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Nitrosative Stress, Cellular Stress Response, and Thiol Homeostasis in Patients with Alzheimer's Disease

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ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disorder with cognitive and memory decline, personality changes, and synapse loss. Increasing evidence indicates that factors such as oxidative and nitrosative stress, glutathione depletion, and impaired protein metabolism can interact in a vicious cycle, which is central to AD pathogenesis. In the present study, we demonstrate that brains of AD patients undergo oxidative changes classically associated with a strong induction of the so-called *vitagenes*, including the heat shock proteins (HSPs) heme oxygenase-1 (HO-1), HSP60, and HSP72, as well as thioredoxin reductase (TRXr). In inferior parietal brain of AD patients, a significant increase in the expression of HO-1 and TRXr was observed, whereas HO-2 expression was decreased, compared with controls. TRHr was not increased in AD cerebellum. Plasma GSH was decreased in AD patients, compared with the control group, and was associated with a significant increase in oxidative stress markers (*i.e.*, GSSG, hydroxynonenal, protein carbonyl content, and nitrotyrosine). In AD lymphocytes, we observed an increased expression of inducible nitric oxide synthase, HO-1, Hsp72, HSP60, and TRXr. Our data support a role for nitrative stress in the pathogenesis of AD and indicate that the stress-responsive genes, such as HO-1 and TRXr, may represent important targets for novel cytoprotective strategies. *Antioxid. Redox Signal.* 8, 1975–1986.

INTRODUCTION

Alzheimer's disease (AD) affects more than 2 million Americans and is the major cause of admission to nursing homes. AD, which rarely occurs before the age of 50 years, usually becomes clinically apparent as subtly impaired cognitive function or a disturbance of affect. With time, progressive memory loss and disorientation eventually progress into dementia. Although most cases are sporadic, 5–10% or more are familial. Gross examination of the brain in AD shows a variable degree of cortical atrophy, with narrowed gyri and widened sulci most apparent in the frontal, parietal, and temporal lobes. Microscopically, the features include neurofibrillary tangles (NFTs), neurite (senile) plaques, the central core of which is amyloid-β peptide, derived from the transmembrane amyloid precursor protein (APP), amyloid angiopathy, granulovacuolar degeneration, and Hirano bodies. Importantly, all of these changes are present in the brains of nondemented older individuals but to a much lesser extent (45, 60). The finding that choline acetyl transferase is decreased by 40–90% in the cerebral cortex and hippocampus of patients with AD has led to the hypothesis that AD is consequence of a deficit in the cholinergic system (35).

AD brain has been reported to be under oxidative stress that may play an important role in the pathogenesis and progression of AD (15, 19, 48). Several lines of evidence now

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support a fundamental role for free radical–mediated event in the pathogenesis of the disease.

Amyloid-β peptide (1–42) has been shown to induce protein oxidation in both *in vitro* and *in vivo* studies (7–9, 33, 89, 95). As a result, amyloid-β peptide (1–42) has been proposed to play a central role in the pathogenesis of AD (16, 19). Although the specific mechanism of neurotoxicity induced by amyloid-β peptide (1–42) remains unknown, the chemistry of the single methionine residue at position 35 in the amyloid-β peptide (1–42) has been proposed as one of the mechanisms associated with neurotoxicity (12, 18, 75). Oxidative stress is thus emerging as a critical factor in AD (19, 56). We recently demonstrated that brain from patients with mild cognitive impairment (MCI) demonstrated increased protein oxidation and lipid peroxidation relative to aged-matched control brain (20, 46). Because many researchers consider MCI to be the transition zone between normal cognition and the dementia of early AD, these findings suggest that oxidative stress is fundamental to the progression of AD and not simply a consequence of AD. Therefore, it is imperative to develop biomarkers of oxidative stress in easily accessible tissue in living individuals to learn more about AD, to monitor drug efficacy, and to follow disease progression.

Recent evidence also suggests that nitric oxide may directly or indirectly be involved in neuronal death in AD and other neurodegenerative disorders. Neurotoxic effects of NO might be mediated by oxidative damage as well as by the activation of intracellular signaling cascades. In particular, peroxynitrite, generated by the reaction of nitric oxide (NO) with superoxide at sites of plaques, is a strong oxidant capable of inducing neuronal cell damage. Strong evidence suggests that both p21ras and p21ras-dependent MAP-kinase pathways are strongly induced in AD, and an aberrant expression of p21 is highly colocalized with an aberrant expression of NOS in this condition (50, 51).

Heme oxygenase is a stress-induced protein that has been implicated in defense mechanisms against agents that may induce oxidative injury, and its induction represents a common feature in a number of neurodegenerative diseases (53, 54, 68). Interestingly, the spatial distribution of HO-1 expression in diseased brain is essentially identical to that of pathologic expression of tau (91). In AD cortex and hippocampus, HO-1 has been shown to be overexpressed and colocalized to senile plaques and neurofibrillary tangles (73,74). Successful transduction of the human HO-1 gene into neuroblastoma cells resulted in a stable increase of HO activity associated with a dramatic decrease in the level of *tau* protein. This result demonstrates that expression of *tau* protein and HO-1 may be regulated by oxidative stress in a coordinated manner, thus enforcing the finding that this enzyme plays a pivotal role in the cytoprotection of neuronal cells (89, 91). In addition, another protein, thioredoxin reductase (TRXr), is emerging as critical vitagene involved in brain stress tolerance. As such, it has been demonstrated that TRXr plays an important role in protecting against oxidative stress and in regulating cell growth and cell death (40, 66, 92).

In the present study, the role of the vitagenes HO-1 and TRXr, along with thiol homeostasis and nitrative stress in the brain and peripheral blood of AD patients, was investigated to gain further insight into the NO system, heat shock signal pathways, and the oxidant/antioxidant balance as critical factors operating in the pathogenesis of AD. The role of nitrative stress in the pathogenesis of AD and the importance of therapeutic strategies focusing on antioxidants and/or upregulation of stress-responsive genes, such as heme oxygenase and TRXr, are discussed.

MATERIALS AND METHODS

Chemicals

5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB), 1,1,3,3-tetraethoxypropane, purified bovine blood SOD, NADH, glutathione (GSH), glutathione disulfide (GSSG), β-NADPH (type 1, tetrasodium salt), glutathione reductase (GR; Type II from Bakers Yeast), and *N*(G)-monomethyl-L-arginine (L-NMMA, a nonisoform-specific NOS inhibitor) were from Sigma Chemicals Co. (St. Louis, MO, U.S.A.). All other chemicals were from Merck (Darmstadt, Germany) and of the highest grade available.

Ethical permission

The study was approved by the local Ethics Committee, and informed consent was obtained from all patients.

Patients

Brain samples. Frozen inferior parietal samples were obtained from six AD patients and six age-matched controls for the present study from the Rapid Autopsy Program of the University of Kentucky Alzheimer's Disease Research Center (UK ADRC) that provided autopsy samples with average postmortem intervals (PMIs) of 2.1 h for AD patients and 2.9 h for control subjects (Table 1). All AD patients displayed progressive intellectual decline and met NINCDS-ADRDA Workgroup criteria for the clinical diagnosis of probable AD (60). Hematoxylin–eosin, modified Bielschowsky staining, and 10-D-5, and α-synuclein immunohistochemistry were used on multiple neocortical, hippocampal, entorhinal, amygdala, brainstem, and cerebellum sections for diagnosis. Some patients were also diagnosed with AD plus dementia with Lewy bodies, but the results of this study showed no difference between AD patients with or without the presence of Lewy bodies. Control subjects underwent annual mental status testing and semiannual physical and neurologic examinations, as a part of the UK ADRC normal volunteer longitudinal aging study and did not have a history of dementia or other neurologic disorders. All control subjects had test scores in the normal range. Neuropathologic evaluation of control brains revealed only age-associated gross and histopathologic alterations.

Plasma and lymphocytes samples. Eighteen patients (nine men and nine women), with an average age of

TABLE 1. DEMOGRAPHIC CHARACTERISTICS OF SUBJECTS

Sample (n=6)	*Age (years)*	*Gender (M/F)*	*Postmortem Interval (h)*
Control	85.8 ± 4.1	4/2	2.9 ± 0.23
AD	84.5 ± 5.2	4/2	2.1 ± 0.47

62–83 years were used in the present study. All the patients had progressive cognitive deficits, for at least 18 months. The diagnosis of probable AD was established by following the criteria of the National Institute of Neurological and Communicative Disorders and Stroke Alzheimer Disease and Related Disorders Association (NINCDS-ADRADA) (60). The evaluation of the stage of dementia was assessed by Mini Mental State Examination (MMSE), following the Italian law to receive free cholinesterase inhibitor drugs. All patients of this group were administered drugs under the supervision of specialized outpatient clinics for dementia. Five patients were classified as mild, and 13 patients, as moderate. The diagnosis was also confirmed by computed tomography (CT) or magnetic resonance imaging (MRI) scan that showed a cortical atrophy in all patients. Eighteen subjects without dementia (six men and 12 women), between 64 and 80 years were recruited as controls.

Western blot analysis

Samples of control and AD patients were analyzed for HO-1, Hsp60, iNOS, and TRXr protein expression, as well as carbonyls (DPNH), hydroxynonenals (HNE), and nitrotyrosine protein, by using a Western immunoblot technique, as described previously (26, 27). In brief, an equal amount of proteins (40 μg) for each sample was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred overnight to nitrocellulose membranes, and the nonspecific binding of antibodies was blocked with 3% nonfat dried milk in phosphate-buffered saline. Immunodetection of iNOS and protein nitrotyrosine were performed by using a polyclonal rabbit anti-iNOS antibody [sc-651, Santa Cruz (Santa Cruz, CA, USA); 1:500 dilution in phosphate-buffered saline (PBS), pH 7.5] and polyclonal rabbit anti-nitrotyrosine antibody (06–284, Upstate, Charlottesville, VA, USA), respectively. Immunodetection of HO-1, HO-2, and Hsp72 was performed by using, respectively, a polyclonal rabbit anti-HO-1 (SPA-895) and anti-HO-2 (OSA-200) antibodies (Stressgen, Ann Arbor, MI, USA, 1:2,000 dilution in PBS, pH 7.5) and a monoclonal mouse anti-Hsp70 antibody (SPA-810, Stressgen). When probed for Hsp60 and TRXr proteins, a polyclonal goat anti-HSP60 antibody (sc-1052, Santa Cruz; 1:1,000 dilution in PBS, pH 7.5), and a polyclonal rabbit anti-TRXr-1 antibody (07–613, Upstate) were used, respectively. For immunodetection of HNE, membranes were incubated for 2 h at room temperature with anti-HNE (anti-4-hydroxy-2-Nonenal Michael adducts (393205, Calbiochem, San Diego, CA). Carbonyl groups were estimated with a rabbit anti-dinitrophenyl (DNP) antibody (V0401, DAKO, Glostrup, Denmark; 1:1,000 dilution in PBS; pH, 7.5). All blots were then visualized by using a horseradish peroxidase–conjugated goat anti-rabbit or anti-mouse IgG. A goat polyclonal antibody specific for β-actin was used as a loading control (sc-1615 product of Santa Cruz; 1:1,000 dilution in PBS; pH, 7.5). Immunoreactive bands were scanned by a laser densitometer (LKB Ultroscan XL, Pharmacia, Uppsala, Sweden). Molecular weights of the proteins were determined by using a standard curve obtained with proteins of known molecular weight.

Glutathione and glutathione disulfide assay

GSH and GSSG were measured by the NADPH-dependent GSSG reductase method, as previously reported (27). Lymphocytes were homogenized on ice for 10 s in 100 m*M* potassium phosphate, pH 7.5, which contained 12 m*M* disodium EDTA. For total glutathione, aliquots (0.1 ml) of homogenates were immediately added to 0.1 ml of a cold solution containing 10 m*M* DTNB and 5 m*M* EDTA in 100 m*M* potassium phosphate, pH 7.5. The samples were then mixed by tilting and centrifuged at 12,000 *g* for 2 min at 4°C. An aliquot (50 μl) of the supernatant was added to a cuvette containing 0.5 U of GSSG reductase in 100 m*M* potassium phosphate and 5 m*M* EDTA, pH 7.5 (buffer 1). After 1 min of equilibration, the reaction was initiated with 220 nmol of NADPH in buffer 1 for a final reaction volume of 1 ml. The formation of a GSH-DTNB conjugate was then measured at 412 nm. The reference cuvette contained equal concentrations of DTNB, NADPH, and enzyme, but not sample. For assay of GSSG, aliquots (0.5 ml) of homogenate were immediately added to 0.5 ml of a solution containing 10 m*M* *N*-ethylmaleimide (NEM) and 5 m*M* EDTA in 100 m*M* potassium phosphate, pH 7.5. The sample was mixed by tilting and centrifuged at 12,000 *g* for 2 min at 4°C. An aliquot (500 μl) of the supernatant was passed at 1 drop/s through a SEP-PAK C18 Column (Waters, Framingham, MA) that had been washed with methanol followed by water. The column was then washed with 1 ml of buffer 1. Aliquots (865 μl) of the combined eluates were added to a cuvette with 250 nmol of DTNB and 0.5 U of GSSG reductase. The assay then proceeded as in the measurement of total GSH. GSH and GSSG standards in the ranges between 0 and 10 nmol and 0.010 and 10 nmol, respectively, added to control samples, were used to obtain the relative standard curves, and the results were expressed in nmol of GSH or GSSG, respectively, per mg protein.

HO activity assay

HO activity was determined at the end of each treatment, as described previously (64). In brief, microsomes from harvested cells were added to a reaction mixture containing NADPH, glucose-6-phosphate dehydrogenase, rat liver cytosol as a source of biliverdin reductase, and the substrate hemin. The reaction mixture was incubated in the dark at 37°C for 1 h and was terminated by the addition of 1 ml of chloroform. After vigorous vortex mixing and centrifugation, the extracted bilirubin in the chloroform layer was measured by the difference in absorbance between 464 and 530 nm (ϵ = 40 m*M*/cm).

Nitric oxide synthase assay

NOS activity assay was performed spectrophotometrically by exploiting the reaction of NO with oxyhemoglobin (HbO_2) to form methemoglobin, according to (72). The reaction mixture contained in a final volume of 1 ml: 1 m*M* L-arginine, 1 m*M* $CaCl_2$, 0.1 m*M* NADPH, 12 μ*M* THB4, 5 μ*M* HBO_2, 4 μ*M* FAD, 100 m*M* HEPES (pH 7.5), and 0.3 ml plasma or CSF sample. The enzyme activity was monitored by absorption spectrophotometry by following the controlled oxidation of HBO_2 to methemoglobin. The oxidation of HBO_2 to methemoglobin sensitive to L-NMMA (1 m*M*) inhibition and in the presence of 1 μ*M* SOD and catalase was followed at 411–401 nm in a double-beam spectrophotometer (Perkin-Elmer 559) with a multiple wavelength program at 22°C. NOS activity was measured in the absence and presence of (a) 0.1 m*M* aminoethyl-isothiourea (ITU), which is a specific

iNOS inhibitor (71), and (b) 1 m*M* methyl-L-arginine (L-NMMA), a nonspecific NOS inhibitor that inhibits all three NOS isoforms (44).

Determination of protein

Proteins were estimated by the BCA protein assay method (82) by using bicinchoninic acid (BCA) reagent.

Statistical analysis

Results were expressed as mean ± SEM of n experiments, each of which was performed, unless otherwise specified, in triplicate. Data were analyzed by one-way ANOVA, followed by inspection of all differences by Duncan's new multiple-range test. Differences were considered significant at $p < 0.05$.

RESULTS

Consistent with others who showed oxidative stress and altered thiol status in AD brain (19, 28–30, 86, 87), we demonstrated that peripheral lymphocytes from AD patients showed significantly decreased GSH levels ($p < 0.05$) and corresponding significantly increased GSSG levels ($p < 0.05$) (Fig. 1A and B). These changes significantly decreased the GSH/GSSG ratio in AD lymphocytes compared with controls (Fig. 1C).

Our laboratory previously demonstrated upregulation of protective proteins in cells exposed to oxidative stress (24, 68). Consistent with these prior findings, in the present study, we observed an increased expression of both the antioxidant proteins HO-1 (Fig. 2A and B) and TRXr ($p < 0.01$) (Fig. 3A and B) in the inferior parietal lobule, a region that showed elevated oxidative and nitrative stress (28–30) of AD brains compared with control brains. The increased expression of these two proteins seemed to be consequent to a strong oxidant environment because the constitutive form of heme oxygenase, HO-2, is not increased but reduced in AD brain (Fig. 2C and D), and TRXr is not elevated in cerebellum (Fig. 3C and D), a brain area that is devoid of protein oxidation and amyloid β-peptide–containing senile plaques in AD (38).

In accord with the results described for brain, HO-1 expression has been found significantly elevated in AD plasma and lymphocytes compared with control samples (Fig. 4A–D), and total HO activity was found to be significantly elevated (Fig. 4E). Elevation of HO activity in AD lymphocytes is likely due to increased HO-1 expression (Fig. 4C) occurring in response to a condition of elevated oxidative stress, because we found that HO-2 is not increased but reduced in AD lymphocytes (data not shown).

Reactive nitrogen species (RNS) generation is associated with various pathologic events that contribute to the cell and

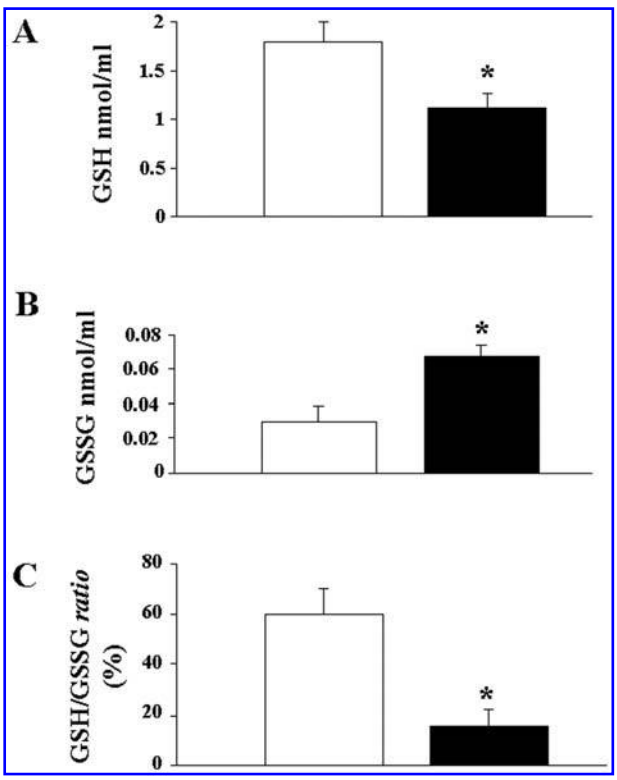

FIG. 1. Thiol status in Alzheimer's disease patients. Lymphocytes from control (white bars) and AD (black bars) patients were assayed for (**A**) GSH and (**B**) GSSG, as described in Materials and Methods. (**C**) The GSH/GSSG *ratio* has been calculated. Data are expressed as mean ± SEM of 18 patients per group. *$p < 0.05$ versus control.

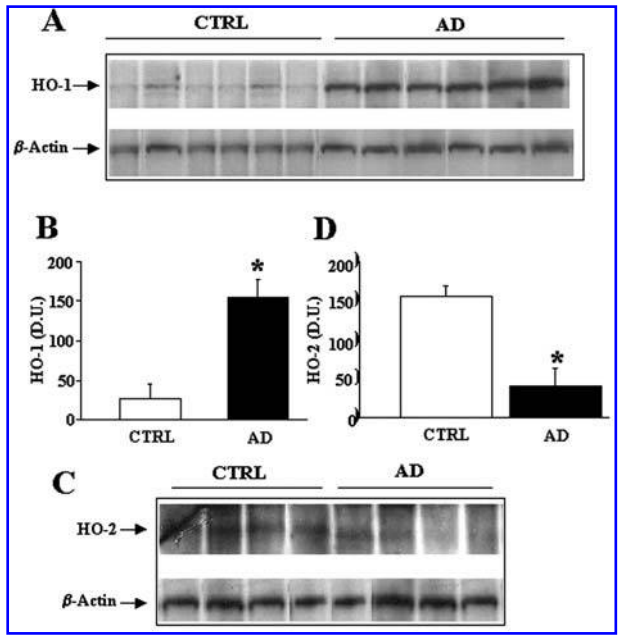

FIG. 2. HO-1 expression in Alzheimer's disease brain (inferior parietal). Brain samples from control and AD patients were assayed for (**A**) HO-1 and (**C**) HO-2 by Western blot, as described in Materials and Methods. (**B**) and (**D**) densitometric evaluation of the immunoblots shown in (**A**) and (**C**), respectively. In (**A**) and (**C**), a representative experiment is shown. In (**B**) and (**D**), data are expressed as mean ± SEM of six patients per group. *$p < 0.01$ versus control. CTRL, control; AD, Alzheimer's disease.

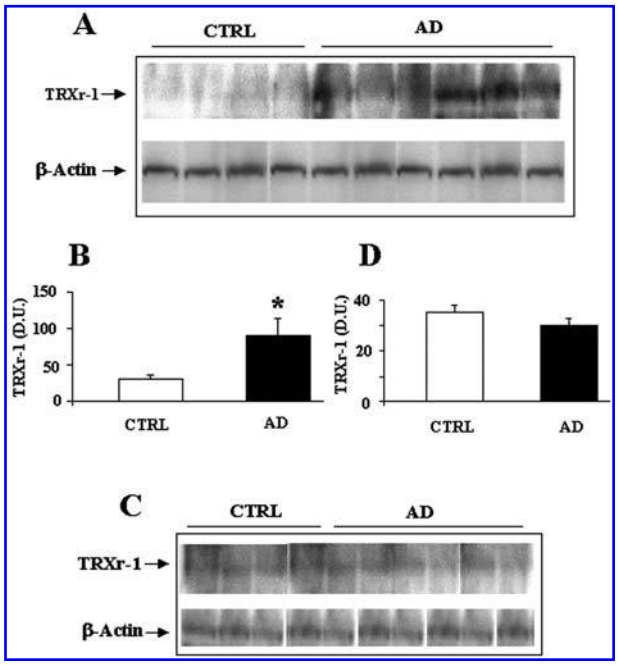

FIG. 3. TRXr in Alzheimer's disease brain. (**A**) Brain (inferior parietal) and (**C**) cerebellum samples from control and AD patients were assayed for TRXr by Western blot as described in Materials and Methods. (**B**) and (**D**), densitometric evaluation of the immunoblots shown in (**A**) and (**C**), respectively. In (**A**) and (**C**), a representative experiment is shown. In (**B**) and (**D**), data are expressed as mean ± SEM of six patients per group. *$p < 0.01$ versus control. CTRL, control; AD, Alzheimer's disease.

tissue damage accompanying neuroinflammation and degenerative damage of the brain. The latter is continually exposed to both exogenous and endogenous sources of NO and NO-derived species. Activated glia secrete RNS products of nitric oxide (NO) metabolism with superoxide radicals ($O_2^{\bullet -}$) to form peroxynitrite anion ($ONOO^-$). At physiologic pH, it protonates to its conjugate acid, peroxynitrous acid, which decomposes with a half-life of less than 1 sec. One of the fastest reactions of $ONOO^-$ is with CO_2/HCO_3^- ($3–5.8 \times 10^4$ M/s at 37°C). Together with the high concentrations of CO_2 (~1.3 m*M*) and HCO^{3-} (~25 m*M*), this reaction is the most probable pathway of $ONOO^-$ decomposition *in vivo* (61).

As far as the contribution of nitrative stress in AD, Fig. 5 shows that NOS-2 expression and activity are significantly elevated in AD lymphocytes compared with controls. Furthermore, because peroxynitrite can, in further reactions, bind to tyrosine residues (5), analysis of AD plasma and lymphocytes indicated elevated 3-nitrotyrosine levels compared with control (Fig. 6). As a corollary of the concept that during stressful conditions, proteins and lipids can undergo oxidative modifications (24, 68), in Figs. 7 and 8, we show that protein carbonyls [assessed immunochemically by detecting the hydrazone product of protein carbonyls with 2,4-dinitrophenylhydrazine (89)] as well as HNE, this latter considered a marker of lipid peroxidation, are elevated in AD plasma and lymphocytes compared with control.

We have previously demonstrated in brain cells that during nitrative stress, the induction of cytoprotective Hsp72 occurs. In view of the evidence indicating that thioredoxine reductase is functional for induction of HO-1 under conditions of nitrative stress (93), we investigated in our experimental conditions the expression of other Hsps, such as Hsp72 and Hsp60,

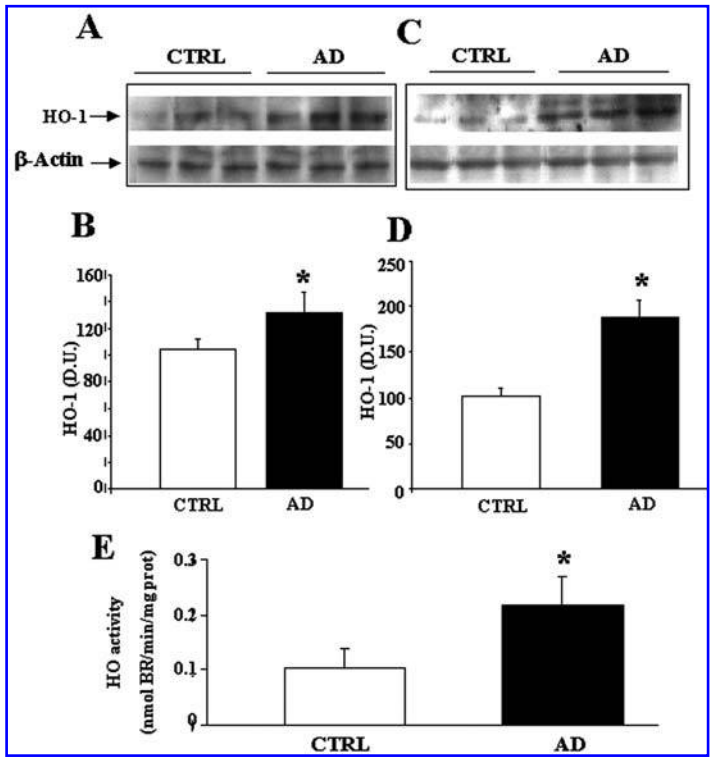

FIG. 4. HO-1 expression in Alzheimer's disease plasma and lymphocytes and HO activity in AD lymphocytes. (**A**) Plasma and (**C**) lymphocytes samples from control and AD patients were assayed for HO-1 expression by Western blot, as described in Materials and Methods. (**B**) and (**D**), Densitometric evaluation of the immunoblots shown in (**A**) and (**C**), respectively. (**E**) HO activity was measured in AD lymphocytes as described in Materials and Methods. In (**A**) and (**C**), a representative experiment is shown. In (**B**), (**D**), and (**E**), data are expressed as mean ± SEM of 18 patients per group. *$p < 0.01$ versus control. CTRL, control; AD, Alzheimer's disease; BR, bilirubin.

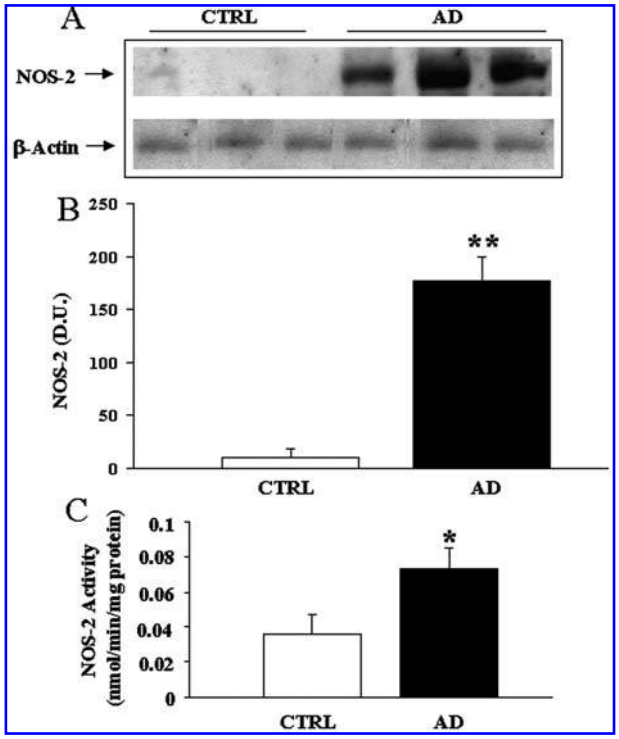

FIG. 5. NOS-2 expression and activity in Alzheimer's disease lymphocytes. Lymphocyte samples from control and AD patients were assayed for NOS-2 (**A**) expression by Western blot and (**C**) activity, as described in Materials and Methods. (**B**) Densitometric evaluation of the immunoblot shown in (**A**). In (**A**), a representative experiment is shown. In (**B**) and (**C**), data are expressed as mean ± SEM of 18 patients per group. *$p < 0.05$ and **$p < 0.01$ versus control. CTRL, control; AD, Alzheimer's disease.

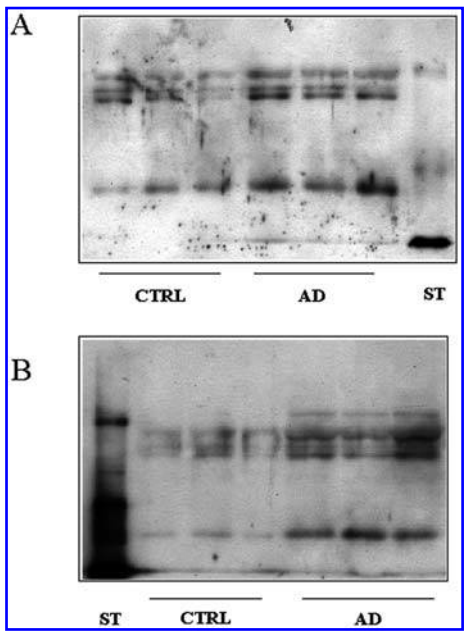

FIG. 6. Nitrotyrosine detection in Alzheimer's disease plasma and lymphocytes. AD (**A**) plasma and (**B**) lymphocyte samples were assayed for nitrotyrosine by Western blot, as described in Materials and Methods. Two representative immunoblots are shown. CTRL, control; AD, Alzheimer's disease; St, standard.

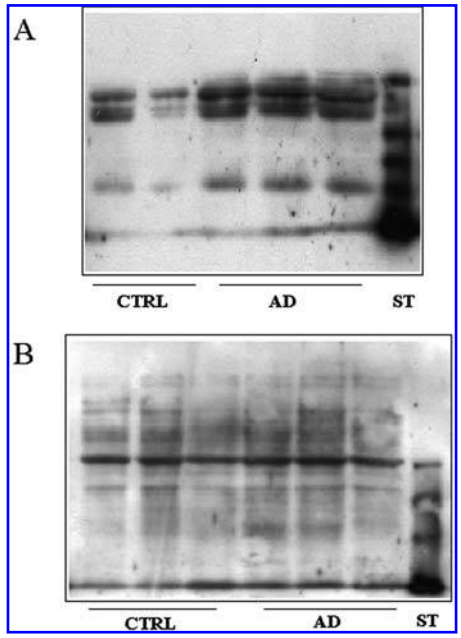

FIG. 7. Carbonyls (DPNH) detection in Alzheimer's disease plasma and lymphocytes. AD (**A**) plasma and (**B**) lymphocytes samples were assayed for carbonyls by Western blot, as described in Materials and Methods. Two representative immunoblots are shown. CTRL, control; AD, Alzheimer's disease; St, standard.

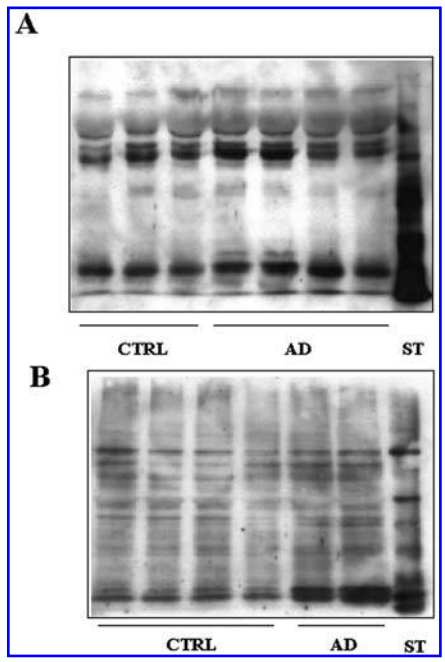

FIG. 8. Hydroxynonenals (HNE) detection in Alzheimer's disease plasma and lymphocytes. AD (**A**) plasma and (**B**) lymphocytes samples were assayed for HNE by Western blot, as described in Materials and Methods. Two representative immunoblots are shown. CTRL, control; AD, Alzheimer's disease; St, standard.

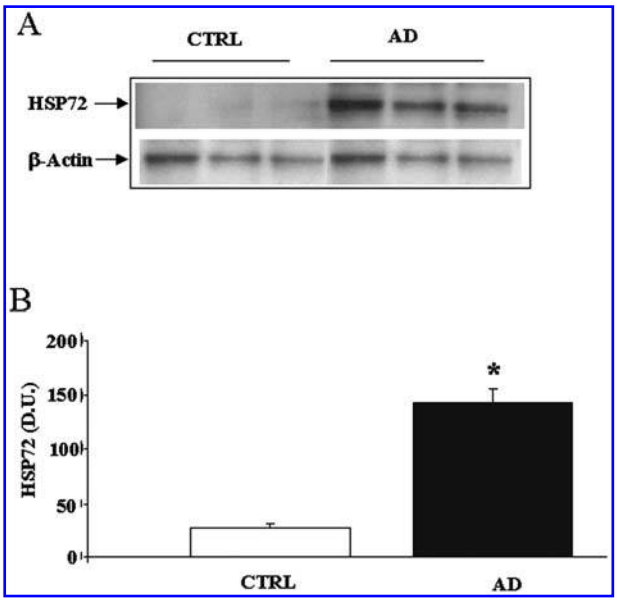

FIG. 9. HSP72 expression in Alzheimer's disease lymphocytes. (**A**) AD lymphocytes samples were assayed for HSP72 expression by Western blot, as described in Materials and Methods. (**B**) Densitometric evaluation of the immunoblot shown in (**A**). In (**A**), a representative experiment is shown. In (**B**), data are expressed as mean ± SEM of 18 patients per group. *$p < 0.01$ versus control. CTRL, control; AD, Alzheimer's disease.

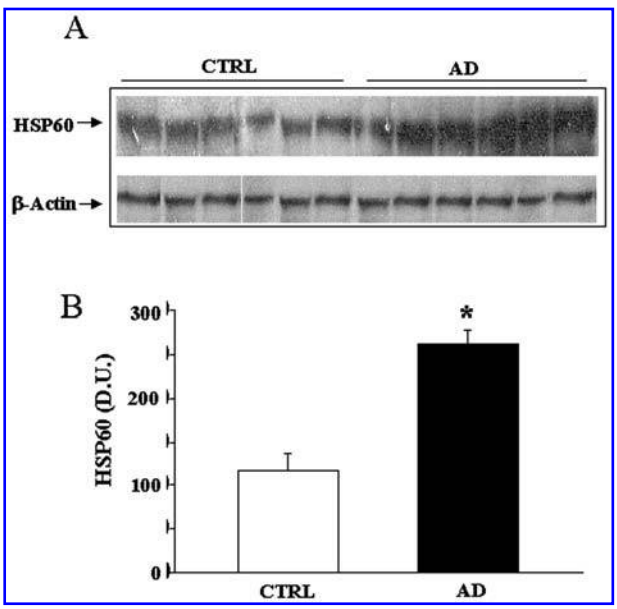

FIG. 10. HSP60 expression in Alzheimer's disease lymphocytes. (**A**) AD lymphocytes samples were assayed for HSP60 expression by Western blot, as described in Materials and Methods. (**B**) Densitometric evaluation of the immunoblot shown in (**A**). In (**A**), a representative experiment is shown. In (**B**), data are expressed as mean ± SEM of eighteen patients per group. *$p < 0.01$ versus control. CTRL, control; AD, Alzheimer's disease.

as well as TRXr. As shown in Figs. 9 and 10, both Hsp72 and Hsp60 are significantly elevated in AD lymphocytes compared with control, whereas TRXr is elevated either in AD lymphocytes or plasma, compared with control (Fig. 11).

DISCUSSION

Alzheimer disease is a progressive neurodegenerative disorder with cognitive and memory decline, speech loss, and personality changes (45). From a neuropathologic point of view, AD is characterized by intracellular neurofibrillary tangles (NFTs), extracellular senile plaques (SPs), the central core of which is amyloid β-peptide (Aβ) derived from amyloid precursor protein (APP) metabolism, and synaptic loss. AD brain has been reported to be under oxidative stress, which may play an important role in the pathogenesis and progression of AD (15, 19, 48).

Advanced glycosylation end products (AGEs) are a family of complex posttranslational modifications that are initiated by condensation of reducing sugars with protein amino groups *via* the Maillard reaction. It is evident that glycation of proteins occurs *in vivo* in aged individuals (52). Oxidative stress increases the frequency of hydroxyl radical–induced autoxidation of unsaturated membrane lipids. Reactive aldehydes, resulting by metal ion-mediated fragmentation of the lipid hydroperoxides can modify proteins through alteration of protein–protein interactions and intermolecular cross-

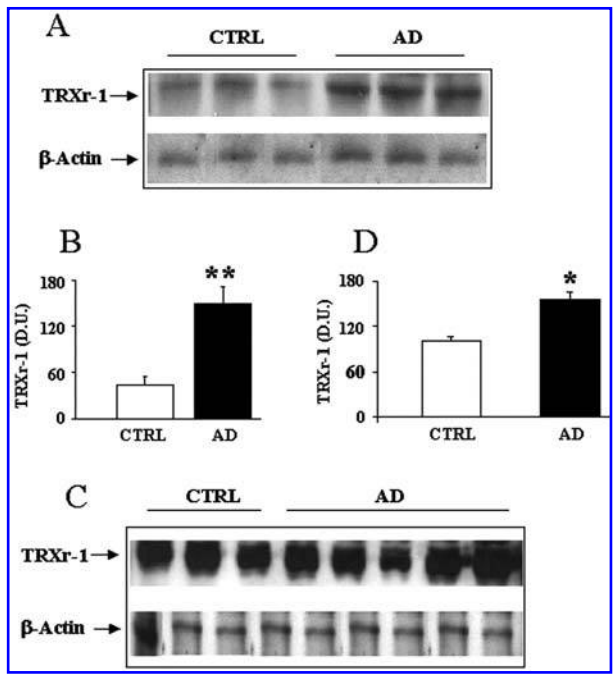

FIG. 11. TRXr expression in Alzheimer's disease lymphocytes and plasma. (**A**) AD lymphocytes and (**C**) plasma samples were assayed for TRXr expression by Western blot, as described in Materials and Methods. (**B**) and (**D**), Densitometric evaluation of the immunoblot shown in (**A**) and (**C**), respectively. In (**A**) and (**C**), representative experiments are shown. In (**B**) and (**D**), data are expressed as mean ± SEM of nine patients per group. *$p < 0.05$ and **$p < 0.01$ versus control. CTRL, control; AD, Alzheimer's disease.

linking. Age modifications and oxidative-stress mechanisms can synergistically accelerate protein damage (9, 17, 24, 33, 56, 69, 83, 88). Several other potential sources of oxidative stress were considered in the pathogenesis of AD. First, the concentration of iron, a potent catalyst of oxyradical generation, is increased in NFT-bearing neurons (79, 80). Second, increased concentrations of iron would result in increased protein modifications, which are catalyzed by metal ions and reducing sugars (81). Third, microglial cells are activated and increased in AD and represent a major source of free radicals (37, 43). Fourth, the increased lipid peroxidation and the resulting membrane disturbances, which are observed in degenerating neurons and neurites, are expected to lead to an influx of calcium, which causes destabilization of cytoskeleton and activation of specific degradative enzymes (19, 59). A decrease of complex IV activity has been reported in the cerebral cortex of individuals who died of AD (47). Although the exact mechanism for this loss of activity is not clear, it is known that this enzyme complex is particularly susceptible to oxidative damage (55, 85). In addition, evidence now suggests that NO metabolism is affected in AD. The glial-derived factor, S-100-β, which is overexpressed in many pathologic conditions, causes induction of iNOS in astrocytes associated with NO-mediated neuronal cell death in a co-culture system (42). Furthermore, amyloid-β is reported to activate NOS in a substantia nigra/neuroblastoma hybrid cell line (62). Analysis of postmortem material has revealed in AD brain the presence of nitrotyrosine, as result of the reaction of $ONOO^-$ and tyrosine residues in protein, which was not detectable in age-matched control brains (39, 67). In addition, by using antibodies specifically directed against iNOS, the presence of this isoform has been demonstrated in NFT-bearing neurons (49). Despite evidence for activation of NO metabolism in AD, analysis of the CSF nitrite + nitrate (stable end products of NO degradation) concentration revealed levels in AD patients comparable to those in controls (90). Although this observation does not dismiss a role for NO/$ONOO^-$ in the etiology of AD, it implies that formation of RNS occurs at a level that not necessarily leads to an increase in CSF RNS concentration.

Amyloid β-peptide, the principal component of senile plaques and the major neuropathologic hallmark of AD, is considered to be central to the pathogenesis of AD. β-Amyloid is a 40- to 42-amino acid peptide that accumulates in the neuritic plaques in AD. The AD brain is under extensive oxidative stress (14, 16, 19). These two observations were joined by a model to potentially account for neurodegeneration in AD brain: the β-amyloid–associated free radical oxidative stress hypothesis of brain cell death in AD (15, 57, 58, 80, 83). In this model, β-amyloid–associated free radicals initiate lipid peroxidation, protein oxidation, reactive oxygen species (ROS) formation, intracellular and mitochondrial Ca^{2+} accumulation, and eventual death of neurons. A prediction of this model is that the antioxidant vitamin E should prevent or modulate these β-amyloid–induced effects on neurons (13, 94). In agreement with this model, this free radical scavenger was shown to block amyloid-β–initiated lipid peroxidation in cortical synaptosomes (84, 94). Further, protein oxidation induced by β-amyloid in astrocyte cultures and assessed by increased protein carbonyl content was abrogated by the more soluble form of vitamin E, trolox (1).

Increasing evidence supports the role of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the pathogenesis of AD. NO has three major role in biologic systems, in that it can be (a) protective, as it has antioxidant properties and can also block expression of adhesion molecules; (b) regulatory, in that it can alter vascular tone and has a role in cell signaling, and (c) deleterious, in that it can react with superoxide to form oxides of nitrogen that can cause DNA damage, lipid peroxidation, and nitration of proteins, thus resulting in changes in three-dimensional structure of proteins and altered activities in important intracellular enzymes. Because of these multiple roles of NO in the CNS, it is not surprising that in certain models of inflammation, a decrease in NO levels can be deleterious (78). Therefore pharmacologic modulation of NOS activity in disease states such as AD must be specifically targeted, both to the specific isoforms of NOS and also to the various cell types involved. Moreover, in addition to manipulation of iNOS activity, certainly a potential role exists for pharmacologic modulators of peroxynitrite metabolism in AD. For example, it has recently been demonstrated that uric acid (a scavenger of peroxynitrite) and FeTMPS (a catalyst specific for the decomposition of $ONOO^-$) resulted in inhibition of inflammatory changes, decreased blood–brain barrier disruption, and less demyelination in mouse models of EAE (32, 41).

In this study, we demonstrated in brain, peripheral lymphocytes, and plasma that oxidative and nitrative stress is evident in AD compared with control subjects. Similar to what is demonstrated in the substantia nigra of Parkinson disease subjects, in which nigral neurons containing cytoplasmic Lewy bodies exhibited in their proximity maximal HO-1 immunoreactivity (73, 96), here we provide strong evidence that HO-1 expression is elevated in AD plasma and AD lymphocytes compared with control (Fig. 4A–D), and total HO activity is correspondingly elevated (Fig. 4E). Elevation of HO-1 expression and activity in AD is likely in response to elevated oxidative stress. This finding is consistent with evidence suggesting that the HO-1 gene is redox regulated and, similar to other antioxidant enzymes (3, 4), this occurs because it contains in its promoter region the antioxidant responsive element (ARE). Therefore, the HO-1 gene undergoes a redox-sensitive modulation by transcription factors recognizing specific binding sites within the promoter and distal enhancer regions of the HO-1 gene (2), such as those responsive to Fos/Jun [activator protein-1 (AP-1)], nuclear factor-κB (NF-κB), and the more recently identified Nrf2 proteins (3, 4). In addition, heme oxygenase-1 is rapidly upregulated by oxidative and nitrosative stresses, as well as by glutathione depletion (21, 65, 77). Given the broad cytoprotective properties of the heat shock response, strong interest now exists in discovering and developing pharmacologic agents capable of inducing the heat shock response (21–23, 25, 26).

An intracellular redox regulator that has been shown to be important for the regulation of redox-sensitive transcription factors is thioredoxin (TRX) (6, 40, 66, 76, 92). When reduced, TRX can oxidatively reactivate inactive transcription factors such as Jun, Fos, AP-1, redox factor-1 (ref-1), and Nrf-2 (93). TRX is usually located in the cytosol, but it translocates into the nucleus in response to various stimuli associated with oxidative stress. TRXr is a flavoprotein that

catalyzes the reduction of oxidized thioredoxin in a NADPH-dependent manner and contains a selenocysteine residue near the C-terminus. TRXr plays an important role in protecting against oxidative stress and in regulating cell growth and cell death. Constitutive TRXr expression has been observed in several cell types of the mammalian body, including neuronal cells after nitrosative stress. Both *in vivo* and *in vitro* studies demonstrated that TRX and TRXr have protective roles against cytotoxicity mediated by the generation of ROS. Consistently, increased vulnerability to oxidation of thioredoxin peroxidase (peroxiredoxin), together with other target proteins, such as ubiquitin carboxyl-terminal hydrolase L-1 (UCH L-1) and α/ATP synthase, have been elegantly demonstrated in the gad mouse brain by redox proteomics (31). Notably, amyloid-β peptide (1–42) *in vitro* and *in vivo* can replicate many of the oxidatively modified proteins found in AD brain (7, 8, 10, 11). In addition, an antisense oligonucleotide directed against APP leads to loss of amyloid-β peptide (1–42), an effect associated with a significant reduction in the level of protein carbonyls for specific proteins, such as aldolase and thioredoxin peroxidase in aged mouse brain (70). By extending our previous findings, we demonstrated in brain of AD patients a significant increase in the expression of vitagenes HO-1, thioredoxin reductase (TRXr), and Hsp60, associated with a decrease in the expression of HO-2. Parallel changes were observed in AD lymphocytes, in which a significant increase in the expression of inducible nitric oxide synthase (NOS-2) was associated with that of the HO-1, Hsp70, and TRXr. Brains of AD patients undergo many changes, such as disruption of protein synthesis and degradation, classically associated with the heat shock response, which is one form of stress response. Heat shock proteins are proteins serving as molecular chaperones involved in the protection of cells from various forms of stress.

Increasing interest has been focused on identifying dietary compounds that can inhibit, retard, or reverse the multistage pathophysiologic events underlying AD pathology. AD involves a chronic inflammatory response associated with both brain injury and β-amyloid–associated pathology. All of this evidence suggests that stimulation of various repair pathways by mild stress has significant effects on delaying the onset of various age-associated alterations in cells, tissues, and organisms. Spices and herbs contain phenolic substances with potent antioxidative and chemopreventive properties, and it is generally assumed that the phenol moiety is responsible for the antioxidant activity. In particular, curcumin, a powerful antioxidant derived from the curry spice turmeric, has emerged as a strong inducer of the heat shock response. In light of this finding, ferulic acid ethyl ester, a modified form of ferulic acid (two molecules of ferulic acid is a component of naturally occurring spice called curcumin) has been shown to protect the primary neuronal cells against amyloid-β–induced toxicity and oxidative stress, suggesting that supplementation of the naturally occurring antioxidant could be an alternative, nutritional approach to reduce oxidative damage and amyloid pathology associated with AD (22, 25, 34, 36, 63, 89), outlining the importance of the vitagene system as a targets for novel cytoprotective strategies useful to alleviate the neurologic impairments associated with neurodegenerative damage present in AD and other oxidative stress–related brain diseases.

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ABBREVIATIONS

AD, Alzheimer disease; ARE, antioxidant responsive element; CTRL, control; D.U., densitometric units; GSH, glutathione; GSSG, glutathione disulfide; HO-1, heme oxygenase-1; HO-2, heme oxygenase-2; Hsp, heat shock protein; iNOS (NOS-2), inducible nitric oxide synthase; NO, nitric oxide; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species; TRX, thioredoxin; TRXr, thioredoxin reductase.

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