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The chance of gender dependency of oxidation of brain proteins in aged rats

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ABSTRACT

The purpose of this study is hopefully to clarify the ambiguity raised in preliminary reports as to gender dependency of oxidative damage in brain proteins. In the current study, we investigated the relation between protein hydroperoxide levels and other protein oxidation parameters. Our study also covered other oxidative stress parameters, such as 4-hydroxynonenal, malondialdehyde, and the redox index in brain tissue of the aged rats. Protein hydroperoxide, 4-hydroxynonenal, thiol levels of male rats were significantly higher than in the female rat group. On the other hand, other oxidative stress parameters were all found to be not different. We suggest that increased total thiol and protein thiol levels found in our study may point to an adaptive reaction to oxidative protein damage. We are of the conviction that the increased thiol groups that we have determined in aged male rats may be a limiting factor in propagation of protein oxidation, as the protein carbonyl, advanced oxidation protein products and nitrotyrosine levels in the brain tissue were unchanged. It has thus been found that gender indeed affects the oxidation of brain proteins and thus its aging; though the extent of the underlying mechanisms affecting brain aging and its etiology are still obscure.

1. Introduction

The brain is particularly vulnerable to oxygen radical damage, through the prevalence of oxidizable polyunsaturated fatty acids in membranes, the presence of redox active metal ions and the high metabolic requirement for oxygen. On the other hand, the brain is rich in microglial cells; an important source of oxygen and nitrogen radicals. In a post-mitotic environment such as neurons, oxidative stress can be used as a marker of age-related deterioration in cellular homeostatic mechanisms (Polidori et al., 2007). The role of reactive oxygen species (ROS) on neurodegenerative diseases (Perry et al., 2002) and on the mechanisms of cell signaling and gene expression control (Allen and Tresini, 2000) is already known. Aging is the major risk factor for Alzheimer's disease, and the accumulation of oxidized proteins in many tissues, particularly the ones formed by cells with low mitotic rate such as brain and muscle is widely considered a hallmark of aging. Neurons have a diminished capacity to deal with redox imbalance, so that even minor stresses can lead to irreversible injury (Polidori et al., 2007). Oxidative damage of proteins in Alzheimer's disease is indicated by high concentrations of several modified amino acids including protein carbonyl (PCO) and nitrotyrosine (NT) residues (Polidori et al., 2007).

Aging studies have almost exclusively been performed with male subjects. A number of studies have shown increases in the post-mitotic tissue concentrations of oxidized proteins as a function of age (Çakatay et al., 2001, 2005; Kayali et al., 2006). Interestingly, not all organs are uniformly susceptibility to protein oxidation (Çakatay et al., 2001, 2005; Kayali et al., 2006, 2007a,b). Oxidative protein damage can be caused by multiple chemical mechanisms, which may selectively target specific proteins (Stadtman, 2004; Sadineni and Schöneich, 2007). Prior studies showed that brain and heart tissue of aged male rats are particularly vulnerable to oxidative protein damage (Çakatay et al., 2001, 2005; Kayali et al., 2007. At the present time it is unknown whether such findings can be generalize to include aged females. The mechanisms underlying such gender-related differences in the lifespan of the subject and the susceptibility to certain diseases are largely unknown (Ehrenbrink et al., 2006). As protein oxidation has been implicated in the aging process and the pathogenesis of many age-related diseases, it will be interesting to know whether there is any difference between male and female aged rats as regards with their abilities to maintenance of the redox homeostasis of the proteins.

The effect of aging on brain-protein oxidation is well documented. However, none of the findings of studies specify

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the effect of gender. The purpose of this study is hopefully to clarify the ambiguity raised in preliminary reports (Dringen et al., 2000; Ehrenbrink et al., 2006; Zhu et al., 2006) as to gender dependency of oxidative damage in brain proteins.

2. Materials and methods

2.1. Experimental animals and procedures

Aged Sprague–Dawley rats (24 months) supplied by the Center for Experimental and Applied Medical Research, Istanbul University, Istanbul, Turkey were used (Çakatay et al., 2001). Rats were divided into two groups as male ($n = 10$) and female ($n = 8$). These animals were housed in conventional wire-mesh cages, four rats per cage, in a room with the temperature regulated at 21 ± 1 °C, humidity 45–50%, and light/dark cycles (12 h). All animals were given *ad libitum* access to natural food, and water by drinking bottle throughout the course of the experiment. They were fed a standard laboratory diet. The experimental procedure used in this study met the guidelines of the Animal Care and Use Committee of Istanbul University.

2.2. Preparation of tissue samples

All animals were anesthetized by a mixture of ketamine and xilazine (i.p. 75 and 10 mg/kg respectively). The rats were decapitated between 9 and 10 a.m., and the brain was quickly removed, washed in cooled 0.15 M NaCl, placed on an ice-cold plate. The brain tissue (except cerebellum, pons, and medulla oblongata) was immediately frozen in liquid N_2 until assay. Brain tissue (200 mg) was homogenized by hand in 2 ml of homogenizing buffer (100 mM KH_2PO_4–K_2HPO_4, pH 7.4, plus 0.1% (w/v) digitonin) in a glass homogenizer to avoid disruptions of nuclear membranes. In this way, contamination by nucleic acids was minimized (Çakatay et al., 2001). Brain homogenates from rats were centrifuged at 5000 × *g* for 10 min, and various analyte determinations were performed in the supernatant fraction.

2.3. Analytical methods

All centrifugation procedures were performed with a refrigerated centrifuge (Jouan G 412). Protein hydroperoxides (P-OOH), PCO, advanced oxidation protein products (AOPP), total thiol groups (T-SH), nonprotein thiol groups (Np-SH), glutathione (GSH), 4-hydroxynonenal (4-HAE), and malondialdehyde (MDA) levels were measured by a spectrophotometer (Heraeus 400, Kendro Laboratory Product, Osterode, Germany). NT measurements were performed by an ELISA system (Organon Teknika, Boseind, The Netherlands).

All reagents were analytical grade. Deionized water was used in analytical procedures. All reagents were stored at +4 °C. The reagents were equilibrated at room temperature for 0.5 h before use.

2.3.1. Assay of protein hydroperoxide levels

P-OOHs were measured by the guanidine-perchloric acid-ferric-xylenol orange method (G-PCA-FOX) (Kayali et al., 2007a). The assay is based on the oxidation of Fe^{2+} by peroxides in the presence of the dye xylenol orange that gives a colored complex with the Fe^{3+} generated. The Fe–XO complex can be measured in the visible absorbance range (560 nm). Proteins were precipitated from 100 μl of the supernatant fraction of the homogenate by the addition of 500 μl of 0.2 M ice-cold PCA. Samples were kept on ice for 5 min and centrifuged at 6500 × *g*. The precipitated proteins were dissolved in 1100 μl of 6 M guanidine hydrochloride. The protein solution was then washed twice with chloroform containing 4 mM butylated hydroxytoluene. After mixing of the washed protein solution, xylenol orange, and ferrous solutions, the absorbance at 560 nm was read after 60 min against a blank containing 6 M guanidine hydrochloride instead of the protein solution. The molar concentration of P-OOHs in the final medium was calculated with the equation $c = A_s/\varepsilon$ using the molar absorption coefficient value of 37,000 M^{-1} cm^{-1}. The coefficients of intra- and inter-assay variations for P-OOH assay were 4.4% ($n = 10$) and 7.9% ($n = 10$), respectively.

2.3.2. Determination of brain tissue protein carbonyl levels

The PCO levels in the homogenates of the brain frontal section taken from aged rats were treated following the Evans et al.'s method (1999). The supernatant was transferred to a plastic tube, left for 15 min at room temperature, and then streptomycin sulphate solution (10% w/v) was added to a final concentration of 1% to precipitate any extracted DNA which could react with DNPH (2,4-dinitrophenylhydrazine) and contribute to the carbonyl level. The solution was mixed and left to stand a further 15 min at room temperature, after which it was centrifuged at 2800 × *g* for 10 min at room temperature. The supernatant was removed and divided equally between two 10 ml plastic centrifuge tubes with the remaining supernatant being reserved for other assays. DNPH (1.6 ml, 10 mM in 2 M HCl) was added to one tube and 1.6 ml of 2 M HCl to the other tube (ratio of supernatant to DNPH solution should be 1:4, v/v). The tubes were then incubated for 1 h on a rotator at room temperature and then the protein was precipitated by adding an equal volume of 20% (w/v) trichloroacetic acid (TCA) to the tubes and leaving them for 15 min. The protein was spun down at 3400 × *g* (10 min, room temperature), the supernatant was discarded, and the pellet was washed with 1.5 ml of an ethyl acetate: ethanol mixture (1:1, v/v) to remove excess DNPH. This procedure was repeated three times. The final protein pellet was dissolved in 1.25 ml of 6 M guanidine hydrochloride and the absorbances of both solutions (DNPH and HCl) were measured at 370 nm from which the PCO content could be evaluated (PCO concentration in nmol/ml: $\Delta A_{370} \times 45.45$, where ΔA_{370} equals A_{370} of DNPH solution—A_{370} of HCl solution). Protein contents were determined on the HCl blank pellets spectrophotometrically using a Folin kit (Sigma Diagnostics, St. Louis, MO, U.S.A.). The coefficients of intra- and inter-assay variations for carbonyl assay were 4.2% ($n = 12$) and 9.6% ($n = 10$), respectively.

2.3.3. Assay of advanced protein oxidation products

Spectrophotometric determination of brain AOPP levels was performed by the modification of Witko-Sarsat's method (1992). Samples were prepared in the following way: 200 μl of the supernatant fraction of the homogenate was diluted 1:5 in PBS, 10 μl of 1.16 M potassium iodide was then added to each tube, 2 min later followed by 20 μl acetic acid. The absorbance of the reaction mixture was immediately read at 340 nm against a blank containing 2000 μL of PBS, 100 μl of KI, and 200 μl of acetic acid. The coefficients of intra- and inter-assay variations were 1.6% ($n = 10$), and 2.4% ($n = 10$), respectively. The chloramine-T absorbance at 340 nm being linear within the range of 0 to 100 μmol/l, AOPP concentrations were expressed as micromoles per liter of chloramine-T equivalents.

2.3.4. Analysis of nitrotyrosine levels

We determined brain NT levels with a commercially available ELISA kit (Northwest Life Science Specialties, Vancouver, Canada). This test is a simple "sandwich" ELISA using a plate bound capture antibody to NT and a biotinylated secondary tracer antibody. Addition of streptavidin-peroxidase followed by tetramethylbenzidine facilitates color development directly proportional to the NT present in the sample. Actual concentration of NT in samples was determined from the standard curve. The calibration curve was approximately linear in the range of 2–1500 nM when plotted on a log-linear basis.

2.3.5. Determination of total thiol, nonprotein, and protein thiol fractions

Aliquots of 250 μl of the supernatant fraction of the homogenate were mixed in 5 ml test tubes with 750 μl of 0.2 M Tris buffer, pH 8.2, and 50 μl of 0.01 M 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). The mixture was brought to 5 ml with 3950 μl of absolute methanol. A reagent blank (without sample) and a sample blank (without DTNB) were prepared in a similar manner. The test tubes were stoppered with rubber caps, color was developed for 15 min and the reaction mixtures were centrifuged at approximately 3000 × *g* at room temperature for 15 min. The absorbance of supernatants was read in a spectrophotometer at 412 nm. Molar extinction coefficient at 412 nm was 13,100 l mol^{-1} cm^{-1} in both T-SH (total thiol) and Np-SH (nonprotein thiol) procedures (Sedlak and Lindsay, 1968).

Aliquots of 250 μl of the supernatant fraction of the homogenate were mixed in 5 ml test tubes with 200 μl distilled H_2O and 50 μl of 50% TCA. The tubes were shaken intermittently for 10–15 min and centrifuged for 15 min at approximately 3000 × *g*. 200 μl of supernatant was mixed with 400 μl of 0.4 M Tris buffer, pH 8.9, 10 μl DTNB was added, and the sample was shaken on a shaker. The absorbance was read within 5 min of the addition of DTNB at 412 nm against a reagent blank with no supernatant fraction of the homogenate. The protein bound thiol groups (P-SH) were calculated by subtracting the Np-SH from T-SH (Sedlak and Lindsay, 1968).

2.3.6. Assay of glutathione and glutathione disulfide

Cayman's (Cayman Chemical Company, Ann Arbor, MI, U.S.A.) glutathione (GSH) assay kit utilizes enzymatic recycling method, using glutathione reductase, for the quantification of GSH. The thiol group of GSH reacts with DTNB (5-5′-dithiobis-2-nitrobenzoic acid, Ellman's reagent) and produces a yellow colored 5-thio-nitrobenzoic acid (TNB). The mixed disulfide, GSTNB that is concomitantly produced, is reduced by glutathione reductase to recycle the GSH and produce more TNB. The rate of TNB production is directly proportional to the concentration of GSH in the deproteinized sample. Measurement of the absorbance of TNB at 414 nm provides an accurate estimation of GSH in the sample. Glutathione disulfide (GSSG) was measured according to the kit instructions after derivatization of GSH with 2-vinylpyridine.

2.3.7. Calculation of the redox index

The brain tissue redox index (RI) is calculated according to the following formula; ([GSH] + 2[GSSG]/2[GSSG] × 100) (Pansarasa et al., 2000).

2.3.8. Malondialdehyde and 4-hydroxynonenal analysis

Lipid peroxidation status was determined in the supernatant fraction of the homogenate samples with a commercial kit (Oxis Health Products, Inc., Portland, OR, U.S.A.). The LPO-586™ assay is based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole, with MDA and 4-HNE. One molecule of either MDA or 4-HNE reacts with 2 molecules of N-methyl-2-phenylindole to yield a stable chromophore with maximal absorbance at 586 nm. Actual concentrations of MDA and 4-HNE in the samples were determined separately from the standard curves.