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Ozone oxidative preconditioning: a protection against cellular damage by free radicals

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THERE is some anecdotal evidence that oxygen-ozone therapy may be beneficial in some human diseases. However so far only a few biochemical and pharmacodynamic mechanisms have been elucidated. On the basis of preliminary data we postulated that controlled ozone administration would promote an oxidative preconditioning preventing the hepatocellular damage mediated by free radicals. Six groups of rats were classified as follows: (1) negative control, using intraperitoneal sunflower oil; (2) positive control using carbon tetrachloride (CCl_4) as an oxidative challenge; (3) oxygen-ozone, pretreatment via rectal insufflation (15 sessions) and after it, CCl_4; (4) oxygen, as group 3 but using oxygen only; (5) control oxygen-ozone, as group 3, but without CCl_4; group (6) control oxygen, as group 5, but using oxygen only. We have evaluated critical biochemical parameters such as levels of transaminase, cholinesterase, superoxide dismutase, catalase, phospholipase A, calcium dependent ATPase, reduced glutathione, glucose 6 phosphate dehydrogenase and lipid peroxidation. Interestingly, in spite of CCl_4 administration, group 3 did not differ from group 1, while groups 2 and 4 showed significant differences from groups 1 and 3 and displayed hepatic damage. To our knowledge these are the first experimental results showing that repeated administration of ozone in atoxic doses is able to induce an adaptation to oxidative stress thus enabling the animals to maintain hepatocellular integrity after CCl_4 poisoning.

Key words: Ozone, Oxidative stress, Preconditioning, Free radicals, Antioxidant defence system

Introduction

Ozone (O_3) has been used as a therapeutical agent for the treatment of different, apparently nonrelated diseases and beneficial effects have been observed in cerebrovascular ischaemia,[1] chronic ulcers,[2] arteriosclerosis obliterans,[3] retinitis pigmentosa,[4] humoral immunity deficiency,[5] hepatic steatosis,[6] and heart ischaemia.[7] In spite of these encouraging results obtained with ozonetherapy, its clinical use remains controversial due to the scarce knowledge of the biochemical and pharmacodynamic mechanisms which underlie its therapeutic action and the efficacy in such heterogeneous pathologies. Last but not least, O_3 has been associated with environmental pollutions and to different pathologies.[8-10] These factors have contributed to scepticism and prejudice of official medical authorities, delaying the acceptance of ozonetherapy. In order to provide scientific support to the aforementioned clinical data, some experimental strategies have been developed in order to increase our knowledge concerning its probable mechanisms of action. On the basis of the oxidant properties of O_3 and on the possibility that specific cell sensors activated by lipid oxidation products (LOP) may upregulate the antioxidant system, we postulate that O_3 may induce an adaptation to oxidative stress. Moreover, not only O_3 could induce tolerance to itself but it could prepare the host to face physiopathological conditions mediated by reactive oxygen species (ROS). With the aim to demonstrate the capability of O_3 to promote an oxidative preconditioning process, we induced hepatocellular damage with a single dose of carbon tetrachloride (CCl_4), which is a recognized organic agent able to produce a cellular injury through generation of free radicals.[11]

Materials and Methods

Animals and sample preparation

Adult female Sprague-Dawley rats (220-250 g) 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

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libitum. O_3 was generated by an OZOMED equipment manufactured by the Ozone Research Center (Cuba) and was administered by rectal insufflation. O_3 obtained from medical grade oxygen was used immediately and it represented only about 3% of the gas (O_2 + O_3) mixture. The O_3 concentration is measured by using an UV spectrophotometer at 254 nm and is very precise. The ozone dose is the product of the O_3 concentration (expressed as mg/l) by the gas (O_2 + O_3) volume (l). By knowing the body weight of the rat the O_3 dose is calculated as 1 mg/kg. Rats received 15 ozone treatments, one per day, 4.4–5.0 ml with O_3 concentration of 50 µg/ml before challenge with CCl_4. After the last ozone treatment, rats received CCl_4 (1 ml/kg) by intraperitoneal administration of a solution of 10% CCl_4 in sunflower oil. The animals were euthanized by ether anaesthesia, 24 h after receiving CCl_4. Immediately after, blood samples were obtained from the abdominal aorta and mixed with 3.8% sodium citrate, used as an anticoagulant, for biochemical determinations. Afterwards, some representative samples of different liver portions were taken for histopathological studies and tissue homogenates. Liver homogenates were obtained using a tissue homogenator Edmund Bulher LBMA at 4°C. The homogenates were prepared by using a 50 mM KCl/histidine buffer pH 7.4, 1:10 (w/v) and were spun down with a Sigma Centrifuge 2K15, at 4°C and 8500 × **g** for 20 min. The supernatants were taken for biochemical determinations.

Treatment schedule

The protocol consisted of six experimental groups (*n*=60). (1) negative control group treated only with sunflower oil by intraperitoneal route; (2) positive control group using 1 ml/kg of 10% CCl_4 solution; (3) ozone (O_3) group receiving 15 O_3 treatments (1 mg/kg) + CCl_4 (1 ml/kg); (4) oxygen (O_2) group with 15 O_2 treatments (26 mg/kg) + CCl_4 (1 ml/kg); (5) O_3 control group with 15 O_3 treatments (1 mg/kg); (6) O_2 control group with 15 O_2 treatments (26 mg/kg).

Biochemical determinations

The biochemical parameters were determined by spectrophotometric methods using an Ultraspect Plus Spectrophotometer from Pharmacia LKB. Aspartic alanine transaminase (ASAT) and cholinesterase (CHEase) levels were measured in plasma using standard commercial kits produced by Boehringer Mannheim. In liver, homogenates were assayed for total superoxide dismutases (Cu/Zn and Mn SODs) activity determining the capacity of the enzyme in inhibiting the autoxidation of pyrogallol by 50%.[12] The catalase concentration was measured through the catalytic activity which promotes the reduction of hydrogen peroxide (H_2O_2) to oxygen and water.[13] The phospholipase A activity was determined according to a standard procedure.[14] Lipid peroxidation was assessed by reading thiobarbituric acid-reactive substances (TBARM).[15] The determination of the activity of calcium-dependent ATPase (Ca-ATPase)[16,17] and glucose-6-phosphate dehydrogenase (G6PD)[18] were carried out as described. After precipitation of thiol proteins using 10% TCA the reduced gluthatione levels (GSH) were determined in supernatants of 10% w/v homogenates.[19] The proteins were measured by a standard Coomassie Blue method.[20]

Histological study

Samples of rat liver were taken and fixed in neutral 10% formalin, processed and embedded in paraffin. The histological sections were stained with haematoxylin and eosin. In addition, neutral lipids were demonstrated with oil red staining in frozen sections. From the sections embedded in paraffin and after a previous study of the microscopic alterations, the cells with ballonic degeneration were counted at the zone III of the Rappaport acini. Ten fields were taken, at random, per animal with a magnification of 250 × and the count made in a blind way by two pathologists. From the frozen sections stained with oil red, the damage area by lipidosis was calculated in other 10 fields per animal, using a morphometric software system.[21]

Statistical analysis

The statistical analysis was started by using the OUTLIERS preliminary tests for detection of error values. Afterward, the Anova method (Single Way) was used followed by homogeneity variance test (Bartlett-Box). In addition, a multiple comparison test was used (Duncan test); values are expressed by the mean ± standard error of mean ($n = 10$ per group). Different letters indicate a statistical significance of at least $P < 0.05$.

Results

Figure 1 shows the mean values ± SEM of the biochemical parameters measured for the first four groups of treatments. Groups treated with either CCl_4 or O_2 + CCl_4, showed a significant increase of ASAT activity in comparison with both the control and O_3 + CCl_4 groups. In contrast CHEase activity for the CCl_4 and O_2 + CCl_4 treatment groups was reduced significantly in comparison with both the O_3 + CCl_4 and control groups. A similar trend has been observed for SODs activity although the O_2 + CCl_4 group has an enzymatic activity significantly lower than the CCl_4 group. Catalase levels increased in CCl_4 and O_2 + CCl_4 treatment groups, while no modifications were observed under O_3 + CCl_4 treatment group with

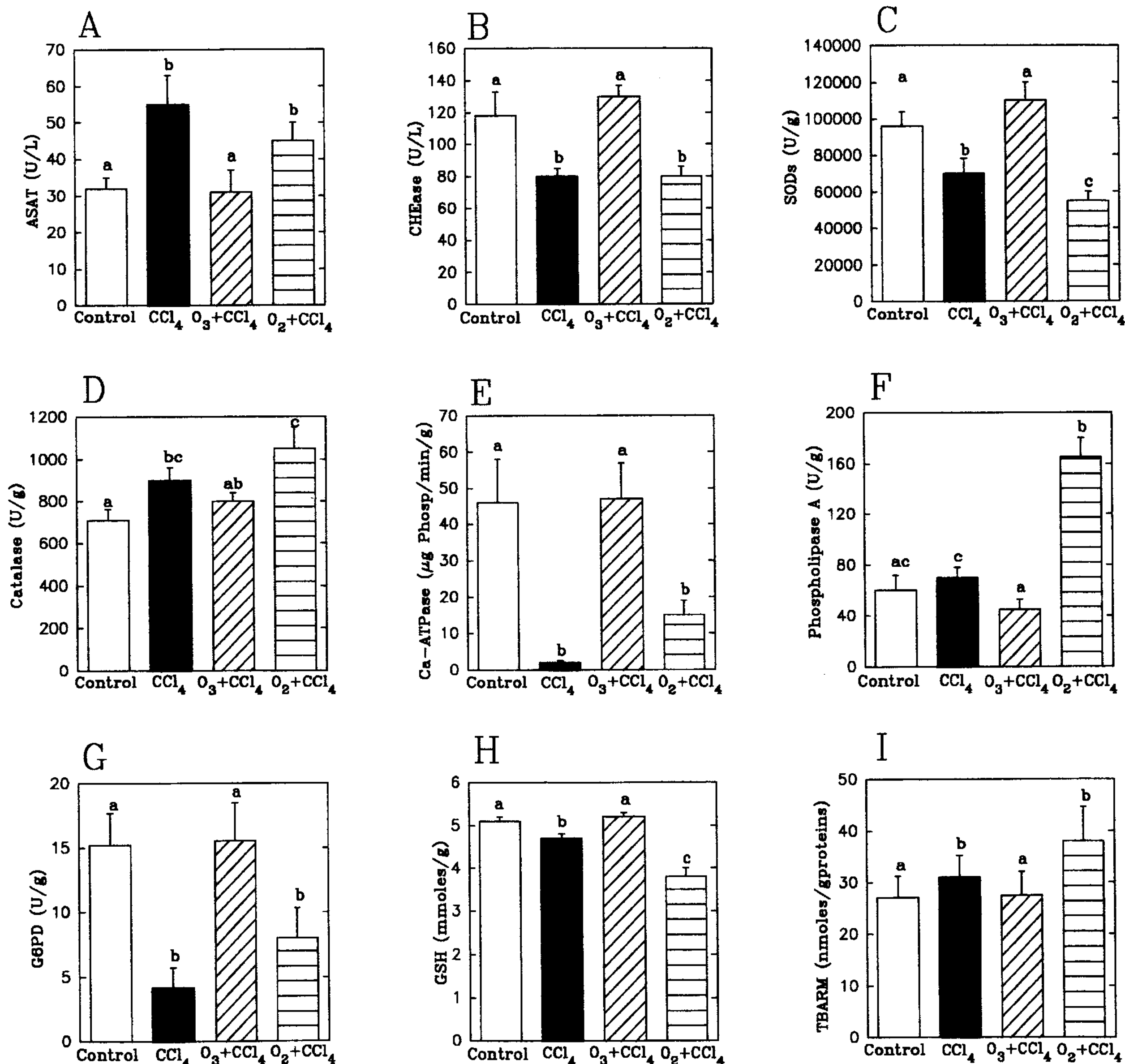

FIG. 1. The effect of ozone oxidative preconditioning in preventing CCl_4 damage in rats. The white and black bars represent either the negative or the positive controls, respectively. The hatched bars represent the situation either after O_3 exposure (diagonal lines) or after O_2 exposure (horizontal lines), respectively, prior to CCl_4. Values represent mean ± SEM.

respect to the control group. Ca-ATPase activity markedly decreased with CCl_4 and the O_2 + CCl_4 treatments in comparison with both the control and O_3 + CCl_4 treatment groups. The phospholipase A concentration in the O_3 + CCl_4 group did not differ from the control group and it was significantly lower in comparison with the CCl_4 and the O_2 + CCl_4 treatment groups. The latter group was markedly increased in comparison with the control group. G6PD was significantly reduced in both the CCl_4 and O_2 + CCl_4 groups, while O_3 treatment prior to CCl_4 maintained the enzyme at a control level. Interestingly, the O_3 treatment was also able to preserve a normal GSH level while the O_2 treatment + CCl_4 markedly reduce it. In line with these results, the TBARM, as an index of lipid peroxidation, was kept under control by the O_3 treatment.

Data from group 5 (O_3) and group 6 (O_2), without the final challenge with CCl_4 are not shown because they remained in the range of the control group, except phospholipase A levels were significantly ($P < 0.05$) higher (80.3 ± 14.2) in the O_2 group than control (60.0 ± 12.0).

Table 1 shows the qualitative results of the general hepatic damage (hepatocellular necrosis, ballonic degeneration, lipidosis and mesenchymal reaction) and the total amount of cells that have undergone ballonic degeneration (BD). In spite of a minimal degree of general hepatic damage and a marked reduction in the amount of cells with BD, values from

Table 1. Evaluation of the general hepatic damage in the three groups challenged with CCl_4

Groups	Degree	BD
CCl_4	1.4	51.0 ± 22.2[ab]
$O_3 + CCl_4$	0.2	6.2 ± 6.2[b]
$O_2 + CCl_4$	2	89.0 ± 33.0[a]

Degree of hepatic damage: 1, slight, 2, moderate, 3, severe.
Values are expressed by the mean ± SEM.

rats treated with O_3 + CCl_4 were not significantly different from those, highly variable, of the CCl_4 treatment group. On the other hand, the morphometric evaluation of the hepatic damage by lipidosis (Table 2) demonstrated a significant reduction ($P < 0.05$) of the damage area in the group treated with O_3 in comparison with the CCl_4 treatment group. No differences were observed between the CCl_4 treatment group and the group treated with O_2 + CCl_4.

Discussion

In order to test our hypothesis that prolonged administration of judicious doses of O_3 may promote the phenomenon of oxidative preconditioning, we had to demonstrate that hepatocytes can become resistant to the damage induced by free radicals after CCl_4 poisoning. The results obtained in the present work fully support this postulation. It is worth remembering that Murry *et al.*[22] in 1986 proposed the concept of 'ischaemic preconditioning' obtained by several cycles of brief coronary occlusion eventually able to minimize myocardial damage after a severe heart ischaemia.

Our experimental results have shown that repeated administration of a gas mixture composed of O_2–O_3 via the colorectal route can induce a sort of cross-tolerance to free radicals released after one single dose of CCl_4. On the contrary, experimental groups (2 and 4) treated with either CCl_4 or O_2 + CCl_4, respectively, displayed a significant cellular damage. These results were well correlated with the histopathological findings in regard to the degree of ballonic degeneration and lipidosis. It must be pointed out that administration of O_3 in rats could be carried out neither by inhalation, due to its toxicity,[23,24] nor by ozonated autohaemotherapy for technical reasons. Nonetheless the colorectal route, although somewhat empirical, is easy, practical, atoxic and has the rationale that ozonated products reach the liver via portal circulation.[25] Taking into account an interspecies factor based on the murine metabolic rate, a correct dose of ozone is 1 mg/kg in the rat and 0.25 mg/kg in humans, respectively. SODs and catalase are recognized scavengers of reactive oxygen species[26] and they have been tested as therapeutic agents against cellular damage induced by ischaemia[27,28] in spite of their low bioavailability and antibody formation. The significant stimulation of endogenous SODs in O_3 + CCl_4 group in comparison with CCl_4 and O_2 + CCl_4 groups, suggests cellular protection most likely through the reduction in the availability of superoxide anion ($\bullet O^-_2$). This result was somewhat expected on the basis of several findings[29–31] reporting increased activities of SOD, catalase and peroxidases after chronic O_3 exposure. It is noteworthy that plants can also express a protective response to O_3[32–34] suggesting that living organisms chronically exposed to O_3 have the option of either programming their death or to react and survive by upregulating the antioxidant defence system capable to readjust the redox balance. Moreover in patients, we and others have found[7,25,35] that calculated, transient oxidative stresses such as those obtained during a cycle of ozonated autohaemotherapy can also induce a state of tolerance characterized by a simultaneous overexpression of SOD, G6PD and possibly a reduction of TBARM levels in plasma. The rectal insufflation of O_3 (group 3) is apparently able to enhance the antioxidant system in a coordinate fashion because the increased activity of catalase on its own (groups 2 and 4) is unable to quench CCl_4 toxicity.

It is known that an increase of intracellular Ca^{2+} concentration plays an important role in the damage and cellular death, representing a critical and early event in the development of toxicity of hepatocytes submitted to oxidative stress.[36] The suggestion that free radicals may affect the activity of the Ca-ATPase, thus contributing to the hepatocellular injury,[37] is well supported by our data because the Ca-ATPase activity was severely inhibited in groups 2 and 4 while the O_3 treated group equalized the control. Indeed it has been previously shown that Ca^{2+}-ATPase enzymes have critical thiol groups rapidly inactivated by ROS.[38,39] Calcium-dependent phospholipase A activity was decreased in the O_3 + CCl_4 treatment group indicating that ozone exerted indirectly a protection against the cellular disruption, mediated by the activation of an enzyme which generates lysophospholipids and other metabolites responsible for cellular lysis. The increased phospholipase A activity in the fourth (O_2 + CCl_4) group suggests the participation of this enzyme in the hepatocellular damage noted in the histopathologic

Table 2. Morphometric evaluation of the hepatic damage by lipidosis

Groups	Damage areas (mm^2)
CCl_4	0.0580 ± 0.0248[a]
$O_3 + CCl_4$	0.0287 ± 0.0237[b]
$O_2 + CCl_4$	0.0512 ± 0.0240[a]

Values are expressed by the mean ± SEM.

studies. CCl_4 poisoning on its own caused only a modest increase of phospholipase A activity with respect to the control, probably due to the moderate character of the oxidative challenge after one single dose of CCl_4. The greater cellular damage observed after oxygen administration plus the hepatotoxic treatment indicates the deleterious effect of hyperoxygenation.[10,11] Sustained levels of GSH and G6PD can be taken as reliable markers of antioxidant defenses in the face of an oxidative challenge. Finally the decrease in hepatic lipid peroxidation obtained in the O_3 + CCl_4 group was in good agreement with the reduction of the lipidosis observed in the histopathologic studies, while under CCl_4 and O_2 + CCl_4 treatments lipid peroxidation levels were raised significantly. Why the O_3 treatment, without the final challenge with CCl_4 (Group 5), did not show a significant increase of antioxidant enzymes may be explained by either the fairly short treatment or, more likely, by the fact that O_3 acts best when there is an ongoing oxidative insult.

In conclusion, the present study contributes to clarify an important pharmacodynamic effect after prolonged ozonetherapy in rats. The phenomenon can be described as an induction of tolerance to O_3 and ROS generated by toxic agents and has been denominated as either 'oxidative preconditioning', or 'oxidative stress adaptation'.[25] Ozonetherapy has been able to preserve liver integrity by inducing either enzymes or activating metabolic pathways that maintain an equilibrated redox balance. High SODs and GSH levels, low peroxidation and a normal Ca^{2+} homeostasis are clear examples of the efficacy of ozonetherapy. We believe that the possibility shown by appropriate ozonetherapy to upregulate the antioxidant system represents a fundamental property of this complementary medical approach and that O_3 comes to typify a unique drug. In fact exogenous administration of antioxidant substances such as ascorbic acid, GSH, *n*-acetyl cysteine, SOD and the like are useful but hardly able to dramatically reverse a chronic oxidative stress. In this sense the improvement of the antioxidant defence is bound to be crucial in cancer,[40] chronic viral infections[41] and neurodegenerative diseases,[42] where the control of endogenous oxidation has gone awry with progressive cell damage. Therefore we should most actively pursue this lead for improving the therapy of these diseases.

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