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Oxidative stress in the brain of reproductive male rats during aging

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ABSTRACT

Reproduction alters the male physiology. We performed a comprehensive study to examine oxidative stress in the brains of male rats with (experienced) or without (naïve) reproductive activity during aging. Oxidative stress was assessed by measuring the activity of catalase, glutathione peroxidase, superoxide dismutase, glutathione S-transferase, aconitase, and aconitase reactivated, and by measuring lipid peroxidation, protein carbonylation, nitrite and nitrate levels, vitamin C levels, and glutathione (total, reduced, oxidized forms) levels in brain tissue, as well as testosterone and estradiol levels in serum. Reproductively active animals exhibited increased testosterone levels and aconitase activity, suggesting an increased metabolism. Increased antioxidant enzyme activities and increased levels of antioxidant compounds were observed, yet damage to biomolecules was also observed in experienced rats. During aging changes in oxidative stress were observed. We found higher activities of antioxidant enzymes, higher amounts of antioxidants, and more damage at six months of age among experienced animals than among naïve animals. Similar antioxidant activities and levels, and damage were found between the groups at twenty-four months of age. These results add comprehensive data regarding changes in oxidative stress during aging, and suggest an explanation for the costs of reproduction.

1. Introduction

Reproduction causes changes in male vertebrates, including morphological, behavioral, and physiological alterations (Miles et al., 2007). Many of these changes are related to brain functions, but some processes remain unclear. Reproductive success incurs some metabolic costs, and previous studies have suggested that reproduction causes oxidative stress. Most studies concerning reproductive activity and oxidative stress have been restricted to invertebrates such as *Drosophila melanogaster* (Wang et al., 2001) and *Caenorhabditis elegans* (Harshman and Zera, 2006), and few studies have addressed vertebrates (Alonso-Alvarez et al., 2004; Arenas-Ríos et al., 2007; Dowling and Simmons, 2009; Wiersma et al., 2004); thus, there is a need for studies addressing more diverse taxa. Studies have typically been limited either to females (lactation, number of litters, size of litter), or have related to male behavior (Hull and Dominguez, 2007; Speakman, 2008).

Behavioral stress in males during reproductive activity can be caused by: (1) contact with a female in heat, which cause alterations in male behavior and physiology; (2) some sexual acts, a kind of exercise, with release of hormones which change male behavior and physiology; (3) refusal of a pregnant female, a kind of social stress for the male; (4) aggressiveness on the part of a female with a brood, a very hard social stress for the male; and (5) brood removal, resulting in better social contact between male and female, restarting the cycle.

In males, steroid hormone concentrations change as a consequence of reproductive behavior and during aging. Testosterone is considered to be a pro-oxidant and estradiol to be an antioxidant compound (Halliwell and Gutteridge, 2007).

The free radical theory of aging is regarded as an important explanation for the aging process (Harman, 1956; Rattan, 2006). This theory defends the importance of the imbalance between reactive oxygen and nitrogen species and antioxidant defenses in the aging process over the lifespan of an animal, resulting in senescence and, ultimately, death.

Mitochondria and peroxisomes are the main source of reactive oxygen species (ROS). This endogenous ROS production can cause oxidative damage such as protein modification, lipid peroxidation, and enzyme inactivation (e.g. aconitase) (Gredilla and Barja 2005; Guevara et al., 2009; Herlein et al., 2009; Razmara et al., 2007; Teixeira et al., 1998). The brain consumes large amounts of oxygen and, consequently, is vulnerable to age-related oxidative stress (Sastre et al., 2003; Serrano and Klann, 2004). Oxidative stress on the brain

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can be dangerous for the organism, as studies have suggested an influence of free radicals in human neurodegenerative diseases (Zecca et al., 2004).

Aconitase (ACO) activity has a role in controlling oxidative processes in the cell including the functioning of the tricarboxylic acid cycle, resulting in induction of oxidation of the electron transport chain components—O_2 to O_2^- reducers (Medvedeva et al., 2002). Mitochondrial ACO is a major target of oxidative damage during aging because it is proximal to the site of free radical formation (Delaval et al., 2004; Tong and Rouault, 2007; Yarian et al., 2006). ACO can be inactivated by some ROS, mainly by superoxide anion, and increased levels of superoxide anion result in a linear increase in the amount of inactive ACO (Gardner et al., 1994; Schapira, 1998; Tórtora et al., 2007). This inactivation occurs by the loss of one iron from the iron–sulfur cluster (Beinert et al., 1996; Han et al., 2005; Tong and Rouault, 2007). Aging associated with ACO inactivation has been associated with decreased life span in Drosophila (Yan et al., 1997).

Aging caused by free radicals can be retarded by antioxidant defenses involving both enzymatic and non-enzymatic mechanisms (Hackenhaar et al., 2009). A number of antioxidant enzymes that act directly on ROS are well known: catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD), and glutathione S-transferase (GST). The main non-enzymatic defense is reduced glutathione (GSH), and there are other mechanisms, such as Vitamin C (Vit C) and other vitamins (Adibhatla and Hatcher, 2010).

The aim of this work was to examine oxidative stress during aging in the brains of male rats which were reproductively actively (experienced) or reproductively inactively (naïve). We examined the levels of some hormones that alters metabolism during reproduction and aging, and which can affect oxidative stress parameters such as antioxidant enzymes and compounds, resulting in oxidative damage to molecules (see Supplementary Fig. 1 for a summary of the cellular antioxidants, oxidants, and related compounds and interactions previously studied).

2. Materials and methods

2.1. Animals

All animal studies were approved by the Animal Ethics Committee of the Universidade Federal do Rio Grande do Sul, Brazil. This study employed 80 Wistar male rats (*Rattus norvegicus*) aged three, six, twelve, and twenty-four months. At one month of age rats were divided into two groups: experienced and naïve ($n = 10$ for each age and group). Experienced rats were maintained in a box with a single female of the same age (1 male and 1 female per box). Naïve rats were grouped with other male rats without any contact with females (5 per box).

Reproduction was considered to have occurred when the females gave birth to litters. Pups were separated from the couple at 21 days of age, i.e., before the initiation of the pubertal stage, which corresponds to an age of 30–70 days for males and 33–42 days for females (Krinke, 2000). Litter size ranged from 3 to 11 pups, and each couple had 8 to 12 litters.

The animal house was kept on a 12 h light/dark cycle at a temperature of 24 ± 1 °C, and animals were provided with standard lab chow and drinking water ad libitum.

2.2. Brain dissection and processing

Animals were sacrificed according to the experimental protocol when they reached three, six, twelve, or twenty-four months of age. All animals were anesthetized using a mixture of ketamine and xylazine (i.p., 75 mg/kg and 10 mg/kg, respectively), and the body weight and length (without tail) were measured. After perfusion using a saline infusion, the brain was removed from the skull, weighed, and immediately frozen in liquid nitrogen for further analysis.

Organ processing was made as previously performed by Hackenhaar et al. (2009). Briefly, brains were processed with manual maceration. The samples were sonicated in 30 mM phosphate buffer (120 mM KCl, 100 mM PMSF, pH 7.4) and centrifuged for 10 min at 3500 g. The supernatant was transferred to a fresh tube and a second centrifugation was performed for 10 min at 15,800 g. The supernatant from the second centrifugation was used for all assays.

2.3. Obtaining blood

Before perfusion, blood was quickly collected by puncturing the left ventricle of the heart. Fresh blood was centrifuged for 4 min at 320 g, and the serum was separated for subsequent radioimmunoassay.

2.4. Hormonal level measurements

The levels of testosterone and 17β-estradiol in serum were estimated by radioimmunoassay using Testosterone RIA DSL-4100® and Estradiol RIA DSL-4100® kits (Diagnostic Systems Laboratories, Inc., Oxford, UK). All assays were independently performed in triplicate.

2.5. Enzyme activities in brain tissue

The assay to measure total superoxide dismutase (SOD) activity was based on a spectrophotometric method that monitors the inhibition of epinephrine autoxidation using absorbance at 480 nm (Misra and Fridovich, 1972). SOD activity was expressed as U/mg of protein, and 1 U was defined as the capacity of the enzyme to inhibit 50% of the epinephrine autoxidation.

GPx activity was evaluated using absorbance at 340 nm to measure the oxidation of NADPH in the presence of reduced glutathione, glutathione reductase, and tert-butyl hydroperoxide (Pinto and Bartley, 1969). GPx activity was expressed as U/mg of protein, and 1 U was defined as the capacity of the enzyme to decompose 1 μmol of NADPH/min.

CAT activity was evaluated by measuring the rate of hydrogen peroxide consumption via absorbance at 240 nm (Aebi, 1984). CAT activity was expressed as U/mg of protein, and 1 U was defined as the capacity of the enzyme to consume 1 μmol of hydrogen peroxide/min.

GST activity was measured by the GST-catalyzed reaction of 1-chloro-2,4-dinitrobenzene with reduced glutathione using absorbance at 340 nm (Tsuchida, 2000). GST activity was expressed as U/mg of protein, and 1 U was defined as the capacity of the enzyme to produce 1 μmol of GS-DNB per minute.

ACO activity was measured at 25 °C using absorbance at 340 nm over time as citrate was converted to alfa-ketoglutarate using $NADP^+$ by 2 units/ml of isocitrate dehydrogenase (Razmara et al., 2007). ACO was fully reactivated (ACOre) by incubation of the samples for 5 min with dithiothreitol, ferrous ammonium sulfate, and reduced glutathione (Razmara et al., 2007; Teixeira et al., 1998). ACO and ACOre activity were expressed as U/g of protein, and 1 U was defined as the amount catalyzing the formation of 1 nmol of isocitrate per minute (Razmara et al., 2007).

All results were normalized against total protein concentration using BSA as a standard (Bradford, 1976). All assays were independently performed in triplicate.

2.6. Oxidative damage assays in brain tissue

As an index of lipid peroxidation, malondialdehyde (MDA) levels were measured by HPLC employing a reverse-phase column (SUPELCOSIL™ LC-18-DB HPLC Column; 15 cm × 4.6 mm, 5 μm), using a flow of 1 ml/min of mobile phase (30 mM monobasic potassium phosphate (pH 3.6) and methanol in a ratio of 82.5:17.5 (v/v)); 20 μL of sample was injected (Karatepe, 2004). The absorbance of the column effluent was monitored at 250 nm. Under these conditions,