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Original Contribution

Effects of oxidative and nitrosative stress in brain on p53 proapoptotic protein in amnestic mild cognitive impairment and Alzheimer disease

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ABSTRACT

Many studies reported that oxidative and nitrosative stress might be important for the pathogenesis of Alzheimer's disease (AD) beginning with arguably the earliest stage of AD, i.e., as mild cognitive impairment (MCI). p53 is a proapoptotic protein that plays an important role in neuronal death, a process involved in many neurodegenerative disorders. Moreover, p53 plays a key role in the oxidative stress-dependent apoptosis. We demonstrated previously that p53 levels in brain were significantly higher in MCI and AD IPL (inferior parietal lobule) compared to control brains. In addition, we showed that in AD IPL, but not in MCI, HNE, a lipid peroxidation product, was significantly bound to p53 protein. In this report, we studied by means of immunoprecipitation analysis, the levels of markers of protein oxidation, 3-nitrotyrosine (3-NT) and protein carbonyls, in p53 in a specific region of the cerebral cortex, namely the inferior parietal lobule, in MCI and AD compared to control brains. The focus of these studies was to measure the oxidation and nitration status of this important proapoptotic protein, consistent with the hypothesis that oxidative modification of p53 could be involved in the neuronal loss observed in neurodegenerative conditions.

Introduction

Alzheimer disease (AD), the leading cause of dementia, involves regionalized features such as neuronal death, synaptic loss, intracellular neurofibrillary tangles, and extracellular amyloid plaques [1]. Although, to date, the mechanism responsible for Alzheimer disease has not yet been identified, several independent hypotheses have been proposed to explain the disease [2–5]. However, none of the hypotheses alone is sufficient to explain the pathological and biochemical alteration in AD. Some of the previous studies showed a role of oxidative stress in development of this neurodegenerative disease [4–9]. Oxidative stress, as well as nitrosative stress, results from an imbalance between oxidants and antioxidants. Oxidants can damage all biological molecules: DNA, RNA, lipid, protein, carbohydrates, and antioxidants. In AD brain, the antioxidant levels were found to be decreased, whereas the protein oxidation (protein carbonyl and 3-nitrotyrosine), lipid peroxidation, DNA oxidation, and advanced glycation end products were found to be increased [4–9]. Also, in mild cognitive impairment (MCI), a transition phase between normal aging and dementia [10], previous studies showed elevated protein oxidation and lipid peroxidation in specific regions of the brain, such as the hippocampus and the inferior parietal lobule (IPL) [11–14]. This strongly supported the thesis that oxidative stress is involved in the progression of AD from an early phase.

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) attack proteins, leading to the formation of protein carbonyls and 3-nitrotyrosine (3-NT). The levels of protein carbonyls and 3-NT reflect the level of protein oxidation in a cell. Protein oxidation causes the loss of protein function, cellular dysfunction, and, ultimately, cell death [11,15–17]. Oxidative damage can be measured by the determination of levels of protein carbonyls, tyrosine nitration, and protein adducts of alkenals such as acrolein and 4-hydroxynonenal, which are themselves reactive products of lipid peroxidation. Tyrosine nitration is one specific form of protein oxidation that is associated with Alzheimer's disease [9,18–20]. Nitric oxide (NO) reacting with the superoxide anion ($O2^{\cdot-}$) forms the product, peroxynitrite ($ONOO^{-}$), known to lead to nitration of tyrosine (3-NT) residues [18,21]. Nitration of proteins results in the inactivation of several important mammalian proteins such as Mn superoxide dismutase (SOD), Cu/Zn SOD, actin, and tyrosine hydroxylase, and likely interferes with tyrosine phosphorylation-mediated cell signaling, as a result of steric effects [17].

Protein carbonyls (aldehydes and ketones, PCO) can arise from direct oxidation of amino acid side chains (His, Pro, Arg, Lys, Thr, etc.), by

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Abbreviations: AD, Alzheimer's disease; HNE, 4-hydroxy-2-nonenal; IPL, inferior parietal lobule; MCI, mild cognitive impairment; NO, nitric oxide; 3-NT, 3-Nitrotyrosine; PCO, protein carbonyls; SOD, superoxide dismutase.

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oxidative cleavage of proteins via the α-amidation pathway, or Michael addition reactions of α-, β-unsaturated aldehydes, such as 4-hydroxy-2-nonenal (HNE), malondialdehyde, and 2-propenal (acrolein), derived from lipid peroxidation [17]. Elevated levels of PCO are generally associated not only with oxidative stress, but also with the disease-resident protein dysfunction [22]. By using a redox proteomics approach, many proteins involved in energy production, pH regulation, and mitochondrial functions were found carbonylated and nitrated in AD inferior parietal lobule [9,15,23–25]. In addition, experiments demonstrated other targets of oxidation in different brain regions, and also that oxidatively modified proteins are prone to inactivation [24].

The tumor-suppressor p53 protein plays an important role in cellular response following DNA damage [26]. p53 binds specific DNA sequences and regulates the expression of target genes which encode the proteins that control cell cycle progression or lead cells to apoptosis [27]. Also, p53 might contribute to apoptosis by a mitochondrial pathway [28]. A close connection between NO and p53 may exist because, on the one hand, p53 accumulates in cells following incubation with NO-releasing compounds [29–32] and, on the other, p53 mediates transcriptional transrepression of iNOS mRNA expression by a negative feedback loop [31,32]. Mutated p53 is unable to exert this function. Moreover, high levels of NO can induce a conformational change of wild-type p53 resulting in impairment of its DNA-binding activity in vitro [32].

We showed recently that the p53 expression was significantly increased in MCI and AD IPL compared to control samples [33]. In addition, one product of lipid peroxidation, HNE, was found to bind significantly to p53 protein in AD IPL, but not in MCI [33]. The results are consistent with the notion of an involvement of p53, an important regulator of apoptosis, in neurodegenerative conditions, and its special link with oxidative stress.

The prior research on p53 from our laboratory dealt with p53 expression in brain of subjects with AD and MCI. The present work expanded the prior study to examine the oxidation status of p53 protein in these neurodegenerative conditions. Therefore, we performed immunoprecipitation experiments to examine 3-nitrotyrosine and protein carbonyl levels in p53 protein in MCI and AD inferior parietal lobule compared to control brains. This research tested the hypothesis that p53 is modified by oxidative and nitrosative stress in MCI and AD, suggesting that alteration of p53 pathway could be involved in neuronal death and in the progression of AD.

Materials and methods

Materials

All chemicals used were purchased from Sigma-Aldrich (St. Louis, MO) with exceptions of nitrocellulose membranes (Bio-Rad, Hercules, CA), electrophoretic transfer system (Trans-blot Semi-dry Transfer Cell; Bio-Rad), anti-p53 monoclonal antibody used for immunoprecipitation and Western blotting (Calbiochem, LA Jolla, CA), and anti-DNP protein adducts polyclonal antibody (Chemicon International, Temecula, CA).

Patients

Frozen IPL samples from MCI, AD, and age-matched controls were obtained from the University of Kentucky Rapid Autopsy Program of the Alzheimer's Disease Clinical Center (UK ADC). The diagnosis of probable AD was made according to criteria developed by the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) and the Alzheimer's Disease and Related Disorders Association (ADRDA) [34]. All AD patients displayed progressive intellectual decline. Control subjects were without history of dementia or other neurological disorders and underwent annual mental status testing and semiannual physical and neurological exams as part of the UK ADC normal volunteer longitudinal aging study. In addition, patients had test scores in the normal range (Table 2). Samples and demographics used for the AD study were described previously [24]. Additional demographic parameters of control, MCI, and AD patients available from medical records are provided in Tables 1 and 2.

Sample preparation

The brain tissues (IPL) from control, MCI, and AD were homogenized in ice-cold isolation buffer containing 10 mM Hepes buffer, 137 mM NaCl, 4.6 mM KCl, 1.1 mM KH_2PO_4, and 0.6 mM $MgSO_4$, as well as proteinase inhibitors leupeptin (0.5 mg/ml), pepstatin (0.7 mg/ml). Homogenates were centrifuged at 14,000 *g* for 10 min to remove debris. The supernatant was extracted to determine the total protein concentration by the BCA method (Pierce, Rockford, IL).

Immunoprecipitations

For immunoprecipitation experiments, 150 μg of protein extracts was resuspended in 500 μl RIPA buffer (10 mM Tris, pH 7.6; 140 mM NaCl; 0.5% NP40 including protease inhibitors) and then incubated with 1 μg of monoclonal conformation-specific antibody against p53 protein (wild-type specific—PAb11) at 4 °C overnight. Immunocomplexes were collected by using protein A/G suspension for 2 h at 4 °C and washed five times with immunoprecipitation buffer. Immunoprecipitated p53 was recovered by resuspending the pellets in loading buffer, and protein was detected by Western blotting.

Western blotting analysis

For immunoblotting analysis proteins immunoprecipitated (30 μl) were electrophoresed through a 10% polyacrylamide gel and transferred to nitrocellulose paper (Bio-Rad Trans-blot Semi-dry Transfer Cell) at 45 mA for 2 h. The membranes were blocked for 1 h at room temperature with blocking solution in 5% nonfat dried milk in phosphate-buffered saline containing 0.01% (w/v) sodium azide and 0.2% (v/v) Tween 20 (PBST) at 4 °C for 1 h. The membranes were then incubated for 2 h at room temperature with primary antibodies: anti-nitrotyrosine polyclonal antibody (3-NT), diluted 1:100 in wash blot, and anti-DNP protein adducts polyclonal antibody (1:100). After three washes for 5 min with wash blot, the membranes were incubated for 1 h at room temperature with IgG alkaline phosphatase polyclonal secondary antibody diluted 1:2000 in wash blot and developed using 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (BCIP/NBT) color developing reagent. Blots were dried and scanned with Adobe Photoshop and quantitated with Scion Image (PC version of Macintosh-compatible NIH Image) software.

Postderivatization of protein

Samples were postderivatized with DNPH on the membrane and probed with anti-DNPH antibody to identify the oxidized proteins. The nitrocellulose membranes where equilibrated in solution A (20% (v/v) methanol:80%(v/v) wash blot buffer) for 5 min, followed by incubation of membranes in 2 N HCl for 5 min. The proteins on blots were then derivatized in solution B (0.5 mM DNPH in 2 N HCl) for exactly 10 min as described by Conrad et al. [35]. The membranes were washed three

Table 1
Characteristics of control and MCI patients (mean±SD)

Demographic variables	Control subjects	MCI subjects
Number of subjects	7	7
Gender (male/female)	3/4	3/4
Postmortem Interval (h)	2.87±1.14	3.125±1.033
Brain weight (g)	1260±120	1120±61
Braak stage	I–II	III–V

MCI, mild cognitive impairment.