings such as trauma, liver resection and transplantation and may lead to local and remote organ failure that compromises the life of the patient and enhances the ratio of morbidity and mortality [1–3].

Several protective strategies have been developed to neutralize and/or attenuate the deleterious effects of I/R in different organs. IPC (ischaemic preconditioning) is considered a rational protective strategy by which brief and repetitive episodes of I/R confer protection against a prolonged ischaemia [3,4]. Previously, Zhao et al. [5] reported that several brief coronary occlusions after 60 min of ischaemia had cytoprotective effects; this new physiopathological event was coined as IPost (ischaemic postconditioning).

Several mediators have been proposed to play a critical role in the protective response of IPC including NO and adenosine [2,6,7]. Interestingly, IPost has also been demonstrated to exert its protective effects through NO and adenosine [8–10].

IPC was subsequently found to be a biphasic phenomenon, with an early phase of protection that develops within minutes of the initial ischaemic insult and lasts 2–3 h and a late (or delayed) phase that becomes apparent 12–24 h later and persists for 3–4 days [11].

Previously, Dana et al. [12] and Zhao et al. [13] reported that transient activation of A1R (adenosine A1 receptors) by CCPA (2-chloro-N6-cyclopentyladenosine), 24 h before an ischaemic insult, induces delayed myocardial protection in rats and this protection is associated with enhanced MnSOD (manganese SOD (superoxide dismutase)) and iNOS (inducible NO synthase) expression. However, the mechanisms underlying the delayed protection with adenosine in hepatic ischaemia are not fully understood. Peralta et al. [14] and others [15] have demonstrated that hepatoprotection induced by IPC is mediated by the activation of A2R. Although, Schauer et al. [16] reported no evidence for a role of A2R in the cytoprotective effects of IPC, they suggested the implication of other adenosine receptor subclasses or additional mediators of IPC that could play a role in such settings. Recently, we have reported that the protective effects of oxidative preconditioning in liver are mediated by the activation of A1R by CCPA [17]. Moreover, experimental evidence demonstrates the involvement of A1R in other organs [12,13,18–20].

The role of ROS (reactive oxygen species) generation in the pathogenesis of liver ischaemia is well known [1,21]. Therefore therapeutic strategies aimed at neutralizing and/or reducing oxidative damage in transplanted organs with the employment and/or overexpression of antioxidant proteins (e.g. SOD) have been considered as rational approaches to diminish liver injury [21–23]; however, none of these strategies have found their way into routine clinical practice. Besides, the outcomes of these interventions are still poor for various reasons, including the failure to reach the main source of ROS formation within the cell, the mitochondrial respiratory chain [1].

Therefore, and based on these considerations, we hypothesized that classical IPC has a protective memory against long-term HI/R and that the transient activation of A1R with CCPA 24 h prior to HI/R is able to mimic the protective effects of IPC by reducing the oxidative damage and lung injury associated with HI/R and this hepatoprotection effect is related to a mechanism involving cellular redox status.

MATERIALS AND METHODS

Animals

Adult female Wistar rats (200–250 g) [CENPALAB (National Center for the Production of Laboratory Animals), Cuba] were used. Rats were maintained on a commercial pellet diet and water ad libitum in a room under normal lighting conditions. All animal experimental protocols were approved by the University of Havana. All animals received humane care in compliance with European Union regulations (Directive 86/609 EEC) for animal experiments.

Induction of HI/R

A model of segmental (70 %) hepatic ischaemia was used [3,24]. The animals were anaesthetized with pentobarbital [60 mg/kg of body weight, i.p. (intraperitoneally)] and placed in a supine position on a heating pad. To induce hepatic ischaemia, laparotomy was performed, and the blood supply to the hepatic left and median lobes was interrupted by placement of a bulldog clamp at the level of the portal vein and hepatic artery. This method of partial-ischaemia prevented mesenteric venous congestion by permitting portal decompression through the right and caudate lobes. Reflow was initiated by removal of the clamp. Animals received 1 ml of sterile saline i.p. and the wound was closed in layers with 4-0 silk and wound staples, then animals were allowed to recover. At the end of the reperfusion period, animals were killed and blood was recollected from the abdominal aorta under pentobarbital anaesthesia. Hepatic and lung samples following reperfusion periods were homogenized in 20 mmol/l KCl/histidine buffer (pH 7.4) and frozen in liquid nitrogen and stored at −70 °C for further analysis.

Experimental protocol

Rats were randomized as follows: (i) group 1, sham (n = 10): animals were subjected to anaesthesia and laparotomy; (ii) group 2, HI/R (n = 15): animals underwent 90 min of partial ischaemia, followed by 24 h of reperfusion; (iii) group 3, sham + CCPA (n = 10): as group 1 plus a single dose of CCPA (0.1 mg/kg of body...
weight, i.p.), an A₁R agonist, 24 h before animals were killed [13]; (iv) group 4, sham + DPCPX (1,3-dipropyl-8-cyclopentylxanthine) (n = 10): as group 1 plus a single dose of DPCPX, an A₁R antagonist, (DPCPX; 0.1 mg/kg of body weight, i.p.) 24 h before animals were killed; (v) group 5, IPC + HI/R (n = 15): animals were subjected to preconditioning induced by 10 min of ischaemia and 10 min of reperfusion before HI/R (as group 2); (vi) group 6, IPC + DPCPX + HI/R (n = 15): as group 5 plus DPCPX (0.1 mg/kg of body weight, i.p.) 24 h before HI/R; (vii) group 7, CCPA + HI/R (n = 15): rats were pharmacologically preconditioned with a single dose of CCPA (0.1 mg/kg of body weight, i.p.) 24 h before HI/R; (viii) group 8, CCPA + DPCPX + HI/R (n = 15): as in group 7 plus a single dose of DPCPX (0.1 mg/kg of body weight, i.p.) 30 min before CCPA (0.1 mg/kg of body weight, i.p.) administration and 24 h before HI/R [12]; (ix) group 9, DPCPX + HI/R (n = 15): rats were treated with DPCPX (0.1 mg/kg of body weight, i.p.) 24 h before HI/R.

**Pharmaceutical compounds**

CCPA and DPCPX were purchased from Sigma and were dissolved in water for injection.

**Biochemical assays**

All enzymatic determinations were performed in triplicate. Evaluation of hepatic damage was performed by determination of ALT (alanine transaminase) and AST (aspartate transaminase) in serum using a commercial kit from Boehringer–Mannheim. For the MPO (myeloperoxidase) assay, MPO activity, as a neutrophil marker, was determined in livers and lungs of the animals at 24 h of reperfusion using an established method as described previously [25]. Determination of MPO activity was quantified using O-dianisidine–H₂O₂ as a substrate for MPO. Maximal chromogen absorption is at 460 nm. For the SOD assay, SOD was measured in tissue using a kit supplied by Randox Laboratories. MnSOD was distinguished from Cu,Zn-SOD (copper/zinc SOD) by assaying in the presence of 2 mmol/l sodium cyanide [26]. Cu,Zn-SOD activity was calculated as the subtraction of MnSOD from total SOD activity. The result was expressed as units/g of protein. GSH and GSSG in tissue and serum samples were measured enzymatically in 5-sulfosalicylic acid–deproteinized samples using a modification [27] of the procedure of Tietze [28]. For the ADA (adenosine deaminase) assay, tissues were homogenized in 10 mmol/l Hepes/KOH buffer (pH 7.4) containing 0.25 mol/l sucrose, 1 mmol/l MgCl₂ and 1 mmol/l 2-mercaptoethanol, on ice. The homogenate was centrifuged at 15 000 × g for 15 min. Tissue ADA activity was determined as described previously [29]. To measure the NO concentration, nitrite/nitrate levels as a measure of NO generation were determined using the Griess reaction by first converting nitrates into nitrates using nitrate reductase (Boehringer–Mannheim). Then the Griess reagent [1 % sulfanilamide and 0.1 % N-(1-naphthyl)-ethylenediamine dihydrochloride in 0.25 % phosphoric acid] was added [30]. Samples were incubated at room temperature (22 °C) for 10 min and absorbance was measured at 540 nm using a microplate reader.

For the LPO (lipid peroxidation) assay, LPO was assessed by measuring the concentration of MDA (malondialdehyde) and 4-HDA (4-hydroxyalkenals) at both tissue (liver and lung) and serum levels. Concentrations of MDA + 4-HDA were analysed using the LPO-586 kit (Calbiochem). In the assay, the production of a stable chromophore after 40 min incubation at 45 °C was measured at a wavelength of 586 nm using a microplate reader. For standards, freshly prepared solutions of MDA bis-(dimethyl acetal) (Sigma) and 5-hydroxynonenal diethyl-acetal (Cayman Chemicals) were employed and assayed under similar conditions. For the TNFα (tumour necrosis factor α) assay, serum concentrations were determined based on the cytotoxicity assay described by Aggarwal et al. [31]. The detection limit of the assay is greater than 20 pg/ml. The total protein was determined using the Bradford method [32]. Unless otherwise stated, all chemicals were obtained from Sigma.

**Statistical analysis**

Values are expressed as means ± S.E.M., and results were analysed and compared by ANOVA, followed by the Student–Newman–Keuls test. P values < 0.05 were considered statistically significant.

**RESULTS**

**Effectiveness of classic hepatic IPC and PPC (pharmacological preconditioning) on delayed HI/R injury**

HI/R (ischaemia for 90 min/reperfusion for 24 h) induced significant (P < 0.05) parenchymal cell injury, as shown by the increase in circulating levels of AST and ALT (Figure 1). IPC as well as an A₁R agonist (CCPA) ameliorated hepatic injury by keeping AST and ALT at the levels of the sham group. In contrast, the A₁R antagonist (DPCPX) totally abolished the protective effects of both preconditionings and significantly (P < 0.05) exacerbated hepatic injury induced by HI/R (Figure 1). A significant increase of TNFα levels in serum was observed in animals subjected to HI/R. CCPA and IPC attenuated the increase observed in HI/R and failed to restore TNFα concentrations to the levels in the sham group. Interestingly, under the experimental conditions of the present study, A₁R-blocking by DPCPX increased significantly (P < 0.05) TNFα levels over all experimental groups (Table 1) and was accompanied by an accentuation of hepatic and lung injury. In line with this result, HI/R caused a 42-fold and 45-fold increase in MPO activity in...
Serum activities of AST and ALT, as markers of hepatic injury after HI/R, were measured in different treatments.

Values are means ± S.E.M. *P < 0.05 compared with the sham group; †P < 0.05 compared with IPC; §P < 0.05 compared with CCPI; ‡P < 0.05 compared with HI/R. U, units.

Figure 1  Serum activities of AST and ALT, as markers of hepatic injury after HI/R, were measured in different treatments.

As shown in Table 2, HI/R caused a significant decrease (P < 0.05) in SOD activity in hepatic tissue with regards to the sham group. PPC and IPC increased the total SOD (P < 0.05) and MnSOD activity (P < 0.05) with regards to all experimental groups including the sham group, whereas cytosolic SOD (Cu,Zn-SOD) was not different from the rest of the groups (P < 0.05). The administration of DPCPX abolished the increase of SOD activity achieved by preconditioning groups; all isoforms of SOD underwent suppression in their activity and were comparable with the HI/R groups (P < 0.05). GSH haemostasis is considered one of the more important features of the redox-status within the cells. In the present study, we have evaluated the extension of the oxidative stress caused by HI/R intra- and extra-cellularly. HI/R leads to a significant reduction (P < 0.05) in the GSH concentration and an accumulation in the GSSG concentration in both compartments (serum and tissue) as compared with the sham and preconditioning groups (Table 3). The transient activation of A1R by CCPI and

Table 1  Changes in the MDA + 4-HDA concentration in tissue (liver and lung) and in plasma, MPO, as a marker of neutrophil infiltration, in liver and lung and NO concentration and TNFα release during HI/R and treatments

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Hepatic levels of MDA + 4-HDA (nmol/g of tissue)</th>
<th>Lung levels of MDA + 4-HDA (nmol/g of tissue)</th>
<th>Serum levels of MDA + 4-HDA (nmol/ml)</th>
<th>Hepatic MPO activity (units/g of tissue)</th>
<th>Lung MPO activity (units/g of tissue)</th>
<th>Hepatic nitrite/nitrate levels (nmol/mg of tissue)</th>
<th>Circulating TNFα levels (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham (group 1)</td>
<td>0.86 ± 0.07</td>
<td>0.09 ± 0.01</td>
<td>0.7 ± 0.18</td>
<td>5.35 ± 0.88</td>
<td>3.19 ± 0.94</td>
<td>0.49 ± 0.01†</td>
<td>&lt; 20 pg/ml</td>
</tr>
<tr>
<td>Sham + IPC (group 2)</td>
<td>0.95 ± 0.09</td>
<td>0.12 ± 0.02</td>
<td>0.85 ± 0.11</td>
<td>6.33 ± 1.09</td>
<td>2.68 ± 0.33</td>
<td>0.89 ± 0.09</td>
<td>22.35 ± 3.09</td>
</tr>
<tr>
<td>Sham + DPCPX (group 3)</td>
<td>1.03 ± 0.1</td>
<td>0.29 ± 0.09</td>
<td>0.9 ± 0.2</td>
<td>6.99 ± 1.68</td>
<td>3.66 ± 1.03</td>
<td>0.55 ± 0.11</td>
<td>24.88 ± 2.55</td>
</tr>
<tr>
<td>HI/R (group 4)</td>
<td>3.29 ± 0.15†</td>
<td>2.34 ± 0.21†</td>
<td>10.9 ± 0.97</td>
<td>228.54 ± 9.54</td>
<td>144.56 ± 9.51</td>
<td>2.99 ± 0.24†</td>
<td>89.25 ± 9.1</td>
</tr>
<tr>
<td>IPC + HI/R (group 5)</td>
<td>1.01 ± 0.08§</td>
<td>0.87 ± 0.14§</td>
<td>2.44 ± 0.43§</td>
<td>23.43 ± 4.05</td>
<td>12.99 ± 2.06§</td>
<td>0.95 ± 0.06§</td>
<td>39.7 ± 1.78§</td>
</tr>
<tr>
<td>IPC + DPCPX + HI/R (group 6)</td>
<td>4.81 ± 0.17†</td>
<td>4.52 ± 0.32‡</td>
<td>12.32 ± 1.05‡</td>
<td>303.54 ± 24.67†</td>
<td>192.23 ± 20.44†</td>
<td>3.37 ± 0.24‡</td>
<td>61.17 ± 6.87‡</td>
</tr>
<tr>
<td>CCPA + HI/R (group 7)</td>
<td>0.94 ± 0.03§</td>
<td>0.15 ± 0.02§</td>
<td>2.91 ± 0.34§</td>
<td>41.79 ± 5.03</td>
<td>4.99 ± 1.23§</td>
<td>1.44 ± 0.05§</td>
<td>44.71 ± 4.11§</td>
</tr>
<tr>
<td>CCPA + DPCPX + HI/R</td>
<td>2.58 ± 0.13†‡</td>
<td>3.18 ± 0.35‡</td>
<td>13.21 ± 1.01‡</td>
<td>126.59 ± 11.25‡</td>
<td>109.64 ± 16.33‡</td>
<td>2.18 ± 0.23‡</td>
<td>76.03 ± 2.54‡</td>
</tr>
<tr>
<td>DPCPX + HI/R (group 9)</td>
<td>6.99 ± 0.28‡</td>
<td>5.59 ± 0.41‡</td>
<td>16.56 ± 1.25‡</td>
<td>414.37 ± 33.04‡</td>
<td>231.54 ± 13.25‡</td>
<td>3.12 ± 0.07‡</td>
<td>97.44 ± 8.21‡</td>
</tr>
</tbody>
</table>

The hepatic A1R activation by CCPA is responsible for protection from HI/R and is a new mediator of the late hepatic IPC. Nitrite and nitrate (NO2−/NO3−) are metabolic products of NO and are often used as markers of NO formation. HI/R significantly increased the levels of hepatic NO2−/NO3− with respect to the sham group (P < 0.05) (Table 1). Both preconditionings restored NO2−/NO3− at levels similar to the sham group. The administration of DPCPX without previous preconditioning augmented the concentration of NO2−/NO3− with respect to HI/R (P < 0.05).
Table 2 Hepatic total SOD and its two isoforms (MnSOD and Cu,Zn-SOD) activities in hepatic homogenate

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Total SOD (units/g of tissue)</th>
<th>MnSOD (units/g of tissue)</th>
<th>Cu/Zn-SOD (units/g of tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham (group 1)</td>
<td>24.75 ± 0.85</td>
<td>11.78 ± 0.59</td>
<td>12.97 ± 0.53</td>
</tr>
<tr>
<td>Sham + CCPA (group 2)</td>
<td>25.36 ± 0.93</td>
<td>12.59 ± 0.99</td>
<td>12.77 ± 1.03</td>
</tr>
<tr>
<td>Sham + DPCPX (group 3)</td>
<td>20.22 ± 1.35*</td>
<td>10.55 ± 1.11</td>
<td>9.67 ± 0.91</td>
</tr>
<tr>
<td>HI/R (group 4)</td>
<td>8.4 ± 0.53*</td>
<td>3.28 ± 0.74*</td>
<td>5.12 ± 0.77*</td>
</tr>
<tr>
<td>IPC + HI/R (group 5)</td>
<td>25.07 ± 0.51§</td>
<td>18.61 ± 1.29§</td>
<td>6.46 ± 1.59§</td>
</tr>
<tr>
<td>IPC + DPCPX + HI/R (group 6)</td>
<td>14.61 ± 0.87†</td>
<td>6.25 ± 1.07†</td>
<td>8.36 ± 1.08*</td>
</tr>
<tr>
<td>CCPA + HI/R (group 7)</td>
<td>29.35 ± 2.11*§</td>
<td>19.84 ± 0.75§</td>
<td>9.51 ± 1.09§</td>
</tr>
<tr>
<td>CCPA + DPCPX + HI/R (group 8)</td>
<td>12.56 ± 1.12‡</td>
<td>5.33 ± 0.84*‡</td>
<td>7.23 ± 1.11‡</td>
</tr>
<tr>
<td>DPCPX + HI/R (group 9)</td>
<td>11.67 ± 0.93*‡</td>
<td>4.12 ± 0.61*</td>
<td>7.55 ± 0.68*</td>
</tr>
</tbody>
</table>

Table 3 Changes in the glutathione concentration in different compartment in HI/R settings

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Tissue levels of GSH and GSSG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GSH + GSSG (μg/g of tissue)</td>
</tr>
<tr>
<td>Sham (group 1)</td>
<td>123.82 ± 19.18</td>
</tr>
<tr>
<td>Sham + CCPA (group 2)</td>
<td>133.25 ± 31.68</td>
</tr>
<tr>
<td>Sham + DPCPX (group 3)</td>
<td>139.98 ± 27.34</td>
</tr>
<tr>
<td>HI/R (group 4)</td>
<td>227.95 ± 22.61*</td>
</tr>
<tr>
<td>HPC + HI/R (group 5)</td>
<td>132.31 ± 14.16*§</td>
</tr>
<tr>
<td>HPC + DPCPX + HI/R (group 6)</td>
<td>182.78 ± 11.79†‡</td>
</tr>
<tr>
<td>CCPA + HI/R (group 7)</td>
<td>146.64 ± 20.46§</td>
</tr>
<tr>
<td>CCPA + DPCPX + HI/R (group 8)</td>
<td>166.67 ± 16.22*‡</td>
</tr>
<tr>
<td>DPCPX + HI/R (group 9)</td>
<td>226.96 ± 25.34*‡</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Serum levels of GSH and GSSG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GSH + GSSG (μg/ml)</td>
</tr>
<tr>
<td>Sham (group 1)</td>
<td>32.55 ± 2.57</td>
</tr>
<tr>
<td>Sham + CCPA (group 2)</td>
<td>41.22 ± 9.21</td>
</tr>
<tr>
<td>Sham + DPCPX (group 3)</td>
<td>55.59 ± 11.24</td>
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<tr>
<td>HI/R (group 4)</td>
<td>92.63 ± 10.1*</td>
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<tr>
<td>HPC + HI/R (group 5)</td>
<td>51.24 ± 8.82*§</td>
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<tr>
<td>HPC + DPCPX + HI/R (group 6)</td>
<td>102.62 ± 15.81†‡</td>
</tr>
<tr>
<td>CCPA + HI/R (group 7)</td>
<td>52.31 ± 5.22*§</td>
</tr>
<tr>
<td>CCPA + DPCPX + HI/R (group 8)</td>
<td>64.19 ± 18.21*‡</td>
</tr>
<tr>
<td>DPCPX + HI/R (group 9)</td>
<td>98.37 ± 10.25*‡</td>
</tr>
</tbody>
</table>

IPC shared the same effects on the GSH concentration of the sham group. Blocking the A1R with DPCPX led to a dramatic decrease in the GSH content and augmented the accumulation of a large quantity of GSSG at both local and systemic levels (DPCPX + HI/R). Protective effects of both preconditioning settings were abrogated by the treatment with DPCPX as shown by the abrupt fall in the GSH content and excessive rise in GSSG content (IPC + DPCPX + HI/R and CCPA + DPCPX + HI/R). The GSH/GSSG ratio showed that glutathione existing in the oxidized form was significantly (P < 0.05) higher in HI/R and DPCPX-treated groups than in preconditioning groups and the sham group.

Considering the critical role of the metabolism of adenosine in the ischaemic and preconditioning scenario, we have evaluated ADA activity. Importantly, animals subjected to HI/R showed a rise in ADA activity with respect to the sham and preconditioned groups.

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ADA activity was measured in different treatment groups.

Values are means ± S.E.M. *P < 0.05 compared with the sham group; **P < 0.05 compared with IPC; ***P < 0.05 compared with CCPA; ¥P < 0.05 compared with HI/R. U, units.

(IPC + HI/R and CCPA + HI/R; P < 0.05) (Figure 2). DPCPX showed an increase in ADA activity over all experimental groups, even above the HI/R group. Unexpectedly, DPCPX alone (sham + DPCPX) increased ADA activity over controls groups (sham and sham + CCPA). Interestingly, the elevation of ADA activity was accompanied by high hepatic and lung injury.

**DISCUSSION**

The present study provides the following important findings: (i) late IPC is able to confer hepatoprotection through a mechanism involving antioxidant balance; (ii) A1R activation protects liver from HI/R and is able to induce delayed hepatic preconditioning and this receptor is involved in the protective effects of IPC in HI/R; and (iii) A1R activation and IPC protect remote organs from long-term HI/R effects.

IPC and CCPA are capable of inducing a delayed hepatoprotection response and of completely protecting the liver from the detrimental effects of the late phase of HI/R, as indicated by a significant reduction in the transaminase activities (Figure 1), and mitigation of neutrophil infiltration (Table 1). Although the selective blocking of A1Rs with DPCPX exacerbated hepatic injury and abrogated the late hepatoprotection achieved by both preconditionings, this fact confirms the participation of this receptor in hepatoprotection settings. Blocking this receptor prevents the protective pathways which would lead to reduction of the ischaemic damage.

Evidence from other groups established a direct implication of A2R but not A1R in the hepatoprotection effects of IPC [14,15]. The difference in the experimental procedures to induce hepatic IPC and the duration of HI/R in the present study may explain this discrepancy. Protective effects of A1R have been reported in various organs, such as heart [12,13], kidney [18] and brain [19,20]. Our results indicate that A1R activation is also involved in the protective effects against HI/R and as a new mediator in late hepatic IPC.

The protective role of A1R is not exclusive to the IPC phenomenon. Recent evidence considered A1R and NO as potential surrogate mediators of the cytoprotective effects of postconditioning [9,10]. Despite the intensive investigation concerning the role of adenosine receptors on pre- and post-conditioning settings, its role is still controversial [33].

The excess of NO has been shown to be deleterious in HI/R [34]. According to Beckman and Koppenol [35], the local balance between NO, superoxide radical and SOD is critical for the understanding of the biological role of NO. The role of NO in IPC has been well-documented [2,13,14]. Peralta et al. [7] demonstrated that adenosine and NO are strong mediators of hepatic IPC. The activation of A1R with CCPA elicits a moderate increase in NO generation (Table 1) and this increase was reflected in the reduction of MPO activity, thus conferring hepatoprotection against HI/R. Previously, it has been confirmed that delayed cardioprotection induced by CCPA increased iNOS expression, this effect was reversed and the delayed cardioprotection was blocked by DPCPX [13].

The protective effects of both preconditionings were associated with an increase in hepatic MnSOD activity by 57.98 % and 68.42 % respectively. DPCPX significantly suppressed this increase in activity (Table 2) and augmented hepatic injury (Figure 1). This observation correlates with the fact that infarct limitation observed 24 h after IPC and CCPA application were accompanied by a significant increase in MnSOD activity [12,36].

The role of antioxidant proteins in late IPC is currently undefined. One of these enzymes is the mitochondrial MnSOD. The importance of MnSOD is highlighted by the findings that, in contrast with the cytosolic Cu,Zn-SOD, MnSOD knockout is lethal to mice [37,38].

The intracellular signalling pathways by which A1R activation caused delayed hepatoprotection and induction of MnSOD in IPC are largely elusive. The capacity of CCPA to induce an enhancement of MnSOD activity seems to be mediated via a PKC- (protein kinase C) and tyrosine-kinase-dependent pathway, because the pharmacological inhibition of PKC attenuated the increase in MnSOD activity and abolished the delayed cardioprotective effects of CCPA [12]. Because the activation of A1R induces the formation of second messengers such as inositol 1,4,5-trisphosphate and activation of phospholipase C with the subsequent generation of diacylglycerol and activation of the PKC superfamily [39–41]. Parson et al. [42] reported that the inhibition of PKC abolished the protective effects of preconditioning induced by CCPA, indicating that PKC acts downstream from A1R in A1R-induced preconditioning. The full activation of all of these mediators promotes the
sequential activation of PKCδ/ε and p38 MAPK (mitogen-activated protein kinase), which might induce the phosphorylation and activation of antioxidant enzymes and iNOS [6,43].

In line with our previous reports [34,44,45], HI/R leads to a decrease in endogenous antioxidant activity after 90 min of in vivo ischaemia. The results of our present study show a decrease in SOD activity (all isoforms) (Table 2) and depletion of GSH content with an accumulation of GSSG in both compartments (tissue and serum) (Table 3). This result suggests an extra- and intra-cellular superoxide sequestration and accumulation, which in the presence of high levels of NO (Table 1) may promote peroxynitrite formation and exacerbation of hepatic and lung damage. The inactivation of MnSOD by peroxynitrite leads to superoxide radical accumulation and hydrogen peroxide formation within the mitochondria [46,47]. The latter can diffuse to the cytoplasm and react with cellular components or may be detoxified by Cu,Zn-SOD, because Cu,Zn-SOD activity did not differ from the sham group (Table 2). These results indicated a high ROS production and LPO generation (Table 1) which overwhelmed the cellular defence mechanism.

The depletion of SOD and GSH observed above can perpetuate the injury by increasing the LPO in both compartments (serum and tissues) following A1R-blocking by DPCPX. This result correlates with other findings, when DPCPX, in a model of cisplatin-induced nephrotoxicity, increased LPO [18]. Previously, Gallos et al. [48] confirmed that A1R-knockout mice increased systemic inflammation and produced acute organ dysfunction, thus enhancing mortality in septic settings. The combination with DPCPX had an exaggerated response on the mortality of mice. These findings and our results confirm the protective role of this receptor in multiorgan failure.

The mechanisms by which the phenomenon of ‘conditioning’ decreases the oxidative stress seem to be shared with the same molecular pathways in pre- and postconditioning settings. Recently, it has been demonstrated that cardioprotective effects of IPPost are strongly associated with a reduction in GSH depletion and mitochondrial hydrogen peroxide, peroxynitrite formation, TNFα release and in vivo LPO production [49–52]. A growing body of evidence also implicates the activation of the PKC superfamily in the cardio- and neuro-protective effects of IPPost [33,53].

As shown in Table 1, HI/R caused negative effects on the lung by increasing MPO and LPO. These results should be due, in part, to an alteration of pro-inflammatory circulating factors such as TNFα. To test this hypothesis, we evaluated the circulating concentration of this cytokine. TNFα is considered as a key mediator in multiorgan failure after HI/R; IPC reduced TNFα release and protected against lung damage mediated by HI/R [54–56]. According to Funakiti et al. [57] the capacity of IPC to attenuate TNFα and ICAM-1 (intercellular adhesion molecule 1) mRNA expression during liver reoxygenation involves a down-regulation of NF-κB (nuclear factor κB). The activation of NF-κB during the reperfusion phase is mediated by the generation of ROS and is controlled by the oxidative balance [58].

In the present study we have demonstrated that CCPA and IPC reduced the TNFα concentration. Blocking A1R exacerbated the release of this cytokine. High MPO activity and MDA + 4-HDA concentrations were also found in the lungs of animals subjected to HI/R (Table 1). These results demonstrated that the decrease in TNFα by both preconditionings could be explained as a consequence of the stimulation and preservation of antioxidants induced by IPC and CCPA.

A postconditioning strategy has also recently been shown to be effective remotely in rodent models of acute myocardial infarction achieved by intermittent episodes of renal ischaemia, and this effect was associated with endogenous adenosine receptor activation [59].

The administration of ADA blocked the protective response of IPC [7]. Andresen et al. [60] reported that DPCPX administration increased the extracellular concentration of adenosine. We suggest that the exacerbation of hepatic and lung injury observed after DPCPX administration and HI/R could be related to the high availability of adenosine to enter into the XO (xanthine oxidase) pathway with subsequent ROS formation, since the inhibition of ADA reduced the formation of hypoxanthine and xanthine (substrates for XO) and blocked ROS generation [61].

Interestingly, DPCPX increased ADA activity over all of the groups including controls. These results support the findings of Andresen [60] and suggest that A1R modulates its own extracellular substrate by mechanisms which may involve a physiological negative feedback system.

This possible negative feedback mechanism displayed by the cells allows the counteraction of the high concentration of accumulated adenosine by the action of DPCPX. The possible excess of adenosine in the extracellular compartment might activate and saturate all subtypes of receptors (A1, A2a, A2b and A3) and engage a complex signal transduction process that ultimately decreases extracellular adenosine levels by increasing ADA activity.

In conclusion, the results of the present study indicate, for the first time, that transient activation of hepatic A1R induces late hepatoprotection. Both preconditionings protected against HI/R injury and this protection was associated with enhanced MnSOD activity and by regulation of the generation of NO. The results of the present study also strengthen the concept that preconditioning at a distance can occur after remote ischaemia and is possibly related, in part, to adenosine. Further investigation should be performed to elucidate how the activation of the A1R triggers molecular mechanisms that lead to the cytoprotective effect against HI/R injury with special attention to MnSOD activity.
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