Free Rad. Res., Vol. 33, pp. 595-605
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Effect of Ozone Treatment on Reactive Oxygen Species and Adenosine Production During Hepatic Ischemia-Reperfusion

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Accepted for publication by Prof. H. Sies

(Received 21 February 2000; In revised form 18 May 2000)

This study investigates whether ozone could confer protection from hepatic ischemia reperfusion by modifying the accumulation of adenosine and xanthine during ischemia. A significant increase in both adenosine and xanthine accumulation was observed as a consequence of ATP degradation during hepatic ischemia. Adenosine exerts a protective effect on hepatic ischemia reperfusion injury since the elimination of endogenous adenosine accumulation with adenosine deaminase increased the hepatic injury associated with this process. On the other hand, the high xanthine levels observed after ischemia could exert deleterious effects during reperfusion due to reactive oxygen species generation from xanthine oxidase. The administration of allopurinol, an inhibitor of xanthine oxidase, attenuated the increase in reactive oxygen species and transaminase levels observed after hepatic reperfusion. Ozone treatment in liver maintained adenosine levels similar to those found after ischemia but led to a marked reduction in xanthine accumulation. In order to evaluate the role of both adenosine and xanthine, we tried to modify the protection confered by ozone, by modifying the concentrations of adenosine and xanthine. The metabolization of endogenous adenosine after ischemia abolished the protective effect conferred by ozone. When xanthine was administered previous to ozone treatment, the protection conferred by adenosine disappeared, showing both postischemic reactive oxygen species and transaminase levels similar to those found after hepatic ischemia reperfusion. Ozone would confer protection against the hepatic ischemia reperfusion injury by the accumulation of adenosine that in turns benefits the liver and by blocking the xanthine/xanthine oxidase pathway for reactive oxygen species generation.

Keywords: ischemia-reperfusion; ozone; liver; reactive oxygen species

Abbreviations: I/R, ischemia-reperfusion; AST, aspartate-aminotransferase; ALT, alanine-aminotransferase; ROS, reactive oxygen species; ADA, adenosine deaminase; XDH, xanthine dehydrogenase; XOD, xanthine oxidase.

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INTRODUCTION

The liver is damaged by sustained ischemia in liver transplantation or in liver surgical procedures, and the reperfusion after ischemia results in further functional impairment[1]. Tissue ATP falls rapidly after ischemia, followed by accumulation of catabolism products such as adenosine, and bases such as hypoxanthine and xanthine[2–4]. In addition, it is known that adenosine and the bases xanthine and hypoxanthine exert opposite effects in ischemia reperfusion (I/R) processes. Adenosine has been known to exert various beneficial biological actions in I/R processes, such as increased blood flow, supression of reactive oxygen species (ROS), and inhibition of neutrophil and platelet activation[5,6]. On the other hand, the accumulation of hypoxanthine and xanthine during ischemia mediates the deleterious effects of reperfusion by the generation of ROS through the activation of the enzyme xanthine oxidase[7]. ROS play a major role in the pathogenesis of I/R[8]. Among different ROS, H_2O_2 has a relative longer half-life and plays a significant role in oxidative stress injury[9]. ROS can directly damage cells through protein alterations, DNA damage, by generating lipoperoxidative chain reactions that promote the breakdown of cell membranes or by the induction of apoptosis[10,11]. Indirectly, some authors propose that the injuring effects of ROS are mediated through secondary effects as vasoconstrictors, or signals for neutrophil adhesion[12].

Preconditioning is a phenomenon which consists in the induction of organ stress to elicit the enhancement of the endogenous defense systems, thus making the organ more tolerant to a subsequent I/R injury. Preconditioning may be achieved by several different techniques, including short periods of ischemia and reperfusion, hypoxia, heat shock or oxidative stress[13–15]. Recently, a strategy based on the use of ozone, an oxidant which could promote an organ stress in order to protect against ischemic disorders has been applied[16–18]. In this sense, the beneficial effect of ozone treatment has been reported in patients with myocardial infarction[16]. In addition beneficial effects of ozone have been described in patients under hypoxic brain conditions[17]. Recently, we have observed the effectiveness of ozone on the injury associated with hepatic I/R in an experimental model of normothermic ischemia in rats[18]. Knowledge of the mechanisms by which the ozone treatment reduced I/R injury could help to provide ways to protect the organ from the damage inflicted by the I/R syndrome. The possibility that ozone could act on nucleotides has been previously proposed in brain[17] and alveolar macrophages[19]. In this sense, it has been suggested that the oxidizing effect of ozone could be beneficial to maintain ATP levels in brain tissue during hypoxia[17]. In addition, it has been shown that ozone leds to a significant increase in the ATP content of alveolar macrophages[19]. Thus, the potential effect of ozone treatment on ATP degradation and the consequent adenosine and xanthine accumulation during hepatic ischemia could be considered.

Taking into account 1) the determinant role of both adenosine and xanthine in I/R injury and 2) the possible effect of ozone on nucleotides, the aim of this work is to stablish whether ozone could offer protection from hepatic I/R injury by the accumulation of adenosine and by blocking the xanthine/xanthine oxidase pathway for ROS generation.

MATERIALS AND METHODS

Surgical Procedure

The study was performed with male Wistar rats weighing between 250 and 300 g. All animals (including controls) were anesthetized with urethane (10 mg/kg, i.p.) and placed in a supine position on a heating pad in order to maintain body temperature between 36°C and 37°C. To

induce hepatic ischemia, laparotomy 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[20]. The studies were performed in concordance with the European Union regulations for animal experiments.

Experimental Design

To study whether ozone could offer protection from hepatic I/R injury by the accumulation of adenosine and by blocking the xanthine/xanthine oxidase pathway for ROS generation, the following experimental groups were performed:

Group 1. Control (n=8): animals subjected to anesthesia and laparotomy plus surgical manipulation (including the isolation of the right hepatic artery and vein vs the left hepatic artery and vein without the induction of hepatic ischemia).

Group 2. Ischemia (I) (n=8): animals subjected to 90 minutes of right-lobe hepatic ischemia.

Group 3. + Ozone treatment (O_3) (n=8): Same as group 2 but with previous ozone treatment before the ischemic period. Ozone was administered by rectal insuflation using an equipment from Biozon, Germany. The O_3 concentration was measured by using an UV spectrophotometer at 254 nm. The administered ozone dose is the product of the O_3concentration by the gas volume. By knowing the body weight of the rat the O_3dose is calculated as 1 mg/kg. Rats received 10 ozone treatments, one per day, of 5.0–5.5 ml at an O_3 concentration of 50 µg/ml[18].

Group 4. + Adenosine deaminase (ADA) (n=16). Same as group 2 and 3, but with a continous infusion of ADA dissolved in bicarbonate-buffered saline (pH=7.4) (0.066 ml/min, i.v.)[21] 15 min before ischemia.

Group 5. + Allopurinol (n=8): Same as group 2 but with prior administration of allopurinol (45 mg/kg, i.v.)[22], an inhibitor of xanthine oxidase, 5 min before the end of ischemia.

Group 6. + Xanthine (n=8): Same as group 3, but with a continous intravenous infusion of 5 mmol/l xanthine dissolved in bicarbonate-buffered saline (pH=7.4) (0.066 ml/min, i.v.)[21] 15 min before ischemia.

Control experiments were performed with the vehicle (O_2) used for the ozone administration. Control animals (as in group 1) were subjected to previous ozone treatment.

Liver samples were obtained after 90 min of ischemia to analyze nucleotides, adenosine deaminase (ADA) and xanthine dehydrogenase/xanthine oxidase (XDH/XOD) activities. In order to evaluate the degree of hepatic injury (evaluated by transaminase levels) and oxidative stress (evaluated by malondialdehyde, MDA, as index of lipid peroxidation and hydrogen peroxide, as index of reactive oxygen species formation), animals subjected to the same experimental procedures as in groups 2, 3, 4, 5 and 6 were subjected to 90 min of reperfusion after 90 min of ischemia. After hepatic reperfusion, blood samples were obtained and processed to determine plasma aminotransferases and tissue samples were taken to determine H_2O_2 and MDA levels.

Biochemical Determinations

Nucleotide analysis

To analyze adenine nucleotide content (ATP, ADP, AMP), adenine nucleosides (adenosine, inosine), and bases (xanthine, hypoxanthine), the livers were freeze-clamped and immediately homogenized in 10 volumes of 3.6 % $HClO_4$. Following homogenization, tissues were allowed to extract for 30 min at 0.5°C, and were centrifuged at 850 g for 15 min. Supernatants were adjusted

to pH 6.0–6.5 and centrifuged at 14000 rpm. Then, 50 µl of the supernatant was injected in a Waters 717 plus Autosampler liquid chromatographic equipment. Nucleotide profiles were obtained using a reversed-phase Spherisorb ODS column (C_{18}, 5 µm particle size, 15 × 0.4 cm; Teknokroma, San Cugat, Spain) coupled to a 600 HPLC system (Waters, Milford, MA) equipped with a Waters 996 Photodiode Array Detector. The absorbance was monitored at 254 nm. Nucleotide separation was allowed to proceed in an isocratic fashion with 100 mM ammonium phosphate (pH 5.5), until ATP, ADP, hypoxanthine, xanthine and AMP were separated. At this point, a mixture of water:methanol (96:4) was introduced into the column, eluting inosine. A mixture of water:methanol (60:40) was introduced after the inosine to elute adenosine[23]. Calibration chromatograms for the standards ATP, ADP, AMP, adenosine, inosine, hypoxanthine, and xanthine were generated by injecting 50 µl of a mixture of known concentrations. The profiles were processed by a Millennium^{32} system.

Hepatic injury

Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using a commercial kit from Boehringer Mannheim (München, Germany).

H_2O_2 measurement

H_2O_2 was determined following the method used by Slezak and cols. in tissue samples[24]. Liver samples were homogenized in a buffer containing 33 mM Na_2HPO_4 and 0.9% KCl, pH 7.4. After centrifugation, the supernanatant was used for H_2O_2 analysis following the method used by Slezak et al in tissue samples.

MDA

Determined by the thiobarbiturate reaction[25]. For this purpose, 1 ml of trichloroacetic acid (20%) was added to 1 ml of homogenate in phosphate buffer 0.067 M at pH 7.4. After mixing and centrifuging, 1 ml of thiobarbiturate (0.67%) was added to the supernatant and boiled for 60 min. After cooling, optical density at 530 nm was assayed.

ADA activity

Tissues were homogenized in 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid-potasium 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 15000 g for 15 min. Tissue adenosine deaminase activity was determined as described in Methods in Enzymatic Analysis[26].

XDH and XOD activity

Tissues were homogenized in 0.1 M Tris, containing 10mM EDTA, 1mM phenylmethylsulfonyl fluoride, and 1mM dithiotreitol. The homogenate was then centrifuged at 15000 g at 4°C and the pellet discarded. The supernatant was chromatographed on Sephadex G-25 80 column in the same buffer at 4°C[27]. The resultant eluate was used for measurement of XDH and XOD activity. Activities were measured spectrophotometrically on the basis of uric acid formation at 292 nm in the presence or absence of 0.60 nM NAD^+, respectively. Xanthine (60 mM) was used as substrate. The kinetic of the reaction was recorded for 10 minutes a 20°C.

Protein measurement

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

Statistics

Data are expressed as means ± SEM. Mean of different groups were compared using a one-way analysis of variance. Student's t test was performed for evaluation of significant differences

between groups. Significance was determined at the 5 % level ($p<0.05$).

RESULTS

Effect of ozone pretreatment on high-energy nucleotides and their breakdown products during hepatic ischemia

As shown in Fig. 1, ATP and ADP levels after ischemia decreased and consequently AMP and all the other products of nucleotide metabolism (adenosine, hypoxanthine, xanthine), were increased with respect to the control group. Ozone treatment leads to ATP and ADP levels significantly higher than those in animals not subjected to this procedure. AMP levels and bases were significantly lower that in the ischemic group without previous ozone treatment. However, adenosine levels were similar to those found after ischemia.

Role of both adenosine and xanthine in the protection confered by ozone

The elimination of endogenous adenosine accumulation after ischemia with ADA in both groups, treated or not with ozone ($I+O_3$+ADA, I+ADA) leads to adenosine levels similar to those found in the control group (Fig. 2). The effects of the manipulation of endogenous adenosine were reflected in changes in hepatic injury, evaluated by transaminase levels after reperfusion. The effectiveness of ozone treatment ($I+O_3$) on the hepatic injury was shown by the reduced transaminase levels with respect to those found in the ischemic group not subjected to this procedure (I). The elimination of endogenous adenosine in both groups, treated or not with ozone ($I+O_3$+ADA, I+ADA) leads to an hepatic injury similar and increased, respectively, with respect to that found in the ischemic group (I).

As shown in Fig. 3, ozone treatment drastically reduced the xanthine accumulation after ischemia. The administration of allopurinol 5 min before the end of ischemia (I+allopurinol) did not modify the xanthine levels, whereas the administration of xanthine previous to ozone treatment ($I+O_3$+xanthine) leads to xanthine levels similar to those found after ischemia. The degree of hepatic injury (transaminases) and the generation of ROS were measured after reperfusion (see Fig. 3). The administration of allopurinol, an inhibitor of xanthine oxidase reduced the hepatic injury and the postischemic ROS generation. However, the administration of xanthine previous to ozone treatment abolished the beneficial effect of ozone on transaminase, H_2O_2 and MDA levels.

We evaluated whether the maintenance of adenosine levels similar to those found after ischemia and the marked reduction in xanthine accumulation induced by ozone could be explained by differences in ADA and/or XDH/XOD activities. As shown in fig. 4, ischemia leads to a significant increase in liver ADA activity levels with respect to control group, but the ozone treatment prevented this increase. There was no change in total XDH+XOD activity in liver after ischemia. However, increased conversion of XDH to XOD occurred as a consequence of ischemia. In control animals, XOD represented 10% of the total enzymatic activity. After ischemia, this proportion increased to 40%. The ozone treatment attenuated the conversion of XDH to XOD, since the XOD represented only 25% of the total enzymatic activity.

DISCUSSION

Adenosine exerts a protective effect on I/R injury since the elimination of endogenous adenosine accumulated after ischemia with ADA (I+ADA) (Fig. 2) leads to an increase in the hepatic injury associated with this process. On

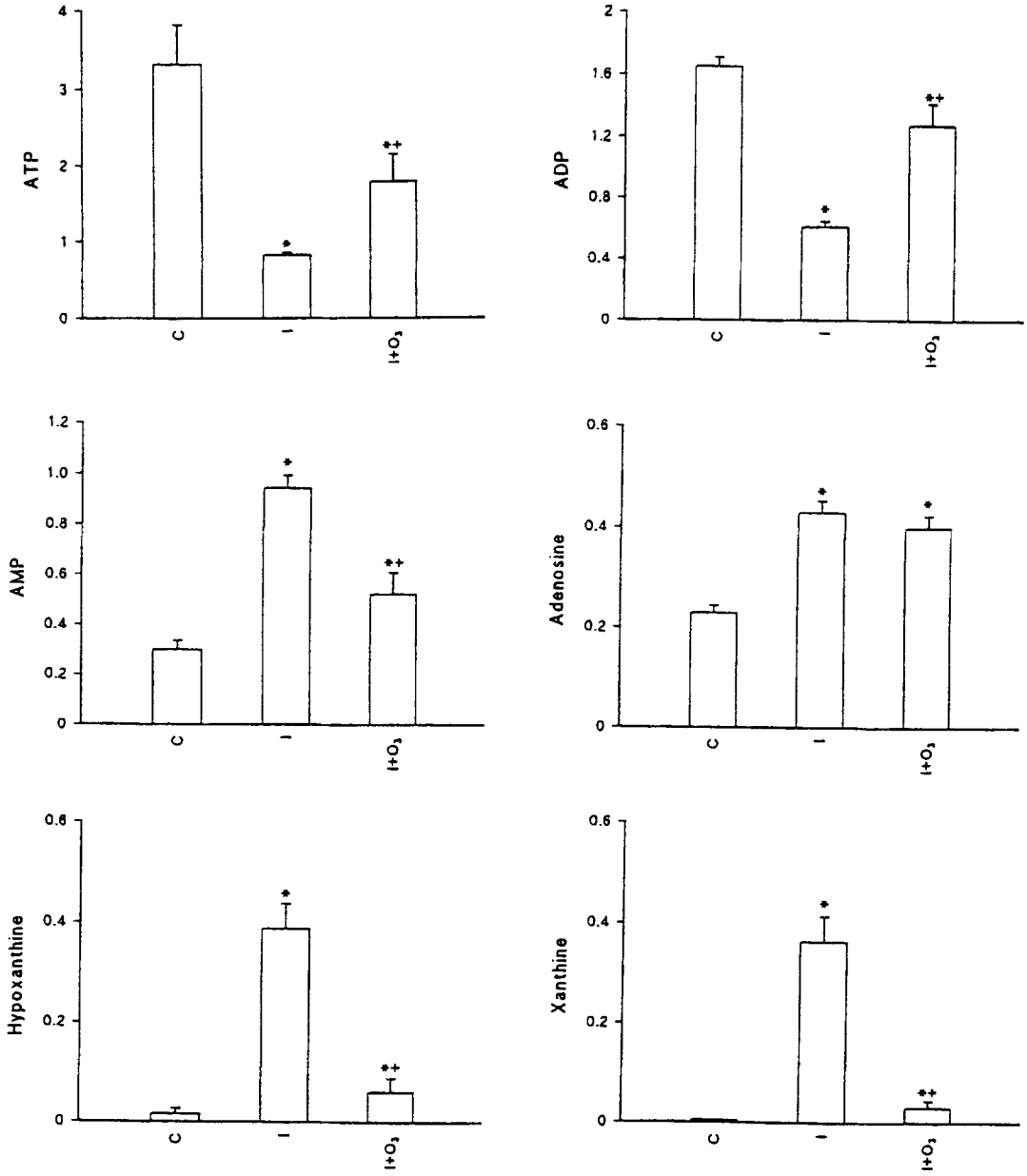

FIGURE 1 High energy nucleotides and their degradation products (ATP, ADP, AMP, adenosine, hypoxanthine and xanthine) after ischemia in controls and in animals treated ($I+O_3$) or not (I) with ozone. *=$p<0.05$ vs. Control (C); $^{+}$=$p<0.05$ vs. Without O_3. Metabolite concentrations are expressed in µmol/g wet wt

the other hand, the high xanthine concentrations found after ischemia (Fig. 3), could exert deleterious effects during reperfusion due to ROS generation from xanthine oxidase, since the

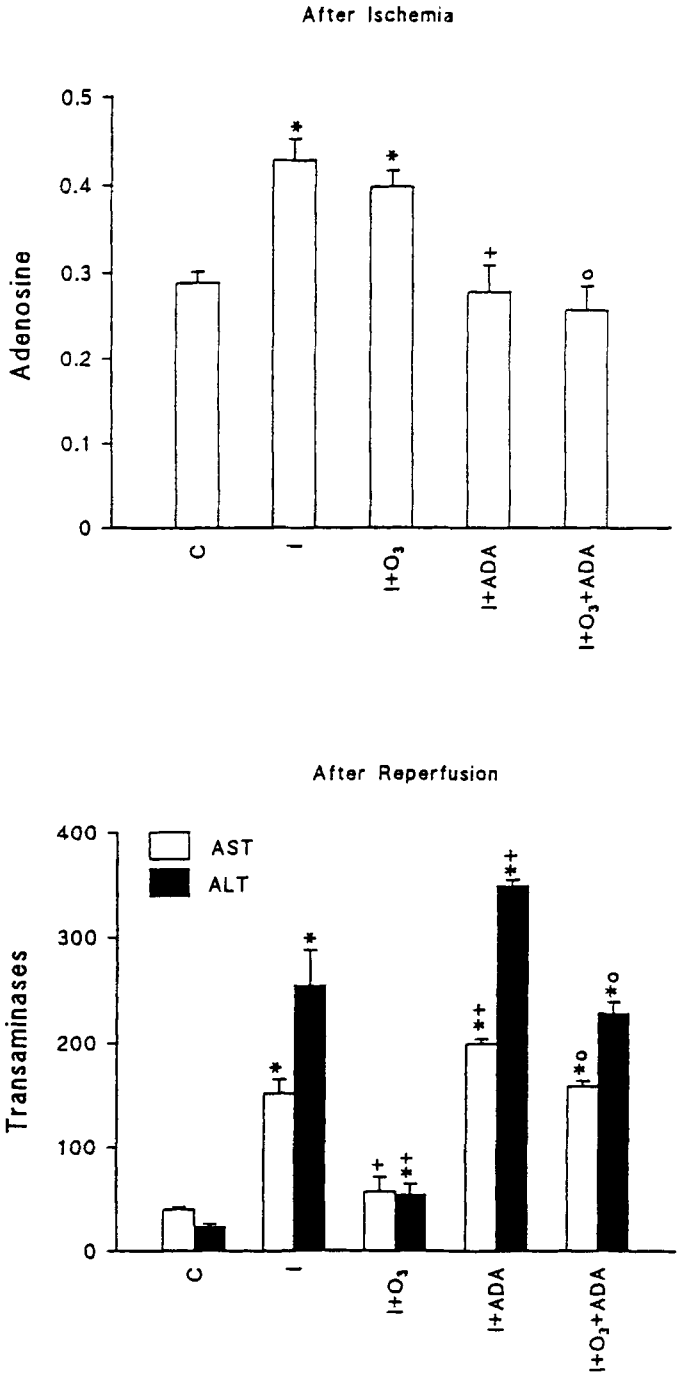

FIGURE 2 Adenosine levels after ischemia and transaminase levels after hepatic reperfusion in the following experimental groups: C: Control, I: 90 min of ischemia; I+O_3: same as I but with previous ozone treatment; I+ADA and I+O_3+ADA: same as I and I+O_3, respectively, but with previous administration of adenosine deaminase. *=$p<0.05$ vs. Control; +=$p<0.05$ vs. Without O_3; o = $p<0.05$ vs. With O_3. Adenosine concentrations and transaminase levels are expressed as mmol/g wet wt and U/l, respectively

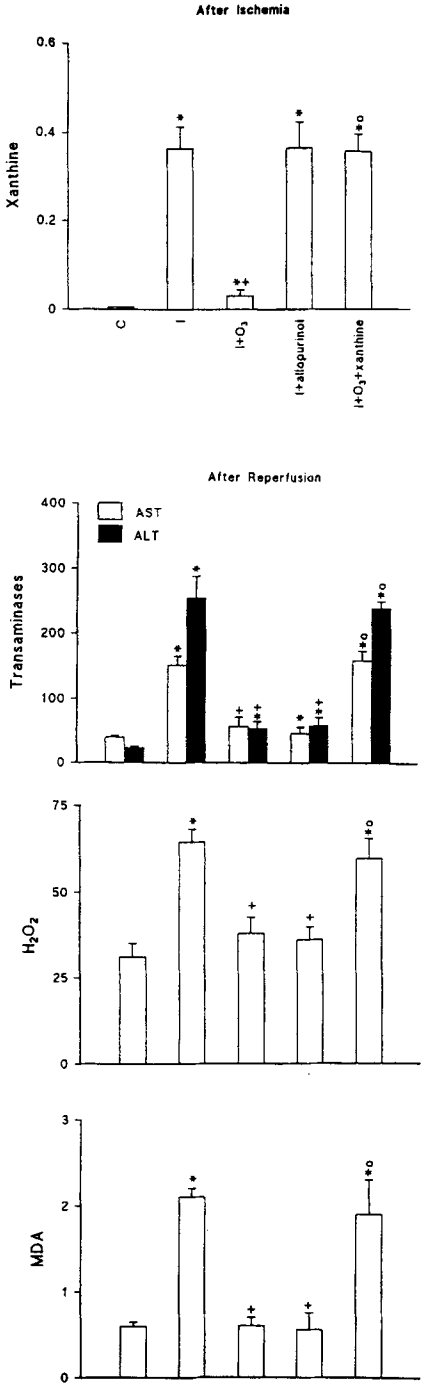

FIGURE 3 Xanthine levels after ischemia and transaminase, H_2O_2 and MDA levels after hepatic reperfusion in the following experimental groups: C: Control, I: 90 min of ischemia; I+O_3: same as I but with previous ozone treatment; I+allopurinol: same as I but with administration of allopurinol; I+O_3+xanthine: same as I+O_3 but with previous administration of xanthine. *=$p<0.05$ vs. Control; $^{+}$=$p<0.05$ vs. Without O_3; o = $p<0.05$ vs. With O_3. Xanthine concentrations, transaminase, H_2O_2 and MDA levels are expressed as mmol/g wet wt, U/l, mmol/g wet wt, and nmol/mg prot, respectively

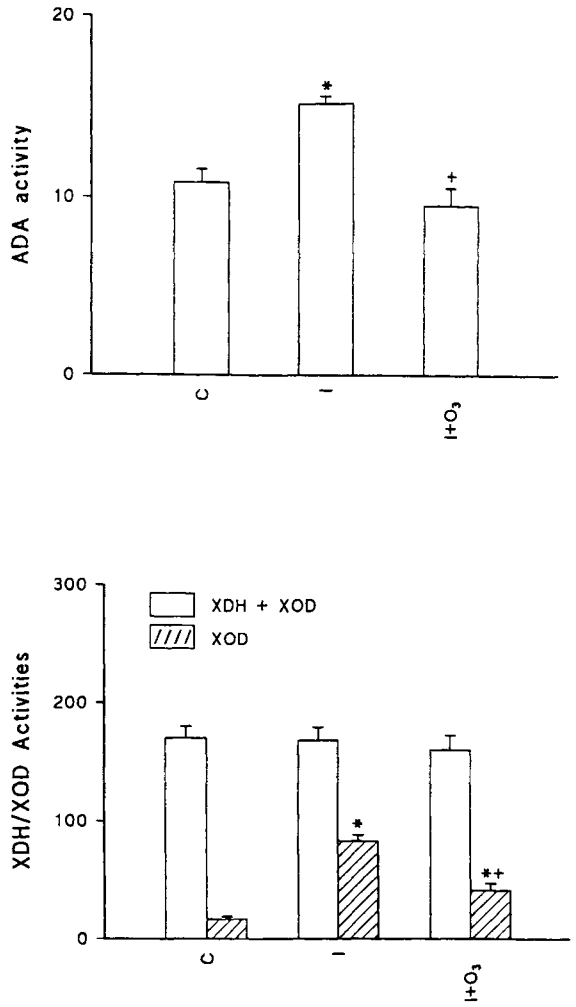

FIGURE 4 ADA and XDH/XOD activities in liver after ischemia in the following experimental groups: C: Control, I: 90 min of ischemia; I+O_3: same as I but with previous ozone treatment. *=p<0.05 vs. Control. +=p<0.05 vs. Without O_3. ADA activity is expressed as U/mg prot, XDH/XOD activities are expressed as μU/mg prot

allopurinol administration, an inhibitor of xanthine oxidase attenuated both the postischemic ROS and the hepatic injury. We suspected that when the xanthine concentration achieves its higher levels, as observed after ischemia (Fig. 3), the deleterious effect of xanthine due to postischemic ROS generation from xanthine oxidase prevails over the beneficial effect of adenosine. Altogether, this could counteract the protection conferred by adenosine. To evaluate our hypothesis that holds that ozone could confer protection against the hepatic I/R injury by maintaining the concentrations of adenosine and reducing the accumulation of xanthine found after ischemia, we tried to modify the protection confered by ozone by modifying the concentrations of adenosine and xanthine. As shown in fig. 2, the metabolization of endogenous adenosine by ADA (I+O_3+ADA) abolished the protective effect conferred by ozone. When xanthine was administered (I+O_3+xanthine) (Fig. 3), the protection conferred by endogenous adenosine disappeared, observing instead an increase in both, the postischemic ROS generation and the transaminase levels after reperfusion similar to those found in the ischemic group (I). Consequently, it could be concluded that ozone by way of a reduction in the accumulation of xanthine after ischemia prevents that the deleterious effects of ROS arising from xanthine oxidase could cancel the protection confered by adenosine on hepatic I/R injury.

It is known that during ischemic conditions, the increase in ADA is responsible for adenosine degradation, leading to an accumulation of hypoxanthine and xanthine[28–29]. It has been reported that ADA inhibitors have protective effects on I/R injury in isolated perfused rat hearts[30–31]. The authors have suggested that the inhibition of ADA activity protects the heart, possibly by an accumulation of adenosine benefitial to the heart and by blocking the xanthine/xanthine oxidase pathway for ROS generation. The attenuating effect of ozone treatment on the increase in liver ADA activity found after ischemia (Fig. 4) could favor the adenosine accumulation while diminishing the accumulation of hypoxanthine and xanthine, thus attenuating the generation of ROS after reperfusion (Fig. 3). However, these differences in ADA activity found between both groups could not completely explain the marked differences in xanthine levels after ischemia. We evaluated the

XDH and XOD activities in liver after ischemia. Under physiological conditions, the enzyme xanthine oxidoreductase (total xanthine dehydrogenase plus xanthine oxidase) is commonly present in its XDH form[32–33]. During ischemia, XDH can be converted to XOD, which catabolizes hypoxanthine into xanthine. It has been suggested that the conversion could involve enzymatic sulfhydryl oxidation or chemical sulfhydryl modifications[34–35]. As shown in Fig. 4, ozone treatment attenuated the conversion of XDH to XOD during hepatic ischemia. This effect could be due to ozone interactions with the sulfhydryl groups of the enzyme. It has been reported that sulfhydryl groups of enzymes are primary targets for ozone[36–37]. Both reductions in ADA and % XOD activities induced by ozone could explain the adenosine accumulation and the lower xanthine levels observed after ischemia.

The effectiveness of ozone treatment has been recently shown in patients with cardiac infarction[16], and in patients under hypoxic brain conditions[17]. In line with these papers, our work reveals that ozone treatment could represent a realistic approach with respect to future clinical applications in liver ischemic disorders.

The results of the present study show that ozone could confer protection against the hepatic I/R injury by the accumulation of adenosine and by blocking the xanthine/xanthine oxidase pathway for ROS generation.

Acknowledgements

Authors thank Dr. Sala-Planell (Clinica Sagrada Familia, Barcelona, Spain) for supplying Biozon equipment. This work was supported by the Fondo de Investigaciones de la Seguridad Social (FIS) through the project 00/0038–1

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