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Original Research Communication

Protein Disulfide Isomerase in Alzheimer Disease

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ABSTRACT

There is a great deal of evidence that places oxidative stress as a proximal event in the natural history of Alzheimer disease (AD). In addition to increased damage, there are compensatory increases in the levels of free sulfhydryls, glucose-6-phosphate dehydrogenase, and NAD(P)H:quinone oxidoreductase 1. To investigate redox homeostasis further in AD, we analyzed protein disulfide isomerase (PDI), a multifunctional enzyme, which catalyzes the disruption and formation of disulfide bonds. PDI plays a pivotal role in both secreted and cell-surface-associated protein disulfide rearrangement. In this study, we show that PDI specifically localizes to neurons, where there is no substantial increase in AD compared to age-matched controls. These findings indicate that the neurons at risk of death in AD do not show a substantial change in PDI to compensate for the increased sulfhydryls and reductive state found during the disease. This suggests that, despite compensatory reductive changes in AD, the level of PDI is sufficiently high physiologically in neurons to accommodate a more reducing environment. Antiox. Redox Signal. 2, 485–489.

INTRODUCTION

AEROBIC METABOLISM presents the challenge of survival in the environment of reactive oxidative by-products (Starke *et al.*, 1997). This oxidative environment induces an array of oxidative defense mechanisms, including glutathione (GSH) and thioredoxin antioxidant systems (Starke *et al.*, 1997; Takagi *et al.*, 1999). Thiols and disulfide bonding play a pivotal role in the maintenance of homeostasis of both GSH and thioredoxin (Sen, 1998; Balijepalli *et al.*, 1999). During periods of oxidative stress, thiol antioxidants provide one of the most important buffering systems to reactive oxygen within cells (Sen, 1998) as well as quenching agents that block the toxicity of oxidatively damaged molecules. Glucose-6-phosphate dehydrogenase (G6PDH), NADPH, and GSH have all been indicated as possible protective responses to redox disequilibrium and oxidative damage related to mitochondria (Hothersall *et al.*, 1982; Russell *et al.*, 1999). Recent evidence indicates that thioredoxin may have been overlooked as a crucial mediator of the antioxidant response of the nervous system (Mansur *et al.*, 1998). In recent studies, protein disulfide isomerase (PDI) was shown to have four thioredoxin domains, two with reactive oxygen sites and two without (Darby *et al.*, 1999), suggesting analysis of whether PDI is altered as a response to increased oxidative stress is warranted.

Oxidative stress is a prominent feature of Alzheimer disease (AD). During the course of AD, there appears to be a compensatory reductive shift in redox balance, as demonstrated

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by increased NAD(P):quinone oxidoreductase 1 (NQO1) (Raina *et al.*, 1999), G6PDH (Martins *et al.*, 1986; Russell *et al.*, 1999), and reactive sulfhydryls (Russell *et al.*, 1999), that proximally parallels the same vulnerable neurons demonstrating increased oxidative stress in AD. Here, we examine PDI to see if it is confined to the same vulnerable neurons displaying oxidative damage as well as whether it is altered in AD.

MATERIALS AND METHODS

Tissue

Hippocampus extending to the entorhinal cortex and temporal neocortex was obtained at autopsy from patients with clinically and histopathologically confirmed AD (Khachaturian, 1985; Mirra *et al.*, 1991) ($n = 13$; ages 69–91; PMI = 2.5–23, Ave: 5.9 hr), as well as from nondemented young and age-matched controls ($n = 13$; ages 3–86; PMI = 4–48; Ave: 19.13 hr) for which the cause of death was known. Tissue was fixed by methacarn (methanol:chloroform:acetic acid in 6:3:1 vol/vol/vol) immersion for 16 hr at 4°C. Tissue was subsequently dehydrated through graded ethanol and xylene solutions and embedded in paraffin. Six-micron-thick sections were prepared and placed on silane-coated slides. Use of human tissue for these experiments was approved by the Institutional Review Board for Human Experimentation at the University Hospitals of Cleveland.

Immunocytochemistry

Monoclonal antibodies to human placenta PDI, HP-24, and to rat liver PDI, RL77 (gifts of C. Kaetzel), were used at dilutions of 1/1,000 to immunostain tissue. 5E2, a monoclonal antibody to τ (gift of K. Kosik), was used at a dilution of 1/1,000 to indicate neurofibrillary tangles. Following hydration, sections were immunostained by peroxidase-antiperoxidase procedure (PAP) with 3,3′-diaminobenzidine (DAB) as cosubstrate (Sternberger, 1986).

The specificity of the monoclonal HP-24 antibody for PDI in human brain was determined by adsorption with 0.5 mg/ml of purified bovine liver PDI (Sigma, St. Louis, MO) prior to application to the section.

Quantitative analysis of the relative intensity of immunostain was determined using a Quantimet 570C Image Processing and Analysis System (Leica, Cambridge Ltd., Cambridge, UK) linked to a COHU solid-state camera mounted on a Leitz LaborLux 12 ME ST microscope. Three cases of AD and four controls were evaluated by measuring the optical density of the immunostained neurons in three adjacent 20× fields for each case. Neurons were manually outlined and the optical density for that area recorded.

Immunoblotting

Samples dissected from autopsied brains and stored frozen at −80°C were homogenized in 10 volumes of Tris-buffered saline and centrifuged at $10{,}000 \times g$ for 10 min. The supernatants, 30 μg protein per lane, were subjected to 10% SDS-PAGE and transferred to an Immobilon-P membrane (Millipore, Bedford, MA). The membrane was then incubated with the antibody to PDI (1/1,000) or where the primary antibody was omitted. The resultant antigen–antibody complexes were detected by using the indirect peroxidase method with DAB as cosubstrate.

Quantitative evaluation of the immunostained bands was with a pdi Digital Image Analysis System (Huntington Station, NY).

RESULTS

PDI immunoreactivity was abundant in large pyramidal neurons with both antibodies, HP24 and RL77. The intense cytosolic immunoreactivity was similar for controls as well as in cases of AD (Fig. 1). Paralleling the widespread distribution of oxidative damage to select pyramidal neuronal populations, both neurons with and without neurofibrillary tangles showed PDI. Aged individuals without AD also had PDI staining of vesicles similar to that found in AD. Quantitative evaluation of the intensities confirmed that immunorecognition was essentially identical ($p = 0.545$) (Fig. 2). Verifying the specificity of the immunoreaction, HP24 stain-

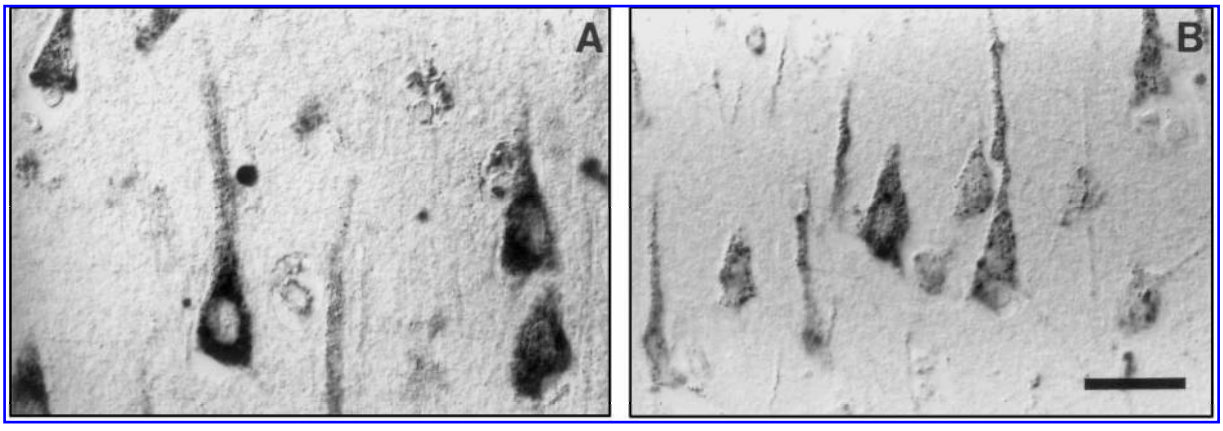

FIG. 1. PDI immunoreactivity with monoclonal HP24 is confined to large neurons in cases of AD (A) as well as controls (B). Scale bar, 25 μm.

ing intensity was greatly reduced by prior absorption with PDI (Fig. 3) and similar staining was not noted with omission of the primary antibody (data not shown). Although PDI was unchanged in AD, it was most pronounced in neurons vulnerable in AD, the same neurons found to have increased oxidative damage in AD (Nunomura *et al.*, 1999). We could discern no pattern of differences in our results based on agonal status, cause of death, or postmortem interval between the control or AD group.

Immunoblots of grey matter showed that both the antibodies recognized a single 56 to 61 kDa band corresponding to the reported molecular weight for PDI (Fig. 4). A minor band at 55 kDa was also recognized with antibody HP24. Quantitative densitometric analysis showed the reflective density for AD cases was essentially identical to that found for control cases ($p = 0.235$) (Fig. 5).

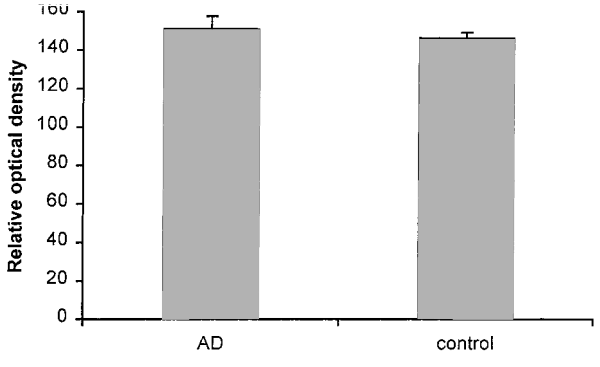

FIG. 2. Densitometric analysis of the intensity of PDI immunoreactivity by monoclonal antibody HP24 in neurons from cases of AD ($n = 3$) and control cases ($n = 4$). A total of 30–40 neurons were analyzed from the AD and control cases ($p = 0.545$).

DISCUSSION

PDI is multifunctional protein that catalyzes thiol–disulfide interchanges that result in the formation, reduction, and rearrangement of both secreted and cell-surface-associated protein (Darby *et al.*, 1999; Jiang *et al.*, 1999). The role of PDI in redox control at the cell surface was demonstrated in recent studies (Zai *et al.*, 1999; Jiang *et al.*, 1999; Ferrari and Soling, 1999). As a response to increased extracellular reductive environment, PDI may help re-establish redox homeostasis by rearranging and forming disulfide bonds (Klappa *et al.*, 1997; Song *et al.*, 1997; Zai *et al.*, 1999; Ferrari and Soling, 1999). The primary site of intracellular reductive equivalents, glutathione (Hothersall *et al.*, 1982; Di Simplicio *et al.*, 1998; Duffy *et al.*, 1998; Sen, 1998) is also involved in major disulfide breakage and formation reactions and probably re-establishes mitochondrial redox balance.

The localization of substantial levels of PDI to vulnerable neurons may be due to the high protein secretion rate of these cells as well as the physiological assault of these cells by abundant reactive oxygen due to their high metabolic rate. The role of PDI in promoting the shift in disulfide bond formation may specifically involve secreted and cell-surface proteins, some of which have redox-sensitive, post-translational modifications (Capellari *et al.*, 1999). The

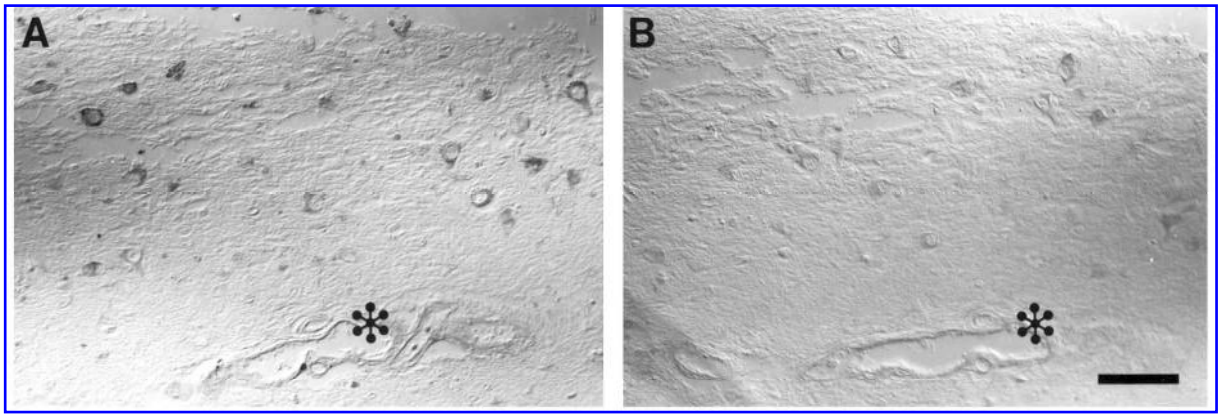

FIG. 3. Neuronal HP-24 immunoreactivity (A) was greatly reduced by absorption with 5 mg/ml PDI (B). Adjacent serial sections with the same vessel landmark (*) indicated in each. Scale bar, 100 μm.

lack of a significant increase in intracellular PDI in AD may reflect the high physiological level of PDI maintained in neurons, and that is sufficient to maintain redox balance even in the midst of increased oxidative insult. Therefore, while changes in G6PDH (Martins *et al.*, 1986; Russell *et al.*, 1999), reactive sulfhydryls (Russell *et al.*, 1999), and NQO1 (Raina *et al.*, 1999) parallel increased oxidative damage in AD, compensatory increased PDI may not be required. It might be argued that the lack of PDI increase may be, in part, responsible for the significant increase in oxidative damage that characterizes the neuronal cell body in AD, the site of most PDI in the brain. Alternatively, post-translational alterations in PDI, unknown at this time, may alter PDI activity without requiring an increase at the protein level. Further investigation is required to resolve the latter issues.

It is also intriguing to consider that AD and other neurodegenerative diseases are associated with major protein conformational changes that are important in the formation of abnormal fibrils characterizing AD, and that the high levels of PDI noted here may parallel and reflect the site of those changes.

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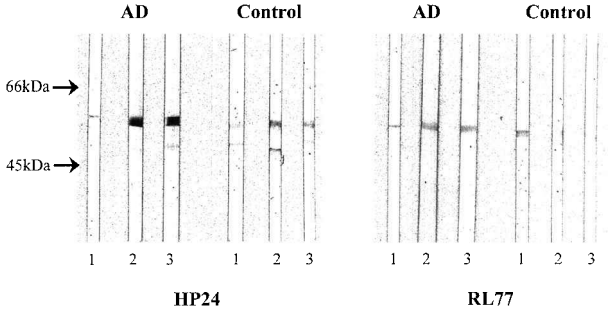

FIG. 4. The antibodies to PDI recognize a major band at 56 to 61 kDa with a minor band of 55 kDa in the case of HP24 and a single band of 56–61 kDa in the case of RL77 in immunoblots of human grey matter from three cases of AD and three control cases. No significant difference was noted between AD and control cases. No bands were recognized if the primary antibodies were omitted (data not shown).

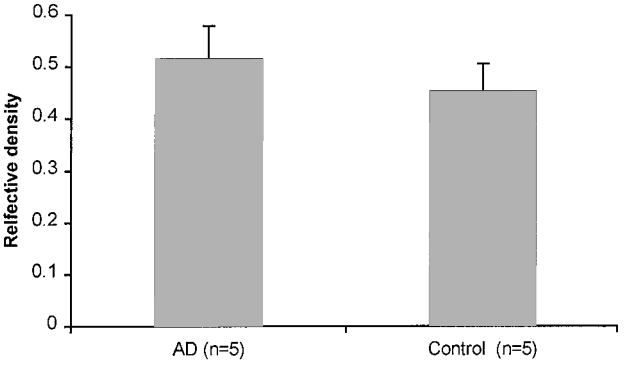

FIG. 5. Quantitative analysis of PDI immunoreaction in immunoblots of human grey matter from AD cases (n = 5) and control cases (n = 5). Values represent average reflective densities (± SEM) of bands recognized by monoclonal antibody HP24 (p = 0.235).

ABBREVIATIONS

Alzheimer disease (AD); 3,3′-diaminobenzidine (DAB); glucose-6-phosphate dehydrogenase (G6PHD); glutathione (GSH); NAD(P): quinone oxidoreductase 1 (NQO1); peroxidase-antiperoxidase (PAP); protein disulfide isomerase (PDI); SDS polyacrylamide gel electrophoresis (SDS-PAGE).

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