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MITOCHONDRIAL DYSFUNCTION IN THE HIPPOCAMPUS OF RATS CAUSED BY CHRONIC OXIDATIVE STRESS

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Abstract—The aim of this study was to analyze the effects of chronic oxidative stress on mitochondrial function and its relationship to progressive neurodegeneration in the hippocampus of rats chronically exposed to ozone. Animals were exposed to 0.25 ppm ozone for 7, 15, 30, or 60 days. Each group was tested for (1) protein oxidation and, manganese superoxide dismutase (Mn-SOD), glutathione peroxidase (GPx) and succinate dehydrogenase (SDH) activity using spectrophotometric techniques, (2) oxygen consumption, (3) cytochrome *c*, inducible nitric oxide synthase (iNOS), peroxisome proliferator-activated receptor γ Co-activator 1α (PGC-1α), B-cell lymphoma (Bcl-2), and Bax expression using Western blotting, (4) histology using hematoxylin and eosin staining, and (5) mitochondrial structure using electron microscopy. Our results showed increased levels of carbonyl protein and Mn-SOD activity after 30 days of ozone exposure and decreased GPx activity. The SDH activity decreased from 7 to 60 days of exposure. The oxygen consumption decreased at 60 days. Western blotting showed an increase in cytochrome *c* at 60 days of ozone exposure and an increase in iNOS up to 60 days of ozone exposure. The expression of PGC-1α was decreased after 15, 30, and 60 days compared to the earlier time Bcl-2 was increased at 60 days compared to earlier time points, and Bax was increased after 30 and 60 days of exposure compared to earlier time points. We observed cellular damage, and mitochondrial swelling with a loss of mitochondrial cristae after 60 days of exposure. These changes suggest that low doses of ozone caused mitochondrial abnormalities that may lead to cell damage.

Key words: mitochondria, oxidative stress, ozone, hippocampus.

INTRODUCTION

An increase in reactive species and the failure of the body's antioxidant systems lead to a state of oxidative stress. Chronic oxidative stress is involved in the development of various degenerative diseases (Rivas-Arancibia et al., 2010; Sasaki et al., 2011).

Nitric oxide (NO) has important physiological functions in the body. Reactive oxygen species (ROS) induce the expression of inducible nitric oxide synthase (iNOS) and the oxidative metabolites of NO in the mitochondria. iNOS activates both oxidative stress and cytokines (Knott and Bossy-Wetzel, 2009). Chronic loss of the redox balance results in the uncoupling of endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS) and the overexpression of iNOS, generating an increase in the production of NO, which forms peroxynitrite in the presence of oxidative stress and contributes to an increase in both oxidative and nitrosative stress (Valko et al., 2007). Moreover, the endogenous antioxidant defenses, such as catalase, glutathione peroxidase, thioredoxin, and superoxide dismutase (SOD), play an important role in the redox balance. SOD catalyzes the reduction of superoxide radicals to hydroxyperoxide, and the enzyme manganese superoxide dismutase (Mn-SOD), which is located primarily in the mitochondria (Okado-Matsumoto and Fridovich, 2001), has important antioxidant functions. The hydrogen peroxide produced by SOD is removed by glutathione peroxidase (GPx) and catalase (Panfili et al., 1991).

The mitochondria are organelles mainly responsible for the production of ATP (Campbell and Mahad, 2011) with a complex series of enzymes called the electron transport chain (ETC), producing the energy needed for cellular functions. Research has shown that the decline in the activity of some complexes of the ETC leads to a decrease in ATP production (Szibor and Holtz, 2003); for example, decreased activity of succinate dehydrogenase (SDH) (Complex II of the respiratory

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Abbreviations: Apaf-1, apoptosis protease-activating factor; Bcl-2, B-cell lymphoma; BSA, bovine serum albumin; DDC, diethyldithiocarbamate; DNPH, 2,4-dinitrophenylhydrazine; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; eNOS, endothelial nitric oxide synthase; ETC, electron transport chain; GPx, glutathione peroxidase; H&E, hematoxylin and eosin; HEPES, 4-(2-hydroxyethyl-1-piperazineethane sulfonic acid; iNOS, inducible nitric oxide synthase; Mn-SOD, manganese superoxide dismutase; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; NBT, Nitro-blue Tetrazolium; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; PBS, phosphate saline buffer; PGC-1α, peroxisome proliferator-activated receptor γ Co-activator 1α; RC, respiratory control; SDH, succinate dehydrogenase; SOD, superoxide dismutase.

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chain) causes a decrease in ATP production, resulting in irreversible cell damage (Beal, 1998).

On the other hand, the mitochondrial biogenesis requires the coordinated expression of two cellular genetic systems: nuclear and mitochondrial. The expression of peroxisome proliferator-activated receptor γ Co-activator 1α (PGC-1α) is evidence of the energy requirements of the cell. PGC-1α has been implicated in multiple biological responses related to energy homeostasis, and if the energy levels decrease, its expression is increased (Fernandez-Marcos and Auwerx, 2011), activating mitochondrial biogenesis. Mitochondria are critical for cellular metabolism because all cells depend on the production of ATP. The formation of ATP consumes a large amount of oxygen, leading to an increase in superoxide radical formation in the mitochondria (Liu et al., 2002; Andreyev et al., 2005).

The increased production of free radicals and reactive oxygen and nitrogen species can activate different cell death pathways involving proteins in the B-cell lymphoma (Bcl-2) family. The Bcl-2 family includes both antiapoptotic (Bcl-2) and apoptotic (Bax, Bak and Bok) members (Susnow et al., 2009). Bax is essential in the mitochondrial pathway of apoptosis (Wei et al., 2001). This protein is located in the cytosol of healthy cells; activation of the apoptotic process induces its relocation to the mitochondria (Guy et al., 2001). The mechanism by which Bax causes apoptosis is proposed to be through interaction with components of the mitochondrial permeability transition pore (Desagher and Martinou, 2000). Antonsson et al. (1997) and Basañez et al. (1999) have suggested that Bax can directly form pores that allow the release of pro-apoptotic proteins into the cytosol. On the other hand, formation of the mitochondrial transition pore allows the release of cytochrome *c* from mitochondria, which binds to apoptosis protease-activating factor (Apaf-1) and activates caspase pathways that play an important role in cell death (Li et al., 1997).

Previous studies by our laboratory have shown that the oxidative stress state caused by ozone produces progressive neurodegeneration in the hippocampus of rats (Rivas-Arancibia et al., 2010). The aim of our study was to analyze the effect of oxidative stress caused by chronic exposure to low ozone doses on mitochondrial function and its relationship to the process of progressive neurodegeneration in the hippocampus of male rats.

EXPERIMENTAL PROCEDURES

Animal care and handling were performed in accordance with Norma Official Mexicana NOM-036-SSA2-2002, the National Institute of Health Guidelines for Animal Treatment and the Ethics Committee of the Facultad of Medicina at the Universidad Nacional Autónoma de México.

One hundred and eighty Wistar rats weighing 250–300 g were individually housed in acrylic boxes with free access to water and food (Purina, Minnetonka, MN, USA) and kept in a clean air room. They were randomly divided into two experimental groups ($n = 36$). Group 1, the control group, was exposed to an air stream free of ozone for 30 days. In previous experiments, controls for each of the times were found to have no differences. Group 2 contained experimental animals that were exposed daily to 0.25 ppm of ozone for 4 h and were subdivided into four randomly chosen subgroups. Each subgroup ($n = 36$) received one of the following treatments: 7, 15, 30 or 60 days of ozone exposure. Immediately after the ozone exposure was completed, animals were returned to their home cages. Two hours after the last air or ozone exposure, all the animals were deeply anesthetized with 50 mg/kg of sodium pentobarbital, sacrificed, and the hippocampus of each animal was dissected and analyzed by one of the following techniques: (1) spectrophotometric techniques ($n = 6$) for evaluation of oxidized proteins, Mn-SOD activity, and glutathione peroxidase activity; (2) mitochondrial isolation and verification of mitochondrial integrity, followed by analysis of SDH activity and western blot analyses ($n = 18$) for cytochrome *c*, iNOS, PGC-1, Bcl-2, and Bax using the isolated mitochondria; (3) hematoxylin and eosin (H&E) staining ($n = 6$); and (4) electron microscopy ($n = 6$) to study the ultrastructural changes of the mitochondria.

Ozone exposure

Animals were placed inside a chamber with a diffuser connected to a variable-flux ozone generator (5 L/s). The same chamber was used for treating the control group, with airflow free of ozone. The procedure has been described elsewhere (Pereyra-Muñoz et al., 2006; Rivas-Arancibia et al., 2010).

Protein carbonyl content

Protein carbonyl content in the tissue was determined by the method of Reznick and Packer (1994). The assessment of carbonyl formation was conducted on the basis of the formation of the protein hydrazone by reaction with 2,4-dinitrophenylhydrazine (DNPH). The hippocampal homogenates were incubated overnight with 10% streptomycin sulfate to remove nucleic acids and centrifuged at 21,000*g* at 4 °C for 40 min. The hippocampus homogenates were then treated with 10 mM DNPH (in HCl 2.5 M) for 1 h at room temperature; 10% trichloracetic acid was added, and the samples were centrifuged at 2500*g* at 4 °C for 10 min. The pellet was washed three times with ethanol:ethyl acetate (1:1), dissolved with 6 M guanidine hydrochloride (in phosphate buffer 20 mM, pH 7.4), incubated for 10 min at 37 °C, and centrifuged at 5000*g* at 4 °C for 3 min to remove insoluble material. The absorbance was measured at 370 nm. The protein carbonyl content was expressed as nmol carbonyl/mg protein using the molar absorption coefficient of DNPH (22,000 M^{-1} cm^{-1}). The total protein concentration was obtained by comparison with the optical density at 280 nm in blank tubes prepared in parallel (treated only with HCl) and a standard curve of bovine serum albumin (0.25–2 mg/mL) prepared in 6 M guanidine-HCl.