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Protective effect of pregnanolone against lipoperoxidation and free radicals generation induced in hypothalamus of ovariectomized rats submitted to CO_2 exposure

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Abstract

Several studies support an association between gonadal hormones and oxidative state. This study aimed to determine the consequence of the absence of ovarian hormones on the oxidative status of animals submitted to acute stress induced by CO_2 inhalation. We also evaluated the effect of pregnanolone administration upon the oxidative status in distinct brain structures of ovariectomized (OVX) rats exposed to CO_2. Female rats were divided into intact and OVX and exposed or unexposed to CO_2. Oxidative status was evaluated by 2′,7′-dichlorofluorescein (DCF) assay, assessment of malondialdehyde (MDA), as an indicator of lipoperoxidation (through the thiobarbituric acid-reactive substances assay, TBARS), and the total antioxidant reactivity (TAR). Both DCF and TBARS were increased in the hypothalamus of animals submitted to OVX and stress. Nevertheless, free radical production and MDA levels were not affected in either condition alone. In the cerebral cortex, lower MDA levels were observed in OVX animals. Pregnanolone administered to rats submitted to CO_2 + OVX resulted in reduced MDA levels and free radicals production in hypothalamus. We suggest that ovarian hormones may protect the hypothalamus against oxidative stress, particularly when the animals are submitted to challenges. Pregnanolone may protect, at least in part, the hypothalamus of OVX rats from oxidative stress.

Keywords: Pregnanolone; Ovariectomy; CO_2; Free radicals; Lipid peroxidation; Rats

1. Introduction

Reactive oxygen species (ROS), which include hydrogen peroxide and other chemical forms known as free radicals, such as superoxide anion, hydroxyl, and peroxyl radicals, are produced as part of normal and essential biological processes (Halliwell and Cross, 1994). Diverse antioxidant systems, both enzymatic and nonenzymatic, operate to control excessive levels of ROS (Halliwell and Cross, 1994). A cell is generally able to maintain an appropriate balance between oxidants and antioxidants under normal conditions. When this balance is perturbed and shifts toward oxidative stress, either by an increase in oxidants or a decrease in antioxidants, the cell becomes more susceptible to injuries, such as trauma (Sies, 1997).

The nervous tissue is highly susceptible to injury caused by lipid peroxidation, probably due to its high oxygen consumption rate and its high content of easily oxidable substrates, mainly polyunsaturated fatty acids and catecholamines (Sandhir et al., 1994). Extensive experimental evidence suggests that peroxidative degradation of neuronal membrane phospholipids occurs following central nervous system (CNS) trauma (e.g., Hall and Braughler, 1986). In addition, increased generation of ROS has been induced after exposure to stressful situations. Immobilization stress is followed by an increase in free radical levels, especially in lipoperoxidation, in plasma and many brain structures, such as the cerebral cortex, cerebellum, hippocampus, and midbrain, when compared with the unstressed controls (Sosnovsky and Kozlov, 1992; Liu et al., 1994). In addition, a decreased activity of the antioxidant

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enzymes copper/zinc superoxide dismutase and glutathione peroxidase has also been observed in the brain of rats treated with glucocorticoids (GCs), the adrenal steroids secreted during stress (McIntosh et al., 1998a). Stress may, thus, impair antioxidant defenses, leading to oxidative damage, considerably changing the balance between oxidative and antioxidant factors in brain (Sosnovsky and Kozlov, 1992; Liu et al., 1994).

Experimental evidence suggests that sex hormones play a role in oxidative stress. Recently, Proteggente et al. (2002) found that levels of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) and of other modified DNA bases are higher in males than in females. Significant effects of gender on hydrogen peroxide production in plasma have also been reported, with men having greater values than women (Lacy et al., 2000). In addition, ovariectomy (OVX) has been shown to cause an increase in peroxide production by liver and brain (Borras et al., 2003; Ozgonul et al., 2003). Estrogens have antioxidant properties and can inhibit lipid peroxidation in vitro, which might contribute directly to their neuroprotective effect (Arteaga et al., 2003), and the antioxidant mechanism of estrogen protection has been suggested to be independent of receptor binding (Sawada et al., 1998; Howard et al., 2001). Other steroids have been suggested to participate in protecting brain tissue against oxidative stress induced by different injuries. For example, dehydroepiandrosterone supplementation greatly reduces oxidative damage in synaptosomes isolated from diabetic rats (Aragno et al., 2000), and dehydroepiandrosterone sulfate added to the medium demonstrated a neuroprotective effect against ischemic neuronal injury in vitro (Kaasik et al., 2001). These studies have suggested a therapeutic value of ovarian hormones in diseases associated with oxidative stress.

Such evidence supports the association between steroid hormones, particularly estrogens, and oxidative state. Increased oxidative status may possibly happen in brain regions involved in responses to stress exposure. A situation used as a stressor in some studies is CO_2 inhalation (Barbaccia et al., 1994, 1996, 1998; Argyropoulos et al., 2002). In an attempt to clarify the consequences of the absence of ovarian hormones upon the oxidative stress processes in distinct brain structures, we have evaluated the effect of ovariectomy and of a situation of acute stress on some parameters of oxidative stress. Therefore, the production of free radicals, the lipoperoxidation, as well as the total antioxidant reactivity (TAR) levels, were evaluated in the hypothalamus and cerebral cortex of rats. Since neurosteroids have been suggested to have some neuroprotective effects (Yao et al., 2002; Maurice et al., 2001; Weaver et al., 2000), the second experiment aimed to verify the effects of different doses of pregnanolone, administered intraperitoneally, to OVX animals submitted to stress induced by CO_2 inhalation. Our hypothesis is that exposure to stressors may increase oxidative status in brain regions involved in the stress response, and that pregnanolone may be able to prevent this effect.

2. Materials and methods

2.1. Chemicals

Thiobarbituric acid (TBA) and Trolox were obtained from Merck, 2,2′-azobis (2-amidinopropane) dihydrochloride (ABAP) was from Wako (USA), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), 2′,7′-dichlorofluorescein (DCF), trichloroacetic acid (TCA); 5-amino-2,3-dihydro-1,4-phtalazinedione (luminol), and pregnanolone (5β-pregnan-3α-ol-20-one) were purchased from Sigma (USA).

2.2. Animals and surgery

Adult female Wistar rats (supplied by Centro de Reprodução e Experimentação Animal-ICBS-UFRGS, Brazil) were used, weighing 180–230 g. Animals were housed in groups of five in a 12-h light/dark cycle (lights on at 7:00 a.m.) with water and food available ad libitum. Rats were bilaterally OVX under general anesthesia with halothane (Brot et al., 1997) at least 2 weeks prior to stress procedures. The experimental design was approved by the Committee on Animal Care and Use of the Universidade Federal do Rio Grande do Sul.

2.3. CO_2 challenge test

The exposure to acute stress (CO_2 inhalation) (Barbaccia et al., 1994, 1996, 1998; Argyropoulos et al., 2002) was carried out during the morning hours. Rats were individually placed in a hermetically closed box and exposed for 90 s to a mixture of 35% CO_2 and 65% O_2 (supplied by White-Martins, Brazil) that was delivered into the box (volume capacity of 3.6 l), at the rate of 4 l/min, measured by a GEMÜ flowmeter (GEMÜ, Brazil). Control rats were put in a similar box but not exposed to the gas mixture.

2.4. Drugs

Pregnanolone (Sigma) was used in this study. Pregnanolone was prepared as a suspension in a vehicle of saline containing 0.5% Tween 80 (Reddy and Kulkarni, 1997), at doses of 1 and 10 mg/kg (Wieland et al., 1995; Rodgers and Johnson, 1998). Rats were injected intraperitoneally, 1 ml/kg, 30 min before exposure to CO_2. The control group received the vehicle.

2.5. Tissue preparation

Rats were killed by decapitation 30 min after CO_2 inhalation. The hypothalamus and frontal cortex were dissected out and instantly placed in liquid nitrogen and stored at −70 °C until biochemical measurements, when the tissues were homogenized in 10 volumes of ice-cold phosphate buffer (0.1 M, pH 7.4) containing 140 mM KCl and 1 mM EDTA. The homogenate was centrifuged at 960 × *g* for 10 min, and the

supernatant was used for the determination of malondialdehyde (MDA) level, free radical production, and TAR.

2.6. 2′,7′-Dichlorofluorescein assay

To assess the ROS formation, DCFH-DA was used as a probe. An aliquot of the sample was incubated with DCFH-DA (100 μM) at 37 °C for 30 min; chilling the reaction mixture in ice terminated the reaction. The formation of the oxidized fluorescent derivative (DCF) was monitored at excitation and emission wavelengths of 488 and 525 nm, respectively, using a fluorescence spectrophotometer (Hitachi F-2000). The free radical content was quantified using a DCF standard curve and results were expressed as picomoles of DCF formed per milligram of protein. All procedures were performed in the dark, and blanks containing DCFH-DA (no homogenate) and homogenate (no DCFH-DA) were processed for measurement of autofluorescence (Driver et al., 2000; Sriram et al., 1997).

2.7. Assay of lipid peroxidation

The assessment of MDA was used as an indicator of lipoperoxidation. The aldehyde groups react with two molecules of TBA at low pH and high temperature to form a pink-colored complex; the results were expressed as MDA equivalents per milligram of protein. The formation of TBA-reactive substances (TBARS) was based on the methods

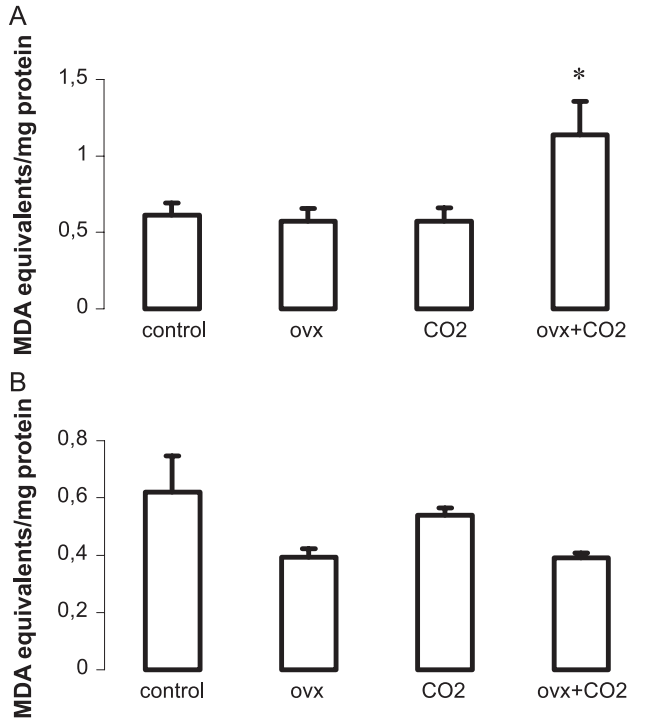

Fig. 1. Effect of CO_2 exposure on lipoperoxidation levels, using TBARS assay, on the hypothalamus (A) and cerebral cortex (B) of intact and OVX female rats. Results are expressed as mean ± S.E.M. of MDA equivalents per milligram of protein ($n = 5$ animals per group). In the hypothalamus (A), there was a significant interaction between OVX and CO_2 exposure [two-way ANOVA, $F(1,16) = 6.52$, $P < .05$]. * Significant difference from other groups (Duncan multiple range test, $P < .05$). In the cerebral cortex (B), there was a significant effect of OVX [two-way ANOVA, $F(1,16) = 8.404$, $P < .02$].

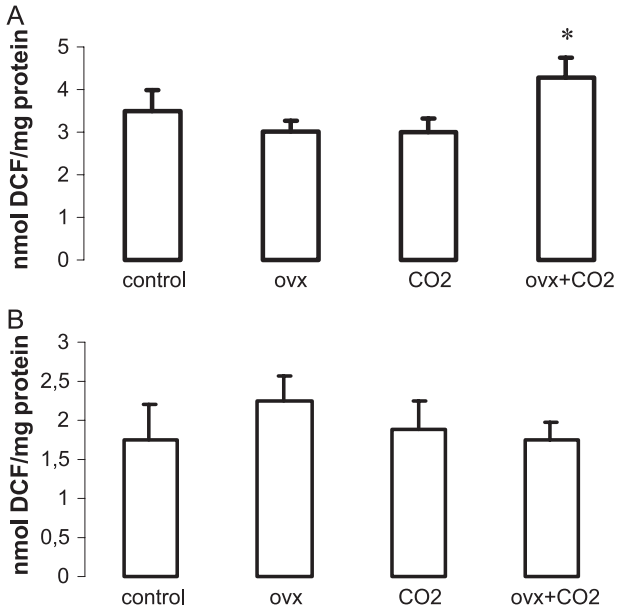

Fig. 2. Effect of CO_2 exposure on free radicals production, using DCFH-DA as a probe, on the hypothalamus (A) and cerebral cortex (B) of intact and OVX rats. Results are expressed as mean ± S.E.M. of nanomoles DCF per milligram of protein ($n = 5$ animals per group). In hypothalamus (A), there was a significant interaction between OVX and CO_2 exposure [two-way ANOVA, $F(1,16) = 5.22$, $P < .05$]. * Significant difference from just OVX and just CO_2-exposed groups (Duncan multiple range test, $P < .05$).

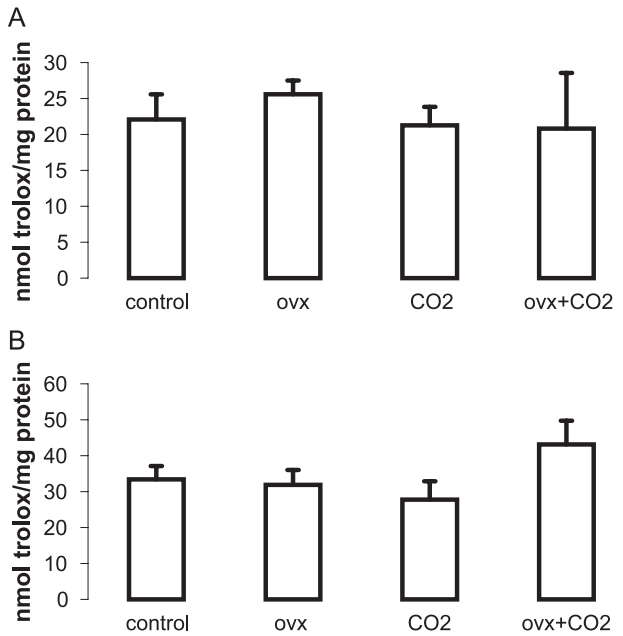

Fig. 3. Effect of CO_2 exposure on TAR on the hypothalamus (A) and cerebral cortex (B) of intact and OVX female rats. Results are expressed as mean ± S.E.M. of equivalents of Trolox per milligram of protein ($n = 5$ animals per group). A two-way ANOVA showed no effect of any treatment ($P > .05$).

described by Buege and Aust (1978). Aliquots of samples were incubated with 10% TCA and 0.67% TBA. The mixture was heated (15 min) in a boiling water bath. Afterward, *n*-butanol was added and the mixture was centrifuged. The organic phase was collected to measure fluorescence at excitation and emission wavelengths of 515 and 553 nm (Yagi, 1998), respectively, and 1,1,3,3-tetramethoxypropane was used as standard.

2.8. Total antioxidant reactivity assay

The reaction mixture contained 2 mM ABAP and 6 mM luminol in glycine buffer. TAR values were determined by assessing the initial decrease of luminescence, calculated as the ratio I_0/I, where I_0 is the chemiluminescence in the absence of additives and I is the chemiluminescence after addition of 20 nM Trolox or the samples (1 μl) (Lissi et al., 1995). TAR values were expressed as equivalents of Trolox concentration per milligram of protein.

2.9. Protein assay

The total protein concentrations were determined using the method described by Lowry et al. (1951) with bovine serum albumin as the standard.

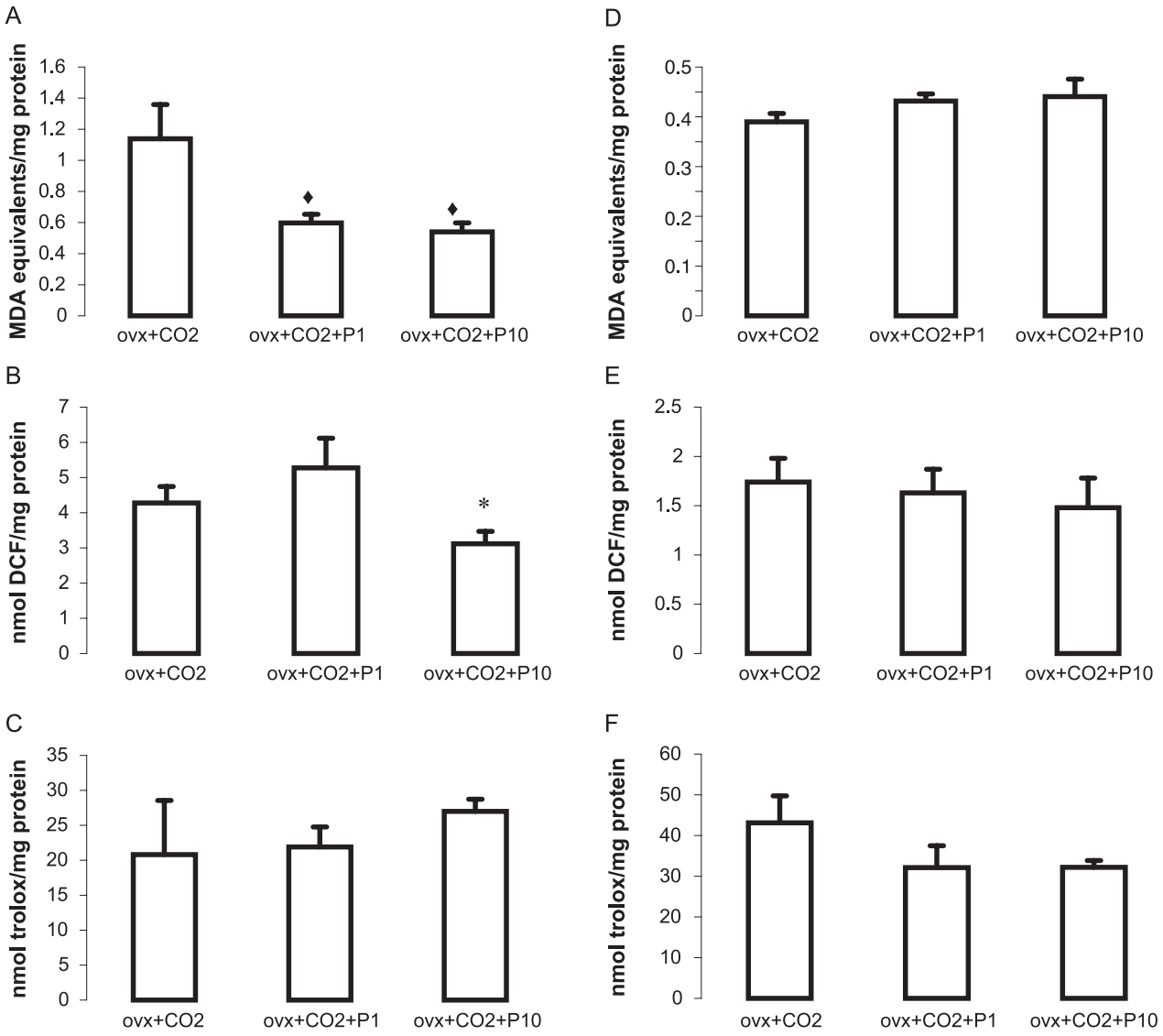

Fig. 4. Effect of intraperitoneal pregnanolone (1.0 and 10 mg/kg; P1 and P10) or vehicle administration, 30 min before CO_2 exposure, on lipoperoxidation levels, using TBARS assay (A and D), on free radicals production, using DCFH-DA as a probe (B and E), and on TAR, in OVX female rats, on the hypothalamus (A–C) and cerebral cortex (D–F). Results are expressed as mean ± S.E.M ($n=5$ animals per group). ♦Significantly different from control (OVX + CO_2 + vehicle) [ANOVA, $F(2,12)=5.92$, $P<.05$, followed by Duncan's multiple range test, $P<.05$]. * Significantly different from control [ANOVA, $F(2,12)=4.39$, $P<.05$, followed by Duncan's multiple range test, $P<.05$].

2.10. Statistical analysis

Data were analyzed using analysis of variance (ANOVA) with post hoc analysis performed using Duncan's multiple range test. A difference was considered significant when $P < .05$. Results are expressed as mean ± standard error of the mean (S.E.M.).

3. Results

In the hypothalamus, a two-way ANOVA showed a significant interaction between OVX and CO_2 exposure both in the generation of free radicals [$F(1,16) = 5.22$, $P = .036$] and in lipid peroxidation [$F(1,16) = 6.52$, $P = .022$], since both parameters were increased in animals submitted to both treatments (Figs. 1A and 2A), with no effect of the treatments when administered alone. No effect was observed in TAR in these animals ($P > .05$ for both treatments; no interaction was observed) (Fig. 3A). On the other hand, in the cerebral cortex, there was an effect of OVX (two-way ANOVA [$F(1,16) = 8.404$, $P = .010$] (Fig. 1B), since animals that had their ovaries removed presented lower levels of TBARS. No effect of CO_2 exposure was observed in this parameter. Additionally, no effect of any of the treatments was observed upon any of the other parameters of oxidative stress measured (Figs. 2B and 3B).

The second experiment aimed to verify the effects of different doses of pregnanolone, administered intraperitoneally, to OVX animals submitted to CO_2 inhalation. Results are shown in Fig. 4. In the hypothalamus, there was a significant effect of pregnanolone administration on TBARS when both doses (1 and 10 mg/kg) of pregnanolone significantly decreased TBARS levels compared with the group not receiving pregnanolone [ANOVA, $F(2,12) = 5.92$, followed by Duncan's multiple range test, $P < .05$]. Considering DCF evaluation in this structure, there was a decrease in DCF after the administration of the higher dose of pregnanolone when compared with the group receiving no hormone [ANOVA, $F(2,12) = 4.39$, followed by Duncan's multiple range test, $P < .05$]. There were no differences in TAR after pregnanolone administration. In the cerebral cortex, there was no effect of pregnanolone administration in any of the parameters evaluated, as is displayed in Fig. 4D–F.

4. Discussion

The results of the present study support the hypothesis that exposure to an acute stress situation may leave cerebral regions related to the stress response more susceptible to an increase in the oxidative status. There was an association between ovarian hormones and oxidative state, since OVX favored the increase in TBARS levels and DCF production induced by CO_2 inhalation. This effect, however, is specific to localized brain areas, since increased generation of free radicals and increased lipid peroxidation were observed just in the hypothalamus; in the cerebral cortex, lipoperoxidation was decreased after ovariectomy, while no effect was observed after exposure to CO_2 in the parameters evaluated.

CO_2 inhalation is a peculiar stressor in that CO_2 by itself may promote oxidative reactions. Carbon dioxide interacts both with reactive nitrogen species and ROS. Its net effect is prevention or induction of oxidative damage, depending on the medium, aqueous or nonpolar (Vesela and Wilhelm, 2002). CO_2 protects superoxide dismutase against oxidative damage induced by hydrogen peroxide. However, in this reaction, carbonate radicals are formed, which can propagate the oxidative damage (Vesela and Wilhelm, 2002). Nevertheless, in the conditions of the present experiments, CO_2 inhalation by itself did not alter lipid peroxidation or free radical production in control rats.

With respect to oxidative stress, it has been shown that the brain exhibits regional vulnerability to many insults (Baek et al., 1999; Babu and Bawari, 1997; Musavi and Kakkar, 1998; Cardozo-Pelaez et al., 1999). This differing vulnerability could be attributed to a greater antioxidant capacity and/or to the presence of higher activity of antioxidant enzymes in these areas (Lores Arnaiz et al., 1998). These findings also provide evidence to support the idea that stress produces oxidants and that oxidative damage in stress could contribute to degenerative diseases (Emerit et al., 2004). GCs have been shown to increase the vulnerability of different brain regions to metabolic insults, potentially by altering the neuronal defense capacity against oxidative damage (McIntosh et al., 1998a,b). The impairment of antioxidant enzyme defenses in several brain structures has been reported as a possible component of GC-mediated neuroendangerment (McIntosh et al., 1998a,b).

This difference in the sensitivity to the effects of CO_2 exposure observed in animals submitted or not to OVX may be due to the possibility that sexual hormones may influence the response to oxidative stress. Effects of sex hormones on oxidative stress have been reported, particularly estradiol, which has been shown to have antioxidant and neuroprotective properties (Lacort et al., 1995; Niki and Nakano, 1990; Sawada et al., 1998; Howard et al., 2001; Lacy et al., 2000; Ozgonul et al., 2003). The present results, however, suggest that, in some circumstances, ovarian hormones may have prooxidant effects. Other reports from the literature have shown that estrogens may increase lipoperoxidation in some organs (Gomez-Zubeldia et al., 2002; Wyllie and Liehr, 1997). The prooxidant capabilities of some estrogens may be a consequence of the reduction of metal ions, such as Cu(II) to Cu(I), initiating lipid peroxidation. In contrast, at high concentration, the scavenging of oxygen radicals may predominate over lipid peroxidation (Markides et al., 1998). Therefore, in some structures, high and low levels of estrogens could have different effects concerning lipid peroxidation.

Exactly how decreased lipoperoxidation occurs in the cortex of OVX animals, in the absence of any alteration in

free radicals content (evaluated by the DCF test) is not clear. It is possible that, in rats not submitted to OVX, ovarian hormones affect free radicals production in a cyclic way, and since this alteration would not be constant, it is not observed when the animals are sacrificed. Altered lipoperoxidation may still be present, since it may not be so easy to recover from damage to lipids.

In this study, a previous administration of pregnanolone was able to reduce oxidative stress parameters in hypothalamus when OVX animals were exposed to CO_2 inhalation.

Reduced pregnane steroids, such pregnanolone, are potent neuromodulators, which are able to affect a number of membrane receptors, including gamma-aminobutyric acid-A, *N*-methyl-D-aspartate, 5-hydroxytryptamine-3, and sigma-1 receptors, modulating the responses to stress, anxiety, and depression (Maurice et al., 2001; Engel et al., 2001; Engel and Grant, 2001). Therefore, the neuroprotective effect of pregnanolone, preventing stress-induced increases in DCF production and lipoperoxidation, could occur through its modulation of some of these systems.

We conclude that ovarian hormones may protect the hypothalamus against oxidative stress, particularly when the animals are submitted to challenges, such as exposure to acute stress. The effects of acute stress on the induction of oxidative stress, on the other hand, vary according to the brain structure analyzed. In addition, the neuroactive steroid pregnanolone may protect, at least in part, the hypothalamus of OVX rats from oxidative stress, when these animals are submitted to acute stress.

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