OZONE OXIDATIVE PRECONDITIONING IS MEDIATED BY A₁ ADENOSINE RECEPTORS IN A RAT MODEL OF LIVER ISCHEMIA/REPERFUSION

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Abstract

The liver is damaged by sustained ischemia in liver transplantation, and the reperfusion after ischemia results in further functional impairment. Ozone Oxidative Preconditioning (OzoneOP) protected the liver against ischemia/reperfusion (I/R) injury. The aim of this study was to investigate the role of $A_1$ adenosine receptor on the protective actions conferred by OzoneOP in hepatic I/R. By using a specific agonist and antagonist of the $A_1$ subtype receptor (2-chloro N6 cyclopentyladenosine, CCPA and 8-cyclopentyl-1,3-dipropylxanthine, DPCPX respectively), we studied the role of $A_1$ receptor in the protective effects of OzoneOP on liver damage, NO generation, adenosine deaminase activity and preservation of the cellular redox balance. Immunohistochemical analysis of Nuclear Factor-kappa B (NF-$\kappa$B), Tumor Necrosis Factor alpha (TNF-$\alpha$) and Heat Shock Protein 70 (HSP-70) was performed.

OzoneOP prevented and/or ameliorated ischemic damage. CCPA showed a similar effect to OzoneOP + I/R group. $A_1$AR antagonist DPCPX blocked the protective effect of OzoneOP. OzoneOP largely reduced the intensity of the p65 expression, diminished TNF-$\alpha$ production, and promoted a reduction in HSP – 70 immunoreactivity. In summary, OzoneOP exerted protective effects against liver I/R injury through activation of $A_1$ adenosine receptors ($A_1$AR). Adenosine and NO produced by OzoneOP may play a role in the pathways of cellular signalling which promote preservation of the cellular redox balance, mitochondrial function, glutathione pools as well as the regulation of NF-$\kappa$B and HSP-70.
INTRODUCTION

Adenosine is an important component of the purinergic system. Berne [1] demonstrated a vasodilatory effect of adenosine produced during hypoxia. In addition to its cardiovascular actions, adenosine suppresses central nervous system excitability and the generation of superoxide in neutrophils, among other effects. However, one of the primary roles of this nucleoside is cytoprotection [2]. Adenosine serves a paracrine function since it is released in response to ischemic stress and activates cells in the vicinity of its release sites. The nucleoside mediates its cytoprotective action by interacting with adenosine receptors (ARs) which include the following subtypes $A_1$ ($A_1$AR), $A_2$ ($A_2$AR) and $A_3$ ($A_3$AR). In myocardial tissue and central nervous system $A_1$AR and $A_2$AR subtypes are involved in adenosine’s protective ability [3].

Ischemic preconditioning is an inducible potent endogenous mechanism by which repeated episodes of brief ischemia and reperfusion confer a state of protection against subsequent sustained ischemic/reperfusion (I/R) injury [4]. Early reports have shown that the release of adenosine plays a key role in promoting liver protection by ischemic preconditioning (IPC) [5,6]. It has been observed that the infusion of adenosine mimicked IPC, whereas adenosine deaminase and the $A_2$AR antagonist (3,7-dimethyl-1-propargylxanthine) both had inhibitory effects [6]. Nakayama et al have reported that rat pretreatment with $A_2$AR agonist CGS21680 but not with the $A_1$AR agonist N-phenyl-isopropyladenosine, 10 minutes before total hepatic ischemia enhances the tolerance against hypoxia/reperfusion damage [7]. In close relation with adenosine, nitric oxide (NO) is released in liver IPC. Both mediators are able to activate protective mechanisms in normothermic conditions as well in cold ischemia associated with liver
transplantation. The optimal ischemic time window to induce preconditioning in the liver is determined by at least two factors: an adenosine concentration high enough to induce NO generation through the activation of A\textsubscript{2}AR together with a low xanthine concentration to avoid the deleterious effects of this metabolite [8].

Ozone Oxidative Preconditioning (OzoneOP) is a protective mechanism able to induce an adaptation to slight and transient oxidative stress which in turn increase and preserve antioxidant endogenous systems in animal models of hepatotoxicity and hepatic and renal I/R [9-12].

From the biochemical point of view, OzoneOP is very similar to liver IPC [13]. More recently, the effects of OzoneOP on NO production, mitochondrial preservation and manganese-dependent superoxide dismutase (Mn-SOD) activation have been investigated in hepatic I/R [14,15].

Since the metabolism of endogenous adenosine by adenosine deaminase (ADA) abolished the protective effect conferred by OzoneOP [12], and the demonstration that A\textsubscript{1}AR expression is regulated by oxidative stress through activation of Nuclear Factor kappa B (NF-\kappaB [16], present investigation was undertaken to study the role of A\textsubscript{1}AR in the protective efficacy of ozone against liver I/R damage. We investigated the relationship between OzoneOP, A\textsubscript{1}AR activation, NO generation and ADA activity with the cellular redox balance. The effects of OzoneOP on NF-\kappaB (p65 subunit), Tumor Necrosis Factor-alpha (TNF-\alpha) and Heat Shock Protein–70 (HSP-70) were also determined.
MATERIALS AND METHODS

The protocol was approved by the Animal Care Committee of the College of Pharmacy (Havana University). Experimental procedures were carried out in accordance with the guidelines established by the Principles of Laboratory Animal Care (NIH publication No. 86-23, revised 1985).

Animals

Adult male Wistar rats (250-275 g of body weight, 10 animals per group) were used for these studies. Rats were maintained in an air filtered and temperature conditioned (20-22 °C) room with a relative humidity of 50-52%. Rats were fed with standard commercial pellets and water ad libitum.

Surgical procedure

All animals (including controls) were anesthetized with urethane (1 g/kg, i.p.) and placed in a supine position on a heating pad in order to maintain body temperature between 36 and 37 °C. To induce hepatic ischemia, laparatomy was performed, and the blood supply to the right lobe of the liver was interrupted by placement of a bulldog clamp at the level of the hepatic artery and portal vein. Reflow was initiated by removing the clamp. Two rats were manipulated in each experiment and this procedure was repeated five times till to complete 10 rats. The procedure was made for each experimental group in order to maintain comparable experimental conditions and to avoid the interference of factors such as time of animal manipulations and others,
Experimental design

To study the effects of the specific agonist and antagonist of the A1AR (2-chloro N6 cyclopentyladenosine: CCPA and 8-cyclopentyl-1,3-dipropylxanthine: DPCPX, respectively) on the protection conferred by OzoneOP, the following experimental groups were performed.

Group 1. Sham operated (n = 10): Animals subjected to anesthesia and laparatomy plus surgical manipulation (including isolation of the right hepatic artery and vein versus the left hepatic artery and vein without the induction of hepatic ischemia).

Group 2. I/R (n = 10): Animals subjected to 90 min of right lobe hepatic ischemia as described under ‘Surgical Procedure’, followed by 90 min of reperfusion.

Group 3. OzoneOP + I/R (n = 10): Before the I/R procedure (as in group 2), animals were treated with ozone by rectal insufflation at a dose of 1 mg/kg. Nelaton canule (No. 8) was introduced 6 cm into the rectum. The possible damage generated by this procedure was evaluated. Histopathological studies have not shown any injury at the macroscopic and microscopic levels. Rats received 15 ozone treatments, one per day of 5-5.5 ml at an ozone concentration of 50 µg/ml. Ozone was obtained from medical grade oxygen, was used immediately as generated and it represented only about 3% of the gas (O3/O2) mixture. The ozone concentration is measured by using a build-in UV spectrophotometer at 254 nm (accuracy: 0.002 at 1 absorbance unit, repeatability: 0.001 absorbance unit and calibrated with internal standard). The ozone dose is the product of the ozone concentration [expressed as mg/ml by the gas (O3/O2) volume]. By knowing the body weight of the rat the ozone dose was calculated as mg/kg as in our previous reports [9-15].
Group 4. OzoneOP + CCPA + I/R (n = 10): Animals were treated with ozone (as in group 3). Afterwards they received a single dose of CCPA (0.1 mg/kg, i.p.). Twenty-four hours later the rats were submitted to I/R (as in group 2).

Group 5. OzoneOP + DPCPX + I/R (n = 10): Animals were treated with ozone (as in group 3). Afterwards they received a single dose of DPCPX (0.1 mg/kg, i.p.). Twenty-four hours later the rats were submitted to I/R (as in group 2).

Group 6. DPCPX + I/R (n = 10): Animals were treated with DPCPX (as in group 5). Afterwards they were submitted to I/R (as in group 2).

Group 7. CCPA + I/R (n = 10): Animals were treated with CCPA (as in group 4). Afterwards they were submitted to I/R (as in group 2).

Sample preparations

Blood samples were obtained from the abdominal aorta in order to evaluate the degree of hepatic injury. Afterwards, the hepatic right lobe of each animals was extracted and homogenized in 20 mM KCl/histidine buffer pH 7.4, 1:10 w/v using a tissue homogenizer (Edmund Bühler Co., Bodelshausen, Germany) at 4 °C and were centrifuged for 10 min at 12 000 x g. The supernatants were taken for biochemical determinations.

Biochemical determinations

Markers of hepatic injury

Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using a commercial kit from Boehringer Mannheim (Munchen, Germany).
Markers of antioxidant-prooxidant balance in liver I/R

Nitrite/nitrate levels as a measure of NO generation were determined by the Griess reaction by first converting nitrates to nitrites using nitrate reductase (Boehringer Mannheim Italy SpA, Milan, Italy). Then the Griess reagent (1% sulphanilamide, 0.1% N-(1-naphtyl)-ethylenediamine dihydrochloride in 0.25% phosphoric acid) was added [17]. Reduced and oxidized glutathione (GSH and GSSG respectively), were measured enzymatically in 5-sulfosalicylic acid-deproteinized samples using a modification [18] of the Tietze’s procedure [19]. Lipid peroxidation was assessed by measuring the concentration of malondialdehyde (MDA) and 4-hydroxyalkenals (4-HDA). Concentrations of MDA + 4HDA were analyzed using the LPO-586 kit obtained from Calbiochem (La Jolla, CA, USA). In the assay, the production of a stable chromophore after 40 min of incubation at 45 °C was measured at a wavelength of 586 nm. For standards, freshly prepared solutions of MDA bis-[dimethyl acetal] (Sigma Chemical Co) and 5-hydroxynonenal diethyl-acetal (Cayman Chemical Ann Arbor, MI USA) were employed and assayed under identical conditions.

Ada activity

Tissues were homogenized in 10 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid-potassium hydroxide (HEPES-KOH) buffer (pH = 7.4) containing 0.25 M sucrose, 1mM MgCl$_2$ and 1mM mercaptoethanol, at 0 °C. The homogenate was centrifuged at 15 000 g for 15 min. Tissue adenosine deaminase activity was determined as described in Methods of Enzymatic Analysis [20].
**Protein measurement**

Total protein concentration in liver homogenates was determined using a commercial kit from Bio-Rad Munich, Germany.

**Immunohistochemistry of NF-κB (p65 subunit), TNF-α and HSP-70**

Liver fragments were harvested during autopsy and immediately fixed in buffered formalin (pH 7.2) for paraffin embedding and Hematoxylin-Eosin staining. Sections (5 µm) used for immunohistochemistry were mounted on syalinized slides and exposed to 56 °C for 20 minutes, afterwards the specimens were xylene dewaxed, rehydrated, rinsed and washed in PBS (7.4) for 30 minutes. Once endogenous peroxidase was quenched, the specimens were washed in PBS and treated for 10 minutes with Dako target retrieval high pH solution equilibrated at 99 °C in order to enhance antigens expression. Next tissue samples were blocked for 25 minutes using Dako protein block-serum free (Dako Corp. Carpinteria, CA, USA) and independently incubated for 30 minutes with the following antibody (obtained from Santa Cruz Biotechnology, CA, USA): rabbit polyclonal antibody against rat NF-κB p65 (1:250), goat polyclonal antibody against rat TNF-α (1:150) and goat polyclonal antibody against rat HSP-70 (1:250). All the antibodies were pre-diluted in Dako diluent with background reducing solution before incubation. Experiment controls were invariably included and consisted in non ischemic liver fragments (sham-operated), isospecies pre-immune serum and antibody blocking peptide (for TNF-α, Santa Cruz Biotechnology). The immunohistochemical reaction was carried out using Dako LSAB + system HRP kit according to the manufacturer’s instructions. Peroxidase chromatogenic reaction was developed with DAB, followed under the microscope and
stopped by immersing the slides in distilled water. The slides were finally dehydrated through graded ethanol, cleared in xylene and mounted.

**Statistical analysis**

The statistical analysis was started by using the outliers’ preliminary tests for the detection of error values. Afterward, homogeneity variance test (Bartlett-Box) was used followed by the ANOVA method (one-way). In addition, a multiple comparison test was used (Duncan test); values were expressed by the mean ± S.E.M. (n = 10 per group). The significance level was set at p<0.05.

**RESULTS**

*Effects of CCPA and DPCPX on the protection conferred by OzoneOP in liver I/R*

**Figure 1**

As shown in Fig. 1A, the degree of hepatic damage induced by 90 min of ischemia followed by 90 min of reperfusion significantly increased (p < 0.05) in the group subjected to I/R as evaluated by levels of AST and ALT. OzoneOP prevented and ameliorated the ischemic damage as assessed by the activities of ALT and AST, respectively. CCPA did not modify the effects of OzoneOP on transaminase activities (OzoneOP + CCPA + I/R), and it provided a protective effect similar to the OzoneOP + I/R group. A₁AR antagonist DPCPX blocked the protective effect of OzoneOP. There was a significant increase in the transaminase activities in the group treated with the A₁AR antagonist as compared to the I/R group. CCPA administered 24 hours before I/R provided significant protection, and transaminase activity values in CCPA-treated rats were similar to OzoneOP + I/R and OzoneOP + CCPA + I/R groups.
The effects of CCPA and DPCPX on NO generation and ADA activity were in agreement with the transaminase activities. Agonist of A1AR did not change ADA activity in the presence of OzoneOP but NO production was reduced at control level in OzoneOP + CCPA + I/R group. Nevertheless, CCPA increased NO levels in CCPA + I/R with regard to sham operated group probably due to differential expression of NOS isoforms. Antagonist DPCPX increased NO and ADA concentrations and annulled the protective actions of OzoneOP.

Figure 2

MDA + 4 HDA is an index of hepatic damage associated with lipid peroxidation. The results are shown in Fig. 2. There was a significant increase (p < 0.05) of lipid peroxidation in I/R. OzoneOP maintained MDA + 4 HAD generation to sham-operated levels in OzoneOP + I/R. CCPA showed a similar effect to OzoneOP + I/R. In contrast, DPCPX blunted the protection conferred by OzoneOP and increased MDA + 4HAD in a similar way to I/R group.

Influence of OzoneOP, CCPA and DPCPX on glutathione (reduced and oxidized) generation

Table 1

The results of total glutathione (GSH + GSSG) concentrations are shown in Table 1. A depletion of GSH and a significant increase in GSSG were observed in the I/R group. OzoneOP (OzoneOP + I/R) prevented the GSH depletion and attenuated GSSG increase. There was a raise of GSH in OzoneOP + CCPA + I/R group in comparison with all treatments, whereas the GSSG levels did not change in OzoneOP + CCPA + I/R with regard to sham-operated group. In line with these results, GSH/GSSG ratio was
higher. In contrast, A1AR antagonist DPCPX abolished OzoneOP and CCPA effects on reduced glutathione concentrations. GSH/GSSG ratio showed that glutathione existing in the oxidized form was significantly (p < 0.05) higher in I/R, OzoneOP + DPCPX + I/R and DPCPX + I/R groups in comparison with OzoneOP + I/R, OzoneOP + CCPA + I/R, CCPA + I/R and sham-operated groups.

Influence of OzoneOP on NF-κB (p65 subunit), TNF-α and HSP -70

First, it was not observed expression of the protein by hepatocytes, sinusoidal and Kupffer cells in fragments harvested from sham-operated groups (Fig. 3 A,D,G ). This suggest that the para-surgical and surgical stresses were not sufficient to induce NF-κB activation nor translocation to the nucleus, TNF-α was not found to be constitutively expressed by any hepatic resident cell in the samples derived from the sham ischemic animals as well as HSP-70 did not appear to be expressed by the liver cells in sham-operated rats. This is in line with the above described findings in that surgery did not seem to impact liver cells strong enough as to trigger a reactive phenotype.

In contrast, as shown in Fig. 3 B for the I/R samples, p65 appeared expressed in the cytoplasm of hepatocytes while showing differences in the intensity of the staining in a topographic selected manner. It means that most of the intensively staining hepatocytes appeared in pericentral location in a radiated pattern. Nuclear staining, which suggests p65 translocation, was only detected in such areas were the damages were more intense but without exhibiting a consolidated necrosis. In this scenario OzoneOP largely reduced the intensity of the p65 expression (Fig. 3 C) without achieving a complete inhibition of its expression suggesting a regulatory effect of NF-κB by OzoneOP. On the other hand, this finding may suggest that p65 up-regulation is a quickly emergent
activation mechanism following hepatic I/R stress and confirm that it is up-stream to TNF-α activation.

I/R clearly induced the expression of TNF-α (Fig. 3 E) which mostly detected in sinusoidal cells and perivascular areas in samples examined. Infiltrating lymphocytes and Kupffer cells were also positive for TNF-α staining, which was in correspondence to damaged areas of the liver parenchyma as judged by the H/E examination (data not shown). In most affected liver areas, hepatocytes turned to produce TNF-α while faintly appearing in the cytoplasm but with a rather enhanced stain in the nucleus. OzoneOP intervention clearly prevented TNF-α production (Fig. 3 F) by the liver cells which is in line with the remarkable preservation of the liver parenchyma architecture and the prevention of the inflammatory recruitment.

I/R provoked a dramatic expression of HSP – 70 in the animals which did not receive OzoneOP (Fig. 3 H). Immunostaining involved the cytoplasm of hepatocytes, sinusoidal and Kupffer cells, parenchymal-infiltrating leukocytes and bile ductus epithelium. In liver samples from rats exposed to OzoneOP (Fig. 3 I) a clear reduction of HSP – 70 was readily detected in terms of intensity, even when a faint staining remained detectable as to establish a difference with samples of the sham ischemic animals. This suggests a regulatory effect of OzoneOP on the expression of HSP-70.

**DISCUSSION**

Hepatic I/R injury associated with liver transplantation and hepatic resection are unresolved problem in clinical practice. [14]. Experimental model used in this work mimics I/R injury similar to liver transplantation procedure and it has allowed clarify some complex processes of liver damage which are not possible to study in humans by ethic principles. Although there are several hypothesis, the precise protective
mechanisms of preconditioning against hepatic I/R injury remain to be elucidated.

Oxygen deprivation is critical for the induction of preconditioning. Other stresses such as hyperthermia [21] or mild oxidative stress [22] enhance the hepatic tolerance to reperfusion injury [22]. In these latter conditions, the hepatoprotective effects are evident up to 48 hours from the treatments, indicating the induction of the late form of preconditioning [23].

In the liver the most investigated molecules involved in the protective mechanism of preconditioning have been: \( \cdot \)NO [24], adenosine [25] and heat shock proteins (HSPs) [26].

Adenosine inhibits leukocyte adhesion, decreases expression of adhesion molecules and inhibits neutrophil and platelet function [27,28]. Adenosine also inhibits free radicals production [29], which are important mediators of cellular damage in the early phase of I/R injury. Although liver cells express both types of adenosine receptors, \( A_1 \) and \( A_{2A} \) [30]; \( A_2 \) receptors have been proposed as the ones associated to IPC [23]. Peralta et al [5] have postulated activation of adenosine \( A_2 \) receptor with subsequent formation of \( \cdot \)NO plays a role in mediating IPC against warm I/R injury. OzoneOP was able to protect against liver I/R damage through a mechanism involving activation of \( A_1 \) adenosine receptors, as shown in the present study (Fig. 1A). This finding is not in contradiction with data from other reports [6,7,25]. First, the preconditioning procedures are quite different. Ozone promote an oxidative preconditioning through slight and transient oxidative stress when rats receive 15 treatments (one each day) of ozone in low doses and by rectal insufflation. IPC is produced when hepatic blood flow supply is interrupted by a brief period of time (5-10 minutes). On the other hand, antagonists were used in different experimental conditions. Peralta et al treated the rats
with DPCPX 5 minutes before preconditioning. In this work, animals were
preconditioned with ozone for 15 days, as described in the experimental design.
Afterwards, the rats were treated with DPCPX, which was administered 24 hours before
I/R.
The protective effects of OzoneOP, mediated by A<sub>1</sub>AR, may be a consequence of a
transient oxidative stress. In line with this idea is important to emphasize that Nie et al
[16] have demonstrated that the expression of the A<sub>1</sub>AR subtype is regulated by
oxidative stress. Oxidative stress induced by certain antineoplastic agents and H<sub>2</sub>O<sub>2</sub> up-
regulates the A<sub>1</sub>AR in hamster ductus deferens (DDT1MF2) smooth muscle cells.
Treatment of these cells with cisplatin, a chemotherapeutic agent that enhances reactive
oxygen species (ROS) generation, increased the level of the A<sub>1</sub>AR by approximately
two fold, as determined by the binding of the antagonist radioligand DPCPX, the
agonist radioligand <sup>125</sup>I-N<sup>6</sup>-2-(4-amino-3-phenyl)ethyladenosine (APNEA) and by
Western blotting. Other inducers of ROS, such daunorubicin, doxorubicin and
mitroxantrone as well as H<sub>2</sub>O<sub>2</sub> were also effective in increasing the A<sub>1</sub>AR [16]. A role
of ROS generation in mediating the increase in A<sub>1</sub>AR expression was supported by the
finding that incubating cells with H<sub>2</sub>O<sub>2</sub> and catalase, an scavenger of ROS, attenuated
the response to H<sub>2</sub>O<sub>2</sub> [2]. The increase in A<sub>1</sub>AR was inhibited when cells were
incubated with actinomycin D, suggesting a role for de novo receptor synthesis in
mediating this process. In line with these results, the protective effects of OzoneOP
were dependent on protein synthesis since cycloheximide, a general protein synthesis
inhibitor, completely blocked the beneficial effects of OzoneOP [5].
A role of NF-κB in the induction of A<sub>1</sub>AR has been reported [16,31]. In cells treated
with cisplatin along with pyrrolidine dithiocarbamate or dexamethasone inhibitors of
NF-κB activation [31], the induction of the A<sub>1</sub>AR by cisplatin was attenuated [16]. In
order to investigate if OzoneOP could modify NF-κB activity, we studied the effects of OzoneOP on this nuclear transcription factor in liver I/R. OzoneOP was able to regulate the enhanced NF-κB activity during hepatic I/R (Fig. 3 C). Ramkumar et al reported that upregulation of the A1AR in some tissues is an adaptative response to counter the oxidative stress [2]. By promoting a slight and transient ROS generation, OzoneOP is able to increase antioxidant endogenous systems [9]. This results in liver protection against oxidative stress during I/R injury.

In contrast to the A1AR, oxidative stress decreased the expression of the A2AR. Treatment of rat pheochromocytoma (PC-12) cells with H2O2 resulted in a significant reduction in the expression of the A2AAR, presumably mediated via activation of NF-κB [32]. Other activators of NF-κB in these cells, such as ceramide and nerve growth factor (NGF), also inhibited A2AAR expression. Although our data clearly showed that A1AR is involved in the protective efficacy of OzoneOP, we can not rule out the possibility that A2AR is also contributing to the beneficial effects seen with OzoneOP.

Thus, by promoting a transient oxidative stress, OzoneOP may increase extracellular adenosine through the control of ADA activity (Fig. 1C). Adenosine can then activate ARs leading to the enhancement of signaling pathways to provide cytoprotection. Oxidative preconditioning mediated by ozone seems to induce expression of the A1AR and possibly A3AR. A3AR has been associated with the induction of antioxidant enzymes. Activation of the A3AR in the rat basophilic leukemia (RBL) cells led to an increase in the activities of superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase, along with a reduction in malondialdehyde, a marker of lipid peroxidation [33]. All these results are in line with the effects of OzoneOP, since OzoneOP raised superoxide dismutase, catalase, glutathione peroxidase and reduced
MDA + 4-HDA concentrations in hepatotoxicity mediated by CCl₄, liver I/R and diabetes respectively [9-15,34-36].

OzoneOP and CCPA favored antioxidant-prooxidant balance. They protected against the increase in lipid peroxidation and transaminase activities (Figs. 1 and 2). These results indicate that OzoneOP and the agonist of A₁AR CCPA avoided the deleterious effects of lipid oxidation, which is associated to tissue damage during I/R. Glutathione is a ubiquitous intracellular antioxidant that plays a key role in the defense against oxygen free radicals. The intracellular oxidation of GSH to GSSG is protective of enzyme sulfhydryl groups and vital membrane components [37]. OzoneOP and the activation of A₁AR with CCPA avoided GSH depletion. CCPA showed higher GSH/GSSG ratio (Table 1) in comparison with all treatments as a result of the prevention of oxidative stress promoted by I/R injury. These results were in correspondence with the reduction of lipid peroxidation which suggests the preservation of membrane integrity by both treatments (OzoneOP and the CCPA agonist).

The effects of OzoneOP on adenosine and NO have a particular importance in cellular signaling processes. Carini et al [23] have proposed that both biomolecules are involved in the mechanisms leading to the development of hepatocyte resistance to I/R injury following early and late hepatic preconditioning. These protective mechanisms include: preservation of energy sources, mitochondrial functions, pH, ion homeostasis as well as to reduce oxidative injury and caspase activation [23]. OzoneOP actions preserved mitochondrial integrity [15] and reduced generations of protons and lactate concentrations by anaerobic glycolysis [38].
Oxidative stress is increasingly recognized as an important cause of liver reperfusion injury [16]. OzoneOP showed a significant amelioration of liver oxidative damage occurring during reoxygenation [9-15,38]. An increased antioxidant capability as well as a decreased generation of ROS by intracellular sources of inflammatory cells might account for this effect. Indeed, OzoneOP increase the hepatic reduced glutathione content (Table 1). Furthermore, decreased generation of both xanthine and xanthine oxidase is also evident in livers submitted to OzoneOP [5,13] and likely contributes to ameliorate oxidative stress on reperfusion. It is possible that the activation of p38 MAPK is responsible for increasing the resistance of preconditioned liver to oxidative stress. These results suggest that cells preconditioned by ozone show a decreased susceptibility to apoptosis. This could be explained by the amelioration of oxidative damage and energy losses mediated by OzoneOP.

NF-κB has also been implicated in the regulation of hepatocyte response to proapoptotic stimuli. OzoneOP was able to regulate NF-κB (Fig. 3 C) and its activity may be associated with a control of cellular redox balance, NO generation, GSH preservation and cytprotection. It is possible that a transient stimulation of NF-κB by OzoneOP might prevent a subsequent sustained NF-κB activation during reperfusion [23].

Post-ischemic tissues produce TNF-α which is a crucial inducer of apoptosis in the liver [39] OzoneOP abolished the increase of TNF-α mainly released from Kupffer cells, thus attenuating the liver injury following hepatic I/R. It is probable that OzoneOP prevents the systemic release of liver-associated TNF.
Late hepatic preconditioning is considered a second window of protection and it is evident 24 hours after the application of a preconditioning and can last for up to 2-4 days. Hepatic late preconditioning may be induced by different stimuli including oxidative stress [22]. Although the present knowledge of the mechanisms responsible is quite preliminary some molecules have been implicated: HSPs, heme oxygenase (HO)-1 (HSP332A) and NO [21,22]. In the rat liver, tolerance to ischemic injury has been associated with production of various inducible HSPs. OzoneOP regulated HSP-70 (Fig. 3I). The induction of HSP, particularly HSP-70, might be relevant for the development of tolerance to reperfusion damage, because this HSP bind apoptotic factors preventing caspase activation [40].

It is conceivable that the expression of other proteins might also contribute to the development of late hepatic preconditioning. Inducible NO synthase, Mn-superoxide dismutase (SOD-2), cyclooxygenase 2 and aldose reductase have been suggested [41-43]. OzoneOP may play an important role in the late preconditioning since it was able to increase Mn-SOD in liver I/R as well as regulate aldose reductase activity in pancreas submitted to streptozotocin treatment [15,34].

In summary, OzoneOP exert protective effects against liver I/R injury through activation of A1AR. In analogy to IPC, adenosine and NO produced by OzoneOP may play a role in the cell signalling pathways which promote preservation of cellular redox balance, mitochondrial function, glutathione pools, regulation of NF-κB and HSP-70, among other effects. OzoneOP may be considered as a pharmacologic liver preconditioning which might be particularly relevant for improving liver transplantation. The finding that the effects of OzoneOP are mediated by A1AR allows
considering other potential medical applications for the ozonetherapy mainly in cardiovascular and central nervous systems.
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Table 1. Glutathione concentrations in hepatic tissue in different experimental conditions

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>GSH (µg/g Tissue)</th>
<th>GSSG (µg/g Tissue)</th>
<th>GSH +GSSG (µg/g Tissue)</th>
<th>Ratio GSH/GSSG</th>
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<tr>
<td>Sham-operated</td>
<td>86.84 ± 3.82</td>
<td>39.21 ± 4.05</td>
<td>126.1 ± 4.67</td>
<td>2.22</td>
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<td>I/R</td>
<td>20.82 ± 2.28</td>
<td>202.01 ± 2.94</td>
<td>222.83 ± 2.38</td>
<td>0.103</td>
</tr>
<tr>
<td>OzoneOP + I/R</td>
<td>101.19 ± 3.92</td>
<td>63.49 ± 5.88</td>
<td>164.69 ± 4.26</td>
<td>1.59</td>
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<tr>
<td>OzoneOP + CCPA + I/R</td>
<td>132.28 ± 8.41</td>
<td>32.97 ± 3.61</td>
<td>165.25 ± 6.58</td>
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<tr>
<td>OzoneOP + DPCPX + I/R</td>
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</tr>
<tr>
<td>DPCPX + I/R</td>
<td>51.98 ± 3.94</td>
<td>219.42 ± 6.61</td>
<td>271.39 ± 9.14</td>
<td>0.24</td>
</tr>
<tr>
<td>CCPA + I/R</td>
<td>95.25 ± 4.91</td>
<td>24.57 ± 3.59</td>
<td>119.8 ± 3.8</td>
<td>3.88</td>
</tr>
</tbody>
</table>

Ischemia/reperfusion (I/R). 90 min of ischemia followed by 90 min of reperfusion. OzoneOP, ozone oxidative preconditioning; CCPA, 2-chloro N6 cyclopentyladenosine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine. CCPA and DPCP were administered after OzoneOP and 24 hours before I/R procedure. Data shown are at least the result of three independent experiments. Each value is the mean ± SEM from 10 rats. Mean values having different superscript letters indicate significant difference (p < 0.05) between groups within the same set.
Captions for figures

Figure 1. (A) Plasma activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT); (B) hepatic tissue levels of nitrite/nitrate (NO\textsubscript{2}/NO\textsubscript{3}); (C) hepatic tissue for adenosine deaminase (ADA) activity. (I/R), 90 min of ischaemia followed by 90 min of reperfusion; OzoneOP, ozone oxidative preconditioning; CCPA, 2-chloro-N6-cyclopentyladenosine; DPCPX, 8 cyclopentyl-1,3-dipropylxanthine. CCPA and DPCPX were administered 24 hours previous to I/R procedure. Data shown are at least the result of three independent experiments. Each value is the mean ± SEM from 10 rats. Mean values having different superscript letters indicate significant difference (p< 0.05).

Figure 2. Hepatic tissue levels of malondialdehyde + 4-hydroxyalkenals. Data shown are at least the result of three independent experiments. Each value represents the mean ± SEM from 10 rats. Mean values having different superscript letters indicate significance difference (p < 0.05) between groups.

Figure 3. Immunohistochemistry of liver slides. Data shown are representative at least of 7 rats. Sham-operated (A, D, G); animals submitted to anaesthesia and laparatomy; I/R, 90 min of ischemia followed by 90 min of reperfusion (B, E, H); OzoneOP, animal preconditioned with ozone and subjected to I/R (C, F, I); p65 subunit of NF-κB, nuclear factor kappa B; TNF-α, tumor necrosis factor alpha; HSP-70, heat shock protein-70- Arrows indicate the reaction intensity between each mediator with its antibody.
Figure 1
Figure 2