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# OXIDATIVE STRESS CAUSED BY OZONE EXPOSURE INDUCES β-AMYLOID 1–42 OVERPRODUCTION AND MITOCHONDRIAL ACCUMULATION BY ACTIVATING THE AMYLOIDOGENIC PATHWAY ☆

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**Abstract—Oxidative stress is a major risk factor for Alzheimer's disease (AD) that has been suggested to be the trigger of AD pathology. However, whether oxidative damage precedes and contributes directly to the intracellular accumulation of beta amyloid 1–42 (βA42) peptide remains a matter of debate. Chronic exposure to low doses of ozone similar to the levels during a day of high pollution in México City causes a state of oxidative stress that elicits progressive neurodegeneration in the hippocampi of rats. Several reports have demonstrated that the mitochondria are among the first organelles to be affected by oxidative stress and βA42 toxicity and act as sites of the accumulation of βA42, which affects energy metabolism. However, the mechanisms related to the neurodegeneration process and organelle damage that occur in conditions of chronic exposure to low doses of ozone have not been demonstrated. To analyze the effect of chronic ozone chronic exposure on changes in the production and accumulation of the βA42 and βA40 peptides in the mitochondria of hippocampal neurons of rats exposed to ozone, we examined the mitochondrial expression levels of Presenilins 1 and 2 and ADAM10 to detect changes related to the oxidative stress caused by low doses of ozone (0.25 ppm). The results revealed significant accumulations of βA42 peptide in the mitochondrial fractions on days 60 and 90 of ozone exposure along with reductions in beta amyloid 1–40 accumulation, significant overexpressions of Pres2 and significant reductions in ADAM10 expression. Beta amyloid immunodetection revealed that there were some intracellular deposits of βA42 and that βA42 and the mitochondrial markers OPA1 and COX1 colocalized. These results indicate that the time of exposure to ozone and the accumulation of βA42 in the mitochondria of the hippocampal cells of rats were correlated. Our results suggest that the accumulation of the βA42 peptide may promote mitochondrial dysfunction due to its accumulation and overproduction.**


**Key words: ozone, oxidative stress, neurodegeneration, Alzheimer's disease.**

## INTRODUCTION

Environmental, dietary and pathological factors have been related to the generation of oxidative stress. Chronic exposure to low doses of ozone ($O_3$) similar to those one would encounter during a day of high pollution in México City and many other large cities around the world causes a state of oxidative stress that produces progressive neurodegeneration in the hippocampi of rats.

Oxidative stress is a major risk factor for Alzheimer's disease (AD) and has been suggested to be the trigger of AD pathology. However, whether oxidative damage precedes and contributes directly to the intracellular accumulation of the beta amyloid 1–42 (βA42) peptide remains unclear. Nevertheless, it has recently been shown that free radicals exacerbate the amyloid pathology of AD (Misonou et al., 2000). Previous studies from our laboratory have shown that the chronic oxidative stress caused by $O_3$ produces progressive neurodegeneration, memory deterioration, motor activity deficits, lipid peroxidation and mitochondrial dysfunction in the hippocampi of rats (Avila-Costa et al., 1999, 2001; Dorado-Martínez et al., 2001; Rivas-Arancibia et al., 2010; Rivas-Arancibia, 2011). Moreover, we have demonstrated a relationship between oxidative stress and βA42 overproduction that causes the extra- and intracellular accumulations of this peptide (Rivas-Arancibia et al., 2011). The extracellular and intraneuronal accumulations of amyloid-beta aggregates have been demonstrated to be involved in the pathogenesis of AD and to affect diverse mechanisms by interacting with or blocking different proteins and extracellular receptors, activating or inhibiting signaling pathways, and ultimately causing cell death and neurodegeneration. However, the precise mechanism of βA42 neurotoxicity is not completely understood.

There are many reports that have demonstrated that mitochondria are targets of βA42, and mitochondrial dysfunction in AD is well documented (Misonou et al., 2000; Manczack et al., 2006; Horowitz and Greenamyre, 2010; Moreira et al., 2010; Rivas-Arancibia et al., 2011).

Previous studies have shown that βA42 can bind to a great number of proteins and extracellular and intracellular macromolecules that affect normal neuronal function due to increases in the production of hydrogen

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*Abbreviations:* AD, Alzheimer's disease; APP, amyloid precursor protein; DAB, 3,3-diaminobenzidine; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MPT, membrane permeability transition; TBS-T, Tris buffer solution; WB, Western blot; βA42, beta amyloid 1–42.

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peroxide, the formation of toxic free radicals, disturbances in $Ca^{2+}$ homeostasis and pathological activation or disruption of neuronal signal transduction pathways (Butterfield and Boyd-Kimball, 2004; Huang et al., 2004; Blurton-Jones and LaFerla, 2006; Demuro et al., 2010; Hernández-Zimbrón and Rivas-Arancibia, 2014). More importantly, mitochondrial dysfunction occurs early in AD, and several hypotheses regarding Aβ mitotoxicity have recently been proposed (Bossy-Wetzel et al., 2004; Canevari et al., 2004; Tillement et al., 2011). The mechanisms that are altered by the βA42 peptide include the following: (1) promotion of the opening of the membrane permeability transition pores (MPT) in isolated brain and liver mitochondria (Moreira et al., 2002), which inhibits respiration and key enzymatic activities (Casley et al., 2002; Tillement et al., 2006); (2) elicitation of an imbalance in mitochondrial fission and fusion that results in mitochondrial fragmentation and an abnormal mitochondrial distribution (Wang et al., 2009; Santos et al., 2010); (3) the βA42 peptide inducing the inhibition of cytochrome c oxidase (also known as respiratory chain complex IV, CcOX, COI or cox) activity in isolated rat and amyloid precursor protein (APP) transgenic mouse brain mitochondria; and (4) copper1-dependent inhibition of human CcOX by dimeric βA in mitochondria from cultured human cells has also been observed (Casley et al., 2002; Hernández-Zimbrón et al., 2012). Together, these events contribute to mitochondrial and neuronal dysfunction.

Further, another group demonstrated that the amyloid beta peptide alcohol dehydrogenase (ABAD)-βA interaction promotes mitochondrial dysfunction and that the inhibition of this interaction reduces βA accumulation and improves mitochondrial function in a mouse model of AD (Lustbader et al., 2004; Takuma et al., 2005; Yan and Stern, 2005; Yao et al., 2011).

Our aim was to demonstrate that the chronic exposure of rats to $O_3$ at 0.25 ppm would cause imbalances in the production of beta amyloids 1–40 and 1–42 that would lead to the progressive accumulation of βA42 peptide in the mitochondria.

## EXPERIMENTAL PROCEDURES

### Animals and animal care

Seventy-two (72) male Wistar rats weighing 250–300 g were individually housed in acrylic boxes within a clean-air box, and food was provided *ad libitum* (NutriCubo, Purina, USA). The control and treated rats were maintained in a temperature- and humidity-controlled environmental bioterium. The animals were maintained and treated in accordance with the Norma Official Mexicana NOM-036-SSA 2-2002, the National Institutes of Health Guidelines for Animal Treatment and the Ethics Committee of the Faculty of Medicine at the National Autonomous University of México.

### General procedure

The rats were randomly separated into six experimental groups ($n$ = 12 per group). Group 1 was composed of animals that were exposed daily to a clear air stream free of $O_3$ for 4 h, and groups 2, 3, 4, 5, and 6 were composed of animals that were exposed for to $O_3$ for 7, 15, 30, 60, and 90 days, respectively. The experimental groups were exposed to 0.25 ppm of $O_3$ for 4 h daily. At the end of the treatment with $O_3$, each group was further separated into half (i.e., six rats per subgroup). One of these subgroups was used for immunohistochemical analyses, and the other was used for cellular fractionation.

### $O_3$ exposure

The animals were placed inside a chamber with a diffuser connected to a variable flux $O_3$ generator (5 L/s) for 4 h daily. This procedure has previously been described (Rivas-Arancibia et al., 1998). Filtered air was used by the $O_3$ generator to produce ozone. The $O_3$ production levels were proportional to the current intensity and to the air flux. The monitor HC-400 Ver. 2.38 0–15 ppm (PCI Ozone & Control System Monitor, Inc. West Caldwell, NJ, USA) was used to measure the $O_3$ concentration inside the chamber throughout the experiment to maintain a constant $O_3$ concentration.

A similar chamber was used for the control group, but $O_3$-free air flux was administered over 30 days. Two hours after the final exposure to clean air or $O_3$, the animals from each group were deeply anesthetized with sodium pentobarbital (50 mg/kg ip; Sedalpharma, Edo. de México, México) and then decapitated.

The hippocampi of six animals from each group were obtained for Western Blot (WB), and the other six animals from each group were transcardially perfused with 4% paraformaldehyde (Sigma–Aldrich Chemie, Germany) in 0.1 M phosphate buffer (J.T. Baker, NJ; PB, Tecsiquim; pH 7.4) for the immunohistochemistry assays.

The brains were postfixed with 10% formaldehyde (J.T. Baker, USA) for 24 h and embedded in paraffin (McCormick, St. Louis, MO, USA). Five-micrometer sagittal slices of the brain containing the hippocampus were obtained using a microtome (American Optical) and mounted on slides.

### Subcellular fractionation

To isolate the cytosolic and mitochondrial fractions, the cells were lysed with a Dounce homogenizer in a buffer containing 20 mM HEPES (pH 7.5), 250 mM sucrose, 20 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.2 trypsin inhibitory units/ml aprotinin, and 20 μg/ml leupeptin at 4 °C. The cell lysates were centrifuged at 600*g* for 10 min to remove the unbroken cells and nuclei. The supernatants were centrifuged at 7000*g* for 10 min at 4 °C, and the resulting pellets were collected as the mitochondrial fractions.

### Western blot (WB)

The expression levels of βA42, βA40 and proteins involved in βA42 production and processing (i.e., ADAM10 and Presenilins 1 and 2) in the mitochondrial fractions were analyzed by gel electrophoresis and WB.