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Increased Neuronal Glucose-6-phosphate Dehydrogenase and Sulfhydryl Levels Indicate Reductive Compensation to Oxidative Stress in Alzheimer Disease

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We analyzed glucose-6-phosphate dehydrogenase, the rate-controlling enzyme of the pentose phosphate pathway and free sulfhydryls, to study redox balance in Alzheimer disease. Glucose-6-phosphate dehydrogenase plays a pivotal role in homeostatic redox control by providing reducing equivalents to glutathione, the major nonenzymatic cellular antioxidant. There is a multitude of evidence that marks oxidative stress proximally in the natural history of Alzheimer disease. Consistent with a role for glutathione in defense against increased reactive oxygen, we found an upregulation of glucose-6-phosphate dehydrogenase together with increased sulfhydryls in Alzheimer disease. These data indicate that reductive compensation may play an important role in combating oxidative stress in Alzheimer disease.

Key Words: **Alzheimer disease; glucose-6-phosphate dehydrogenase; glutathione; oxidative stress; pentose phosphate pathway; redox control; sulfhydryls.**

A spectrum of evidence implicates oxidative stress in Alzheimer disease (AD)[2] pathophysiology (1–3). A major focus in the study of oxidative stress in most systems has been the characterization of damage to cellular components (3, 4) based on the perception that increased reactive oxygen species (ROS) will cause increased damage. However, recent studies have shown that ROS may not only cause oxidative damage but also trigger protective responses (4). This is not surprising if one considers that breaching oxidative defenses is often associated with apoptosis (5).

As a reaction to oxidative stress, cytoprotection is afforded by the upregulation of multiple antioxidant enzyme systems (6–8). Glutathione (GSH), a broad-spectrum antioxidant, participates in a number of cellular defenses, namely, reduction of free radicals (9), removal of toxins (4), and protection from oxidative damage (10). GSH, the most abundant nonprotein thiol in eukaryotes, is also responsible for the regulation of redox state of the cell (11, 12). Recent studies have identified the protective effects of the GSH center upon maintaining the reductive state of thiol groups as well as detoxification of reactive products resulting from ROS attack (10, 13). The increase in reducing power is provided by modulating the activity of the rate-controlling enzyme of the pentose phosphate pathway, namely, glucose-6-phosphate dehydrogenase (G6PDH). An upregulation of G6PDH has been associated with an increase in GSH and inhibition of apoptosis (9, 14), and elevated levels of G6PDH activity correlate with a higher antioxidant capacity (15). G6PDH is the key enzyme in reestablishing steady-state levels of GSH/GSSG ratio at the intracellular level (10, 15, 16), which prevents the S-thiolation of mitochondrial membrane proteins (9, 17). Increased G6PDH and GSH activity in AD found previously by others (15, 18, 19) as well as upregulation of NAD(P):quinone oxidoreductase 1 (NQO1) (20) gave us the impetus to study whether there might be a reductive sulfhydryl compensation in

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[2] Abbreviations used: AD, Alzheimer disease; ROS, reactive oxygen species; G6PDH, glucose-6-phosphate dehydrogenase; NQO1, NAD(P):quinone oxidoreductase 1; PAP, peroxidase–antiperoxidase procedure; 3,3′-diaminobenzidine; FITC, fluorescein isothocyanate; PBS, phosphate-buffered saline; RT, room temperature; TBS, Tris-buffered saline.

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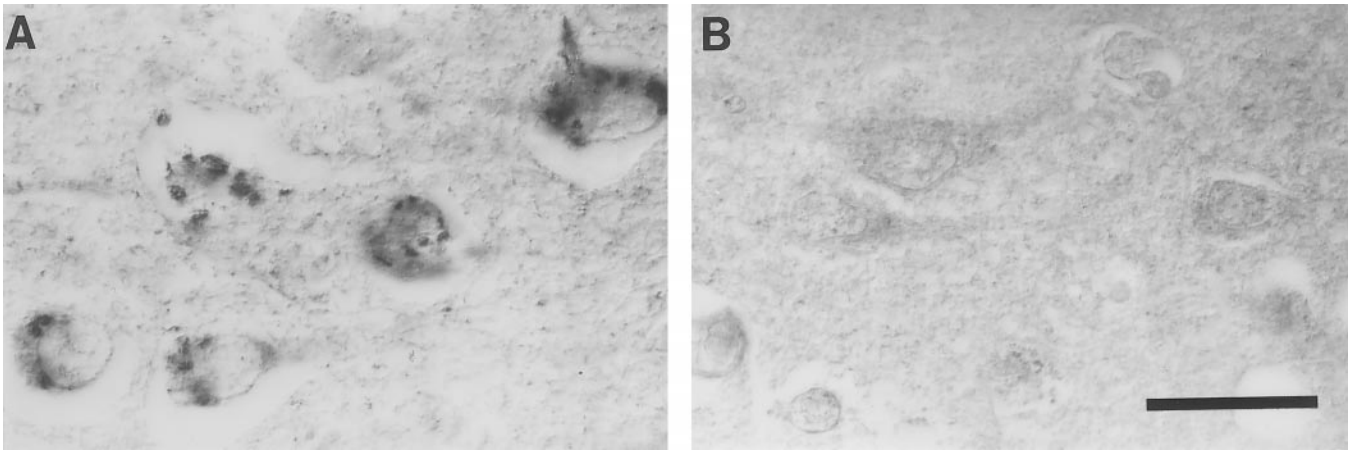

FIG. 1. Cytoplasmic vesicles as well as cytosol of pyramidal neurons in Alzheimer disease are strongly recognized by the antibody to yeast G6PDH (A). In contrast, in control cases, G6PDH immunoreaction was reduced compared to controls and was seldom seen in vesicles (B). Scale bar, 25 μm.

AD that parallels the profile of oxidative damage being limited to the perikaryal cytoplasm of vulnerable neurons in AD.

MATERIALS AND METHODS

Tissue. Hippocampal samples extending to the entorhinal cortex as well as samples from the cerebellum of some of the cases were obtained at autopsy from patients with clinically and histopathologically confirmed AD (21, 22) (n = 17, ages 69–93; PMI = 1–23 h, ave = 6.1 h) as well as from nondemented young and age-matched controls (n = 14, ages 3–86; PMI = 1.75–34.5 h, ave 15.7 h, with 3 cases <6 h). The cause of death was known for each case. Tissue was fixed by methacarn (methanol:chloroform:acetic acid in a 6:3:1, v/v/v) immersion for 16 h at 4°C. Alternatively, tissue from some cases, analyzed for G6PDH only, were fixed in buffered 3.7% formaldehyde. Tissue was subsequently dehydrated through graded ethanol and xylene solutions and embedded in paraffin. Six-micrometer-thick sections were prepared and placed on silane-coated slides.

Immunocytochemistry. Rabbit antiserum to G6PDH isolated from *Saccharomyces cerevisiae* (baker's yeast) (Sigma) or recombinant human G6PDH (23) was used at a dilution of 1/1000 to immunostain tissue that had been fixed in either methacarn or buffered formalin. Yeast G6PDH has 48% identical and 87% similar amino acid sequences compared to human G6PDH. 5E2, a monoclonal antibody to τ (a gift of K. Kosik), was used at a dilution of 1/1000 to indicate neurofibrillary tangles. Following hydration, sections were immunostained by the peroxidase–antiperoxidase procedure (PAP) with 3,3′-diaminobenzidine (DAB) as cosubstrate (24). Specificity of the yeast G6PDH antisera was verified by absorption by incubating the antibody with 10 mg/ml G6PDH isolated from baker's yeast (Sigma Type V or IX) for 16 h at 4°C prior to immunocytochemistry. A high specificity of the antibody to human G6DPH has been already demonstrated (25).

Free sulfhydryls. Following hydration, methacarn-fixed tissue was incubated with 0.01 mM fluorescein-5-maleimide (FITC-maleimide) (Molecular Probes) in PBS for 2 h at RT. After rinsing, sections were incubated with a mouse monoclonal antibody to FITC (Sigma) at a dilution of 1/1000 and developed with the PAP procedure. The specificity of the FITC-maleimide binding was verified by prior treatment of the section with 1 mM *N*-ethylmaleimide (unlabeled) (Sigma) for 2 h at RT and subsequent rinsing prior to the addition of FITC-maleimide. Additionally, the antibody alone was incubated on adjacent sections in each experiment and had no reactivity.

Immunoblotting. For immunoblot analysis, purified G6PDH (Sigma) and gray matter homogenates of AD and control brain were separated by polyacrylamide gel electrophoresis and transferred to Immobilon (Millipore). The blots were blocked in 10% dry nonfat milk in TBS for 1 h at RT and then incubated for 24 h at 4°C with antibody to G6PDH at a dilution of 1/1000. Visualization of immunorecognition was with the indirect peroxidase–antiperoxidase method by using DAB as cosubstrate.

RESULTS

In all the cases, both antibodies showed G6PDH immunoreactivity was limited to large pyramidal neurons and the smooth muscle layer of major vessels. Neuronal G6PDH was present in the cytosol of controls as well as in cases of AD, but in AD, cytoplasmic G6PDH was more intensely stained and also present in vesicles (Fig. 1). Paralleling the widespread distribution of oxidative damage to select pyramidal neuronal populations demonstrated in prior studies, both neurons with and without neurofibrillary tangles showed increased neuronal G6PDH. A few aged individuals without AD, but with a small number of neurofibrillary tangles, also had G6PDH staining of vesicles similar to that found in AD, albeit to a lesser extent. Furthermore, increased G6PDH, like oxidative damage, follows the pattern of neuronal vulnerability, because even large Purkinje neurons of the cerebellum were no more stained for G6PDH in cases of AD than in controls. We could discern no pattern of differences in our results based on agonal status just prior to death or cause of death between the nondemented group and the AD group. Additionally, postmortem interval (1–34.5 h) did not influence our results. Specificity of the antibody for yeast G6PDH was shown by reduction of immunoreaction after prior incubation with G6PDH

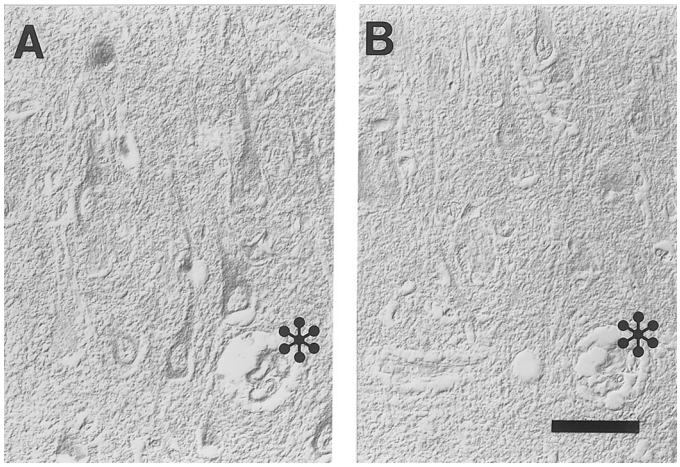

FIG. 2. Pyramidal neurons in Alzheimer disease are recognized by the antibody to yeast G6PDH (A), a reaction that is blocked by prior incubation with yeast G6PDH protein (B). Asterisks (★) marks the same vessel passing through both sections. Scale bar, 50 μm.

protein (Fig. 2). In immunoblots of grey matter, the antisera to yeast G6PDH recognized a single 60-kDa band corresponding to the reported molecular weight for G6PDH that was abolished after absorption (data not shown).

Increased G6PDH should be associated with increased NADPH, redox balance, and sulfhydryls. To address whether this happened in AD, we used *N*-ethylmaleimide to detect reactive sulfhydryls. We found a robust increase in cytosolic sulfhydryls in AD, again limited to pyramidal neurons (Fig. 3). Total blockage of the sulfhydryl sites with prior treatment with unlabeled *N*-ethylmaleimide (Fig. 3B) verified the specificity of this detection method for reactive sulfhydryls. This finding, along with the appearance of increased G6PDH, may be a clear indication of a reductive shift resulting from G6PDH activity.

DISCUSSION

G6PDH is the key rate-controlling enzyme in the pentose phosphate pathway, the primary pathway that generates reductive equivalents and thus effectively acts as a monitor of the intracellular reductive state. Increased activity, as previously shown globally by the work of others (18, 19), is shown here limited to vulnerable neurons in AD consistent with an attempted reductive compensation to oxidative stress that is limited to the same type of neurons. The increase is cytosolic, the expected site of G6PDH, as well as vesicular, suggesting a major change in G6PDH compartmentation in AD. The change in G6PDH may not only modulate redox state but also signal a major shift in metabolism, such as modifying lipid synthesis (26) or signal transduction (27). That mitochondrial abnormalities are a major feature of AD (Hirai *et al.*, unpublished observation) suggests the observed vesicles may be related to mitochondria where G6PDH is lending reductive equivalents. Mitochondrial membrane sulfhydryl groups are susceptible to attack and depletion by H_2O_2 and other ROS (28, 29) during oxidative stress, features that could be compensated by increased GSH. This feature is particularly important in AD since amyloid-β increases mitochondrial oxidative stress (30).

A positive change in the NADPH/NADP ratio generates a more reducing intraneuronal environment (19), which, in addition to altering metabolism, is used by the NADPH-dependent glutathione reductase (9, 31) to produce glutathione. The primary site of cellular reductive equivalents, glutathione (4, 9, 15, 32), functions as an antioxidant (10, 33, 34). Glutathione acts like a scavenger to remove H_2O_2, lipid peroxides and their products like 4-hydroxynonenal, respectively, preventing H_2O_2 from being converted into highly reactive hydroxyl radicals (9), terminating propagated lipid peroxidation, and detoxifying reactive carbonyls.

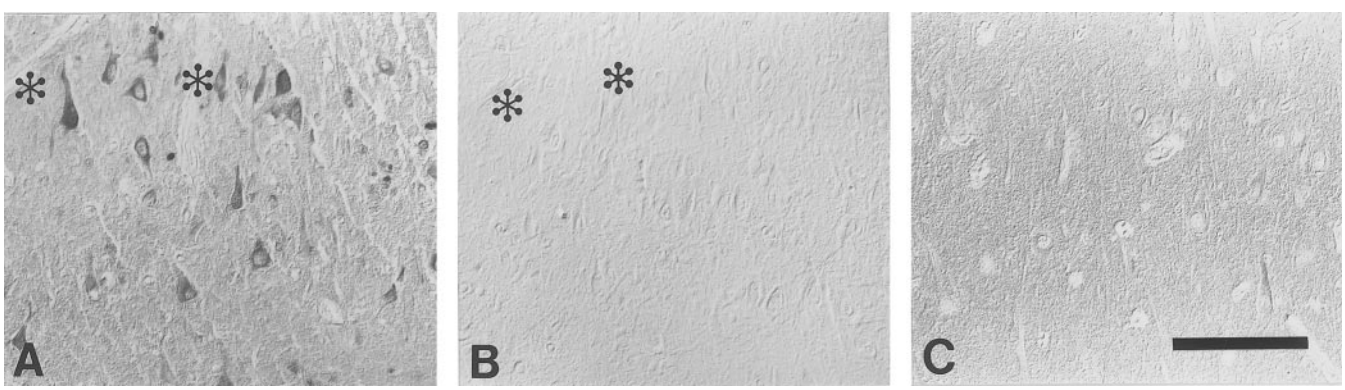

FIG. 3. Free sulfhydryls detected by FITC-maleimide binding was prominent in the cytoplasm in the neurons of cases of Alzheimer disease (A) and blocked by prior pretreatment with unlabeled *N*-ethylmaleimide (B). In contrast, neurons in control cases were essentially unstained (C). Asterisks (*) in A and B mark the same vessel passing through both section. Scale bar, 100 μm.

That we found increased reactive sulfhydryls in the neuronal cytoplasm in cases of AD indicates that G6PDH increases do effectively alter redox balance *in vivo*. The increased sulfhydryls may play a major role in controling reactive oxygen. Furthermore, these findings support not only damage but also metabolic compensation as an early event in AD.

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