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ORIGINAL ARTICLE

Ozone/Oxygen Mixture Modifies the Subcellular Redistribution of Bax Protein in Renal Tissue from Rats Treated with Cisplatin

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Background. Cellular events in cisplatin-mediated nephrotoxicity include apoptosis induction, decreased protein synthesis, changes in the subcellular redistribution of Bax mitochondrial dysfunction, DNA injury, increased lipid peroxidation, depletion of glutathione and decrease in enzymatic activity of renal antioxidant enzymes. In previous papers we have shown that intra-rectal (ir) ozone/oxygen mixture protected and induced a significant recovery in cisplatin-induced renal damage and it was related to a significant increase in the antioxidant system in renal tissue.

Methods. This study was undertaken to examine the effect of the intra-rectal applications of ozone/oxygen mixture in the renal expression pattern of Bax proteins in rats treated with cisplatin. A group of male Sprague-Dawley rats were pretreated with 15 ir applications of ozone/oxygen (1.1 mg/kg) before intraperitoneal injection of cisplatin (6 mg/kg). Another group was treated with five ir applications of ozone/oxygen mixture after cisplatin administration. Serum creatinine was measured thereafter. Subcellular distribution of Bax in renal tissue was analyzed by immunohistochemistry.

Results. Ozone pretreatment prevented the increase in serum creatinine levels and completely inhibited the acute tubular necrosis induced by cisplatin in renal tissue, diminishing the expression of Bax. Ozone treatment after cisplatin application reduced the increase in serum creatinine levels and the renal necrosis, inducing a lesser decrease of the Bax expression in cisplatin-treated kidneys.

Conclusions. Expression of Bax in renal tissue seems to play an important role in the protection and recovery in cisplatin-nephrotoxicity achieved by ozone/oxygen mixture.

Key Words: Ozone/oxygen mixture, Cisplatin-nephrotoxicity, Bax renal expression.

Introduction

Cisplatin (cis-diamminedichloroplatinum) (CDDP) is one of the most effective chemotherapeutic agents used for the treatment of many malignancies. However, nephrotoxicity is an important side effect of this drug, which results from injury to renal tubular epithelial cells and can be expressed as either acute or a chronic syndrome (1).

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The nephrotoxicity of CDDP is associated with cell membrane peroxidation, mitochondrial dysfunction, inhibition of protein synthesis, DNA injury (2,3), expression of pro-apoptotic Bax protein (4) and inhibition of the antioxidant system by pro-oxidant damage to the renal tissue (5). Various studies have demonstrated a protective role for antioxidants and free radical scavengers such as vitamin E, lipoic acid, ebselen, N-acetylcysteine and superoxide dismutase (SOD) in CDDP-induced acute nephrotoxicity (4,6–9).

Previous reports have shown beneficial effects of ozone intra-rectal (ir) applications in CDDP-induced nephrotoxicity (10,11), but the mechanisms of its protective effects

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are still unknown. Ozone oxidative properties involve the generation of an amount of reactive oxygen species (ROS) (12–19) that possess cell-activating and -impairing activities, depending on the amount of generated ROS. An appropriate amount of ROS, usually a small amount, activates phosphokinases and increases the intracellular Ca^{2+} levels, favoring cytoprotective pathways (20). However, a large amount induces hyperoxidation of DNA proteins and lipids, which results in cell impairment (21).

Some apoptosis regulatory proteins relevant to renal pathology have been characterized, which seems to be strongly related with the balance between factors that contribute to survival growth or lethality in renal cells (22). Bax is a Bcl-2-like protein that binds and antagonizes the protective effect of Bcl2 and BclxL, rendering cells more sensitive to death (22). In this sense, the ratio of expression of Bcl2 or BclxL to Bax appears to determine cell fate in an adverse environment (22).

This study hypothesizes that nephroprotection and beneficial effects conferred by ozone in CDDP-induced acute renal damage are associated with changes in the renal expression of Bax. Therefore, we examined the effects of ozone/oxygen mixture in the renal expression of Bax in rats treated with CDDP.

Materials and Methods

Chemicals

Serum creatinine (Cr) was measured spectrophotometrically with the creatinine assay kits purchased from Biological Products Enterprise ''Carlos J. Finlay'', Havana. All reagents used and cisplatin were purchased from Sigma Chemical Co. (St. Louis, MO). Other reagents of analytical grade were obtained from normal commercial sources.

Animals

Male Sprague-Dawley rats (200−250 g) were obtained from the National Center for Laboratory Animal Production (CENPALAB, Havana Cuba). The animals were housed under a 12-h light-dark cycle with room temperature maintained at 25°C, humidity at 60% and food and water ad libitum. The experiments were conducted in accordance with the ethical guidelines for investigations in laboratory animals and were approved by the Ethical Committee for Animal Experimentation of the National Center for Scientific Research, Havana, Cuba.

Experimental Design

Ozone/oxygen mixture (OOM) was generated by OZOMED 01 equipment manufactured y Ozone Research Center (Cuba). OOM was obtained from medical grade oxygen and was used immediately. The ozone concentration was measured by an UV spectrophotometer at 254 nm. OOM was administered to the rats by rectal insufflations (ri) at a dose of 1.1 g/kg. OOM was applied as oxidative preconditioning, which was performed with 15 applications (once daily) of the mixture before CDDP injection and was also evaluated with five applications of the OOM as a treatment after CDDP injection. The volume of insufflated mixture was approximately 9 ml. The expulsion of the gas was prevented by occluding the anus with an appropriate laboratory tissue, immediately after the insufflation of the mixture for the following 60 sec. After that, the characteristic odor of ozone was not detected, which demonstrates that all the applied gas reacted inside the rectum.

CDDP was diluted in saline (pH 7.2) and administered to the rats by a single intraperitoneal injection (6 mg/kg body weight).

In each experiment the rats were divided into three groups of eight rats: 1) control rats that received a single IP injection of saline, 2) rats treated only with CDDP, 3) rats pretreated with 15 applications (once daily) of OOM (30 μg of O_3/ml at a dose of 1.1 mg/kg) before CDDP in the first experiment. In the second experiment the third group of rats was treated with CDDP at the same dose and thereafter five applications (once daily) of OOM (30 μg of O_3/ml at a dose of 1.1 mg/kg) were given.

Five days after CDDP injection, animals were sacrificed by asphyxia in ether. The blood was collected and serum was separated by centrifugation for Cr analysis. The kidneys were perfused in situ via the aorta with phosphate-buffered saline (PBS) (pH 7.4) and then excised for histopathological studies.

Assessment of Histopathological Damage

The left kidneys were fixed in 10% formaldehyde. Tissues were embedded in paraffin and sectioned at 3 and 5 μm. Paraffin-embedded tissue sections, 3-μm thick, were stained with hematoxylin and eosin (H/E). The degree of morphological evidence of renal failure was determined using light microscopy as described by Megyesi et al. (1) with some modifications, and it was performed by an investigator blinded to the treatment protocol.

Immunohistochemistry

Five-μm-thick, paraffin-embedded tissue sections were floated onto APES- (Sigma) coated slides. Slides were deparaffinated with xylene and dehydrated in graded concentrations of ethanol. Endogenous peroxidase was quenched with 3% H_2O_2:methanol (1:1) for 30 min at room temperature. Sections were rinsed in PBS and then blocked with 6% horse serum and 4% BSA in PBS for 1 h at room temperature. Primary antibodies were mice polyclonal anti-Bcl-X S/L (1:150; Santa Cruz Biotechnology, Santa Cruz, CA) and goat anti-Bax (1:150; Santa Cruz Biotechnology). They were diluted in 1% horse serum and 4% BSA in PBS and left overnight at 4°C.

Sections were washed twice for 5 min in PBS, followed by the addition of horseradish peroxidase-conjugated anti-mice IgG and anti-goat IgG (Amersham, Buckinghamshire, UK) at a dilution 1:200 in 4% BSA in PBS for 30 min at room temperature. After washing twice for 5 min in PBS, antibody location was determined with the addition of DAB chromogen (Dako, Glostrup, Denmark): 3% H_2O_2 (130:1) for 10−15 min, and washing with water stopped color development. Sections were counterstained with Carazzi's hematoxylin (Bio-optica, Milano, Italy), dehydrated and mounted in Canada balsam (DPX, Poole, UK). As the negative control, nonspecific mice and goat IgG was used instead of the primary antibody.

Statistics

Results are expressed as mean ± SEM. Significance at the 95% level was established using one-way ANOVA. The presence of significant differences between groups was examined by Duncan's test by means of the SigmaStat statistical software (Jandel, San Rafael, CA).

Results and Discussion

The levels of Cr in the blood serum of rats are presented in Table 1. After CDDP injection, Cr levels increased about 4-fold at day 5 with respect to the control. As we reported before, oxidative preconditioning and treatment with OOM at 1.1 mg/kg body weight before and after CDDP injection, respectively, induced a remarkable decrease in serum Cr levels with respect to the control treated only with CDDP (Table 1).

The aims of the study were to examine the effect of the ir applications of OOM in the renal expression of Bax proteins in rats treated with CDDP. No comparison between the two schemes of applications of ozone are established because they have different purposes, ozone oxidative preconditioning was suggested to prevent CDDP nephrotoxicity, whereas the application of OOM after CDDP was suggested to diminished CDDP nephrotoxicity (10,11).

Table 1. Serum creatinine levels in the experiments

Serum creatinine levels (mM)		
Experimental groups	Experiment 1	Experiment 2
Saline-injected control	62.4 ± 11.98[a]	64.8 ± 12.46[a]
CDDP-injected control	280.7 ± 45.05[b]	207.3 ± 98.11[b]
OOM 1.1 mg/kg and CDDP (6 mg/kg)	130.4 ± 30.41[c]	140.8 ± 8.54[c]

Note: In the first experiment the animals received 15 intrarectal applications of ozone/oxygen mixture (OOM) before cisplatin (CDDP) intraperitoneal injection, and in the second experiment CDDP was administered after OOM.

[a,b,c]Different letters means statistical differences ($p < 0.05$). Data are presented as mean ± standard error of the mean.

Serum Cr was measured with the creatinine assay kits (Biological Products Enterprise 'Carlos J. Finlay', Havana, Cuba).

Considering that only 5% of the OOM corresponds to ozone and 95% to oxygen, the effect of the ri of oxygen in the nephrotoxicity of CDDP was previously examined using both schemes (10,11). In both cases, the ri of oxygen did not modify the biochemical parameters determined in the experiments, suggesting that the results obtained with ir applications of OOM may be ascribed to the effect of the 5% of ozone present in the mixture, according to previous reports (10,11).

Histopathological assessment by H/E staining of CDDP-induced nephrotoxicity was previously reported (21). CDDP-induced nephrotoxicity is a model of acute renal failure affecting the corticomedullary region and induces tubular degeneration, tubular necrosis, epithelial desquamation of renal cells and tubular cast formation (21). In our experiment, similar effects were observed in rats treated with CDDP alone (Figure 1B) (10,11).

Control rats exhibited a normal morphology without any significant change of the renal architecture (Figure 1A). Animals pretreated with 15 applications of OOM before CDDP showed a moderate cellular tumefaction, revealing no significant differences between them and saline-treated rats (Figure 1C). In contrast, in rats treated with CDDP and thereafter with five ri applications of OOM (1.1 mg/kg), the tubular necrosis was slight and to a lesser extent than it occurred in rats treated with CDDP alone. Tubular dilation and cast formation in the tubular lumen were also reduced in this group (Figure. 1D).

Protein immunohistochemistry (Figures 1A–1D) revealed that the distribution of Bax protein was patchy in whole tubuli and isolated tubular cells, including those detached into the tubular lumen, with some tubules showing intense staining while some cells were negative. It is important to point out that an endogenous expression of Bax protein was observed in the control group, similar to previous reports (22). Bax protein expression in this group was slight and confined to the medullary region (Figure 1A).

Widespread expression of Bax protein was noted in the group treated only with CDDP, with both cytoplasmic and nuclear localization, appearing to be more intense in the nucleus.

Bax protein distribution after CDDP injection includes medulla, cortex and corticomedullary junctions (Figure 1B).

The 15 applications of OOM at 1.1 mg/kg before CDDP injection modify the pattern of immunolocalization and intensity of the expression of Bax protein in renal tissue (Figure 1C). Bax protein expression turned to be selective, in both nucleus and cytoplasm, but its expression continues to be more intense in the nucleus, although it is lower than in the CDDP control group. In juxtaglomerular tubules, Bax expression was basal and appeared in both nucleus and cytoplasm.

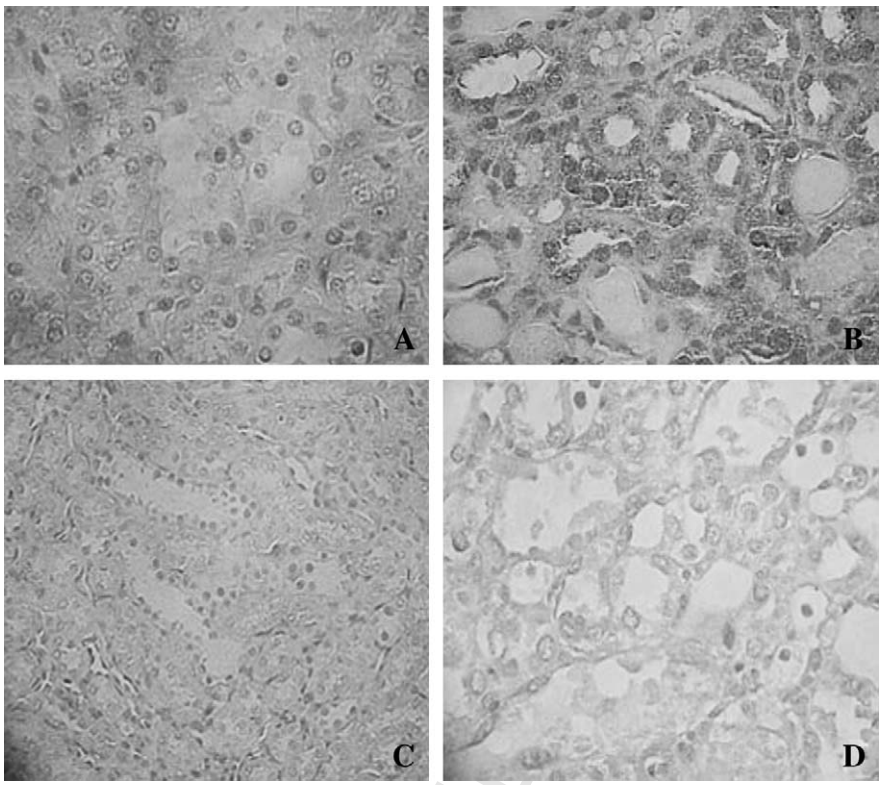

Figure 1. Immunohistochemical staining in rat kidneys (20×). (A) Normal morphology of control rats showing an slight and only nuclear expression of Bax. (B) Widespread expression of Bax was seen in renal tissue treated with cisplatin. Intense cytoplasmic and nuclear expressions were seen, being more intense in nucleus. (C) A selective expression of Bax was observed in renal tissue of rats treated with 15 applications of ozone before cisplatin injection. Bax expression was slight, appearing either in nucleus and cytoplasm, but to a lesser extent than in cisplatin-treated group.(D) Widespread expression of Bax was observed in renal tissue of rats treated with five applications of ozone after cisplatin administration. Intense cytoplasmic and nucleus expression were noticed, being more intense in nucleus; however, Bax expression in the cortex of was slight and similar to the control group.

However, when OOM were applied after CDDP, the changes in Bax expression in comparison with CDDP treated control group were less evident. An intense cytoplasmic and nuclear expression of this protein was noted in medullary and corticomedullary zone, quite similar to the widespread localization of Bax in CDDP treated group, but its expression was basal in the nucleus and cytoplasm of cortex cells.

In previous works we demonstrated the beneficial effect of OOM at 1.1 mg/kg either as preconditioning or treatment in CDDP-induced nephrotoxicity (10,11). Intrarectal ozone therapy prevented the decrease in the renal antioxidant defense system, avoiding the deleterious effect of CDDP. Furthermore, we have reported an increase in the activity of antioxidant enzymes in renal tissue, probably due to the induction of the novo enzyme synthesis rather than activation of the preexisting apoproteins. We also reported an attenuation of the renal tubular damage and enhancement of the regenerative response of the damaged tubular cells (10,11). These effects are accompanied by an evident modification of the expression pattern of Bax protein in renal tissue.

Previous *in vivo* reports have shown an increase in Bax expression after acute renal failure (5), pointing out that vascular smooth muscle cells were the main site of Bax expression in the normal kidney (5). However, Bax expression in our experiment was not ascribed only to muscle cells but dispersed throughout the entire medullary zone. These results were similar to those reported by Cuttle et al. who noted an *in vitro* endogenous expression of Bax in untreated distal cells, as well as in the distal tubule of untreated rat and in human kidneys (22). Our findings corroborated previous reports that noted an increase in the expression of Bax protein in renal tissue after CDDP nephrotoxic insult (2,3).

Considering that Bax is a protein thought to be present in the cytosol in a monomeric form since enforced dimerization leads to its insertion in the mitochondria in response to cytotoxic insults, and taking into account that some authors have observed that CDDP induced the *in vitro* translocation of Bax from the cytosolic to the membrane fraction (3), the nuclear localization of Bax was the most apparent difference in our results.

First, nuclear localization of Bax was associated with the possibility of losing its function as inducer of apoptosis or having an unknown role in the nucleus (23). However, years later, it was noted that nuclear accumulation of Bax was an early event in apoptosis induction in glioma cells (24). As well as when motoneurons are committed to cell death,

a translocation of Bax from the cytosol to organelle membranes and to the nucleus occurs (25).

Nuclear localization of Bax was reported in seven cell lines of human lung cancer without any trace of mutations (26). It was also noted in germ cells (27), increasing the evidence that point out that the Bcl-2 family of proteins might extend their activities to the nuclear compartment. Although [Q1] Bax was detected in the nucleus acomplexed with humanin, an anti-apoptotic peptide encoded in mammalians genomes (28), with protein p53 in human melanoma (29) and small cell lung carcinoma cell lines (4) following CDDP exposure.

Our results suggest that OOM modifies the expression of Bax in renal cells, and this effect may be related to the protection achieved by ozone therapy. Considering that the expression level of each Bcl-2 protein is controlled by transcription, heterodimerization and ubiquitination (20), a broad spectrum of mechanisms should be considered. Recent studies revealed that large amounts of ROS suppressed the expression of Bcl-2, increasing the expression of Bax and the heterodimerization between pro- and antiapoptotic proteins, decreasing the ubiquitination and degradation of proapoptotic proteins (20). Ozone application under controlled conditions may generate an appropriate amount of ROS capable of generating a cytoprotective response, which could include an increase in the ubiquitination and degradation of Bax, in a similar way to what was reported for N-acetylcysteine and pyrrolidine dithiocarbamate (4).

However, a decrease in the expression of Bax protein could also be caused by its heterodimerization with another Bcl-2 protein, such as Bcl-XL. We have observed an increase in Bcl-XL expression in ozone-treated renal tissue, which correlates with a decrease in Bax expression in the same tissue (data not shown), favoring a cellular ratio Bax/Bcl-XL that appears to promote tubular regeneration and cytoprotective pathways in renal cells. Bcl-XL has shown to be involved in a mechanism of protection of renal cells from oxidative stress (22).

Previous reports have shown an attenuation of mitochondrial damage with ozone (30), which could be closely linked with a diminution of the binding of Bax polymers to the outer membrane of the mitochondria. Bax influenced the opening and/or closing of mitochondrial megachannels by permeability transition pores, as well as the release of apoptogenic factors from the mitochondria (31).

In summary, the present study demonstrated that OOM at 1.1 mg/kg reverted and protected from CDDP-induced renal damage by modulation of the Bax protein expression. Further studies are necessary to account for the beneficial effects of OOM in CDDP-induced acute renal failure. Elucidation of such pathways may lead to a better understanding of the mechanisms of ozone therapy and its rational uses either to enhance the recovery and the protection against acute renal failure and to diminish the nephrotoxicity of CDDP, providing clues for the rational design of novel therapeutic interventions.

Uncited references

32,33 and 34.

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