Increase in Expression Levels and Resistance to Sulphydryl Oxidation of Peroxiredoxin Isoforms in Amyloid β-Resistant Nerve Cells*

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Peroxiredoxins (Prxs) are a ubiquitously expressed family of thiol peroxidases that reduce hydrogen peroxide, peroxynitrite, and hydroperoxides using a highly conserved cysteine. There is substantial evidence that oxidative stress elicited by amyloid β (Aβ) accumulation is a causative factor in the pathogenesis of Alzheimer disease (AD). Here we show that Aβ-resistant PC12 cell lines exhibit increased expression of multiple Prx isoforms with reduced cysteine oxidation. Aβ-resistant PC12 cells also display higher levels of thioredoxin and thioredoxin reductase, two enzymes critical for maintaining Prx activity. PC12 cells and rat primary hippocampal neurons transfected with wild type Prx1 exhibit increased Aβ resistance, whereas mutant Prx1, lacking a catalytic cysteine, confers no protection. Using an antibody that specifically recognizes sulfynlated and sulfonylated Prxs, it is demonstrated that primary rat cortical nerve cells exposed to Aβ display a time-dependent increase in cysteine oxidation of the catalytic site of Prxs that can be blocked by the addition of the thiol-antioxidant N-acetylcysteine. In support of previous findings, expression of Prx1 is higher in post-mortem human AD cortex tissues than in age-matched controls. In addition, two-dimensional gel electrophoresis and mass spectrometry analysis revealed that Prx2 exists in a more oxidized state in AD brains than in control brains. These findings suggest that increased Prx expression and resistance to sulphydryl oxidation in Aβ-resistant nerve cells is a compensatory response to the oxidative stress initiated by chronic pro-oxidant Aβ exposure.

A wide body of evidence has implicated oxidative damage in the pathogenesis of Alzheimer disease (AD). AD, the most common form of dementia in the elderly, is characterized by extracellular neuritic plaques containing the amyloid beta (Aβ) peptide1–42 and intracellular neurofibrillary tangles composed mainly of hyperphosphorylated tau protein. Several studies have shown that Aβ exposure increases levels of hydrogen peroxide (H2O2), lipid peroxidation, and protein oxidation (carbonylation) in cultured neurons (2–4). Increased oxidative damage to lipids, DNA, and proteins is also found in AD brains (1). Because the exogenous addition of antioxidants or catalase protects cultured nerve cells from Aβ toxicity (2, 5), it is likely that free radicals play a critical role in Aβ cytotoxicity. Therefore, cellular mechanisms that either prevent or remove reactive oxygen species (ROS) or reverse damage to cellular components after ROS exposure may play a key role in blocking Aβ toxicity.

Although widespread nerve cell death occurs in the brains of AD patients, some neurons are spared, indicating that certain populations of cells survive the same conditions that kill neighboring cells. Early studies had shown that low concentrations of Aβ can actually rescue neurons from stressful conditions (6). However, the mechanism of Aβ resistance is only poorly understood. Previously, a series of Aβ-resistant clones was derived from the rat pheochromocytoma cell line PC12 by growth for 4 months in the presence of high concentrations Aβ and subsequent cloning (2, 5). Aβ-resistant nerve cell clones not only survive exposure to toxic concentrations of Aβ but are also less sensitive to hydrogen peroxide (H2O2) and t-butyl-H2O2 in part due to increased expression and activities of the antioxidant enzymes glutathione peroxidase and catalase (2, 5). Further analysis revealed that Aβ-resistant nerve cells also have an enhanced flux of glucose through both the glycolytic and pentose phosphate pathway (PPP) (7). As a result of enhanced glycolysis and PPP activity, Aβ-resistant cells produce higher levels of reducing equivalents such as reduced nicotinamide adenine dinucleotide (NADPH). NADPH is essential for maintaining glutathione and thioredoxin (Trx) in a reduced state through reactions catalyzed by glutathione reductase and thioredoxin reductase (TrxR), respectively (8). Both the glutathione and TrxR systems are essential for maintaining intracellular protein sulphydryls in a reduced state.

Peroxiredoxins (Prxs) are a widely expressed group of peroxidases that reduce H2O2, peroxynitrite, and a range of organic hydroperoxides using reducing equivalents provided by the Trx/TrxR system (9). Recent studies have indicated that Prxs can also mediate signal transduction processes elicited by...
various growth factors and cytokines (10). Prxs, which exist as homodimers (with the exception of Prx6), use redox-active cysteines to reduce peroxides and are divided into two classes, the 1-Cys and 2-Cys Prxs, based upon the number of conserved cysteines involved in catalysis (9). Typical 2-Cys Prxs, the largest class of Prxs, undergo a catalytic cycle in which the N-terminal cysteine (generally near residue 50) is oxidized by H$_2$O$_2$ to a cysteine-sulfenic acid (Cys-SOH) that then reacts with the C-terminal cysteine (near residue 170) of another subunit to produce an intermolecular disulfide. This disulfide is then reduced by the Trx/TrxR system, completing the catalytic cycle. Formation of the disulfide bond is a slow process, and the sulfenic intermediate is occasionally hyperoxidized to a sulfenic acid (Cys-SO$_2$H) or even a sulfonic acid (Cys-SO$_3$H), resulting in inactivation of Prx peroxidase activity (11). Oxidation of the catalytic cysteine to a Cys-SO$_3$H in Prxs 1–4 is reversed by a thiol-specific antioxidant. In addition, cortical tissue from post-mortem AD patients exhibits increased expression of Prx1, whereas Prx2 exists in a more oxidized (acidic) form in AD brains. These findings suggest that increased Prx expression and the ability to maintain Prxs in a reduced state is part of a specific neuroprotective mechanism that occurs in response to Aβ accumulation. Understanding these mechanisms of Aβ resistance may be important for designing therapies to increase both the antioxidant and reductive capacity of nerve cells in AD patients.

**EXPERIMENTAL PROCEDURES**

**Culture Conditions and Experimental Treatment**—The PC12 clonal cell line, their Aβ-resistant derivatives, and their culture conditions have been previously described (2, 5). Rat primary cortical and hippocampal cultures were prepared and cultured under standard conditions (21). For Aβ treatment, a stock solution (0.5 mM) of the Aβ$_{1–42}$ peptide (Bachem) was freshly prepared in distilled water and allowed to form fibrils for 4 h at room temperature before the addition to cell culture media. Primary cultures were treated with 10 μM Aβ$_{1–42}$ peptide for various time periods as indicated. In some cases primary cultures were co-treated with 1 mM N-acetylcysteine (Sigma). Expression constructs containing FLAG-tagged versions of both wild type and cysteine mutant (C52S) Prx cDNAs were generously supplied by Dr. Hyunjung Ha (Chungbuk National University, Cheonju Korea). PC12 cells and primary cultures were seeded in 35-mm dishes at 5 × 10$^5$ and 2 × 10$^5$ cells/dish, respectively, and co-transfected with either pcDNA or FLAG-tagged Prx expression constructs along with an enhanced green fluorescent protein expression vector (Clontech, Palo Alto, CA) at a 3:1 molar ratio for a total of 4 μg of DNA per dish using Lipofectamine 2000 (Invitrogen) in serum-free media. Plasmid DNA was mixed with 4 μl of Lipofectamine 2000 in Opti-MEM media (Invitrogen) added to PC12 cell cultures, and 6 h later the transfection media was replaced with regular media supplemented with 10% fetal bovine serum. Primary hippocampal neurons were transfected based on a protocol optimized for transfecting this cell type (22). In brief, plasmid DNA was mixed with 8 μl of Lipofectamine 2000 in Opti-MEM media (500 μl total volume) and allowed to form a complex for 20 min at room temperature. The DNA-Lipofectamine complex was then added to the primary hippocampal culture, and after 6 h the transfection media was replaced with Neurobasal media with B27 supplement (Invitrogen) and 0.5 mM l-glutamine. Transfection efficiencies were ~70 and 20% for PC12 and primary hippocampal neurons, respectively. One day after transfection, 20 μM Aβ$_{1–42}$ peptide was added to the experimental dishes, and cell viability was determined at 24-h intervals. Twenty random fields of cells transfected with the Prx constructs (~500 cells total) were scored for green fluorescence at 400× magnification using a Leica DMIRB microscope equipped with a mercury lamp and appropriate filters. Cell viability was assessed by comparing the average number of green positive cells after Aβ treatment versus no treatment. Cell viability data were based on the average of three separate experiments.

**Immunoblotting**—After exposure to various oxidative stimuli, ~1 × 10$^6$ cells were washed twice with phosphate-buffered saline (PBS) and then incubated in ice-cold PBS with 40 mM iodoacetamide for 5 min to prevent thiol-disulfide exchange and inhibit post-lysis oxidation of free cysteines. These cells were harvested as a 0.1 ml of digitonin extraction buffer (10 mM PIPES, pH 6.8, 0.015% (w/v) digitonin, 300 mM sucrose, 100 mM...
TABLE 1
Control and AD patient details

| Patient | Diagnosis | Age | Sex | PMT | PDS* |
|---------|-----------|-----|-----|-----|------|
| C1      | Normal    | 94  | Female | 4.5 | 0    |
| A1      | AD        | 95  | Female | 4.5 | 3    |
| C2      | Normal    | 87  | Female | 6   | 0    |
| A2      | AD        | 86  | Female | 6   | 1    |
| C3      | Normal    | 88  | Male  | 2   | 0    |
| A3      | AD        | 85  | Male  | 4.5 | 1    |
| C4      | Normal    | 69  | Male  | 8   | 0    |
| A4      | AD        | 75  | Male  | 2   | 5    |
| C5      | Normal    | 91  | Female | N/A | 0    |
| A5      | AD        | 90  | Female | 4.5 | 5    |
| C6      | Control   | 92  | Female | 7   | 0    |
| A6      | AD        | 90  | Female | 7.5 | 3    |
| C7      | Control   | 80  | Male  | 12  | 0    |
| A7      | AD        | 77  | Male  | 3   | 5    |
| C8      | Control   | 71  | Female | 11  | 0    |
| A8      | AD        | 72  | Female | 5   | 5    |

Frozen tissue samples were partially thawed, and ~100-mg pieces were removed and minced in 5× weight/volume extraction buffer containing 50 mM Tris, pH 7.5, 2% SDS, and a protease inhibitor mixture. After sonication and centrifugation, supernatants were collected, and protein extract concentrations were determined using the Lowry assay. Tissue protein extracts (20 μg) were analyzed by immunoblotting as described above.

Two-dimensional Gel Electrophoresis and Mass Spectrometry Analysis—Because the type and amount of proteins vary dramatically with the growth state of cultured cells (23), care was taken to grow and plate cells under identical conditions where comparisons of protein content were to be made. Exponentially dividing cultures were dissociated and replated at 5 × 10⁶ cells/100-mm tissue culture plate. The next day 10 μM AB₄₂ was added, and 24 h later the cells were harvested for two-dimensional gel electrophoresis. 0.5 ml of 8 M urea, 4% (w/v) CHAPS, 40 mM Tris, 0.2% Bio-Lyte 3–10 ampholytes (Bio-Rad), and 50 mM dithiothreitol were added to the plate, and the cells were scraped into a microcentrifuge tube. DNase1/RNase (100/50 μg/ml) was added to the lysate and incubated for 30 min at room temperature. After centrifugation at 14,000 × g, the protein amount was determined, and 300 μg of supernatant was loaded onto pH 4–7 isoelectric focusing strips (Bio-Rad) and electrophoresed to 60,000 V·h on a Bio-Rad Protean isoelectric focusing machine. The strip was then applied to the top of an 18-cm Bio-Rad precast 12% acrylamide gel and electrophoresed at 25 milliamps per gel until the dye front was at the bottom of the gel. Gels were fixed in 50% methanol overnight and silver stained according to the method of Shevchenko et al. (24). Gel spots from two-dimensional gels were excised, in-gel-digested with trypsin (24), and analyzed by liquid chromatography electrospray ionization mass spectrometry (LC-MS). Briefly, samples were loaded onto a fused silica capillary column (Picofrit Column, New Object, Woburn, MA) packed with reversed phase material (Zorbax C-18, 5-μm particle size, Agilent, Santa Clara, CA). The mobile phase consisted of aqueous 0.1% formic acid (A buffer) and 0.1% formic acid in 80% acetonitrile/water (B buffer). Elution was achieved by a gradient of 5 to 70% B buffer in 65 min at a flow rate of 150 nl/min. The eluant was electrospayed into a Bruker Esquire 3000 Plus quadrupole ion trap mass spectrometer (Bruker Daltonics, Billerica, MA). Spectra were measured for the three most intense species in each time window. For each mass, MS and MS/MS spectra were recorded. After two sets of spectra, the parent mass was excluded for 1 min. The complete data set for each gel spot was searched using the Mascot algorithm (Matrix Science, London, UK) against a recent release of the non-redundant NCBI data base. Only results that gave significant Mascot scores were reported.

Statistical Analysis—Changes in protein expression levels or cell viability were analyzed by ANOVA followed by Tukey post hoc testing using GraphPad InStat software. A p value <0.05 was considered significantly significant.

RESULTS

Aβ-Resistant PC12 Cells Display Increased Prx Levels—Previous studies have shown that Aβ-resistant clonal populations...
of PC12 nerve cells are resistant to multiple forms of oxidative stress (2, 5, 7, 25). Although part of this resistance is attributable to an up-regulation of antioxidant and glycolytic enzymes, the full repertoire of differentially expressed proteins in Aβ-resistant cells has not been examined. We, therefore, compared protein profiles between an Aβ-resistant clone (PC12r7) and its parental cell line (PC12) using two-dimensional gel electrophoresis and liquid chromatography mass spectrometry (LC-MS) analysis to identify other proteins that may contribute to Aβ resistance. The range of proteins that displayed the most dramatic differences in abundance and pI focused between pH 5.3 and 6.5 with a molecular mass between 15 and 50 kDa. Among this subset of proteins 26 spots were identified that varied between the sensitive and resistant cells (Fig. 1). In agreement with earlier work (7), a number of glycolytic enzymes (Table 2) were up-regulated in the PC12r7 clone. Essentially identical results were obtained with another resistant clone, PC12r1. When PC12 cells were transiently exposed to 10 μM Aβ1-42, the same group of enzymes was up-regulated including triosephosphate isomerase, phosphoglycerate mutase, α-enolase, and aldolase A (Table 2). In addition, several antioxidant proteins were also elevated in the resistant and Aβ-treated cells including biliverdin reductase and Prx6.

Curiously, Prx6 was identified in five different locations in the gels (Fig. 1). Although increased catalase and glutathione peroxidase activity have been detected in Aβ-resistant cells (5), the observation that Prx6 expression was elevated in Aβ-resistant cells was new. Because multiple proteins can reside in the same spot and mask the relative protein abundance of Prx isoforms on silver-stained two-dimensional gels, we examined expression levels of all six Prx isoforms in lysates from both parental and Aβ-resistant PC12 cell lines by immunoblotting with isoform-specific antibodies. Although the same amount of protein per lane was loaded, all Prx levels were also standardized against an actin loading control. As expected, PC12-resistant clones displayed elevated levels of Prx6 compared with the parental cell line (Fig. 2). In addition, elevated levels of Prx1 and Prx4 were also detected in the PC12 resistant lines even when taking into consideration the higher expression levels of actin in the resistant cell lines. The elevated actin levels in the Aβ-resistant PC12 clones is probably a reflection of their flatter, more spread-out morphology and adherent properties compared with the parental cell line (5). Comparison of Prx expression levels based on total protein loaded revealed even higher Prx1, Prx2, and Prx6 expression in the resistant lines relative to the parental cell line. In contrast, the expression levels of Prx3 and Prx5 were similar among all cell lines.

Transfection of Prx1 Increases Resistance of Nerve Cells to Aβ Toxicity—Previous studies have shown that overexpression of Prxs can protect neurons from various forms of toxic stimuli including neurotrophic factor withdrawal-induced apoptosis and H2O2-induced cell death (26, 27). To determine whether increased expression of a single Prx isoform can confer resistance to Aβ, both PC12 cells and rat primary hippocampal neu-
rions were transfected with a FLAG epitope-tagged version of Prx1. As a control, cells were also transfected with a Prx1 mutant that lacks both a catalytic cysteine residue (Prx-C52S) and antioxidant activity. PC12 cells transfected with wild type Prx1 exhibit resistance to Aβ-toxicity over the entire 3-day exposure period (Fig. 3A). In contrast, PC12 expressing the Prx1 cysteine mutant (C52S) exhibit sensitivity to Aβ exposure similar to mock-transfected cells. Western blotting with a FLAG antibody revealed that a 2-day Aβ exposure did not affect expression of either the wild type or mutant epitope-tagged Prx1 compared with untreated cells (Fig. 3B). Rat primary hippocampal neurons transfected with wild type Prx1 also exhibit resistance to Aβ, whereas the cysteine mutant confers no protection (Fig. 3C). These results indicate that increased expression of Prx1 can confer resistance to Aβ toxicity that is dependent on a functional catalytic cysteine residue within the protein.

Prx Are Less Susceptible to Cysteine Oxidation in Aβ-resistant Cells—Analysis of the two-dimensional gel protein profiles of the PC12 parental and resistant cells revealed that both Prx3 (thin arrow) and Prx6 (thick arrows) focused at several different isoelectric points (pI) and, in the case of Prx6, exhibited two different electrophoretic mobilities in the second dimension (Fig. 1). To confirm the differences in Prx isoelectric focusing (Fig. 1), PC12 parental and resistant cells were transfected with a FLAG epitope-tagged version of Prx1 and subjected to two-dimensional gel electrophoresis. Immunoblotting with both Prx3 and Prx6 antibodies revealed that the PC12 parental cell line expressed two 26-kDa proteins that migrate at a lower isoelectric point than the corresponding proteins in the PC12 resistant cells (Fig. 1). The PC12 parental cell line also exhibited a 27-kDa protein that migrates at a lower isoelectric point than the corresponding protein in the PC12 resistant cells (Fig. 1). These results indicate that increased expression of Prx1 can confer resistance to Aβ toxicity that is dependent on a functional catalytic cysteine residue within the protein.

**TABLE 2**
MS identification of proteins in PC12 control, PC12r7, and PC12 cells treated with Aβ
Silver-stained proteins in Fig. 1 were picked, digested with trypsin, and identified by LC-MS using the program MASCOT. Spots that were either present (†) or absent (−) relative to each data set are indicated. Spots that displayed a greater than 2-fold increase (↑) or decrease (↓) relative to each data set are also indicated. Ub, ubiquitin; SOD, superoxide dismutase.

| Spot no. | PC12 | PC12r7 | Aβ | Protein name | Accession no. | Molecular mass | Mascot score | Peptides |
|----------|------|--------|----|-------------|---------------|---------------|-------------|---------|
| 1        | +    | −      | −  | Heat shock protein 27 | 204665        | 22,879        | 131         | 3       |
| 2        | +    | +      | +  | Ribosomal protein S12 | 13928992      | 14,259        | 105         | 2       |
| 3        | +    | −      | −  | Histone H2B | 477676        | 13,851        | 178         | 5       |
| 4        | +    | +      | +  | Ribosomal protein S20 | 59926145      | 17,272        | 66          | 2       |
| 5        | +    | −      | −  | Histone 1 | 30061391      | 13,984        | 211         | 9       |
| 6        | +    | +      | +  | Cu,Zn SOD | 16758810      | 17,113        | 114         | 4       |
| 7        | +    | +      | +  | Heat shock protein 27 | 207012        | 15,700        | 273         | 7       |
| 8        | −    | +      | +  | α-Crystallin | 57580        | 19,945        | 190         | 5       |
| 9        | +    | −      | −  | Peroxiredoxin 6 | 11968132      | 28,303        | 106         | 2       |
| 10       | +    | +      | +  | Peroxiredoxin 6 | 16758348      | 24,803        | 280         | 6       |
| 11       | +    | +      | +  | Peroxiredoxin 3 | 11968132      | 28,303        | 106         | 2       |
| 12       | +    | +      | +  | Heat shock protein 27 | 204665        | 22,879        | 169         | 4       |
| 13       | +    | −      | −  | Peroxiredoxin 3 | 11968132      | 28,303        | 283         | 1       |
| 14       | +    | +      | +  | Heat shock protein 27 | 204665        | 22,879        | 315         | 8       |
| 15       | −    | +      | +  | Histone H2B | 477676        | 13,851        | 338         | 7       |
| 16       | +    | +      | +  | Glyceraldehyde-3-phosphate dehydrogenase | 56188        | 35,813        | 60          | 2       |
| 17       | +    | −      | −  | Lysophospholipase 2 | 16758348      | 24,803        | 110         | 4       |
| 18       | +    | +      | +  | Triosephosphate isomerase 1 | 16758348      | 24,803        | 130         | 4       |
| 19       | +    | −      | −  | α-Enolase | 54035341      | 41,851        | 64          | 2       |
| 20       | +    | −      | −  | Proteasome 28 subunit a | 16758348      | 24,803        | 130         | 4       |
| 21       | +    | +      | +  | Heat shock protein 27 | 204665        | 22,879        | 122         | 3       |
| 22       | +    | −      | −  | Heat shock protein 27 | 204665        | 22,879        | 338         | 7       |
| 23       | +    | +      | +  | Heat shock protein 27 | 204665        | 22,879        | 294         | 5       |
| 24       | +    | +      | +  | Heat shock protein 27 | 204665        | 22,879        | 331         | 1       |
| 25       | +    | −      | −  | Heat shock protein 27 | 204665        | 22,879        | 556         | 13      |
| 26       | +    | +      | +  | Heat shock protein 27 | 204665        | 22,879        | 148         | 6       |
| 27       | +    | −      | −  | Heat shock protein 27 | 204665        | 22,879        | 66          | 2       |
Prx3 isoforms (pI 6 and 6.1) and a 23-kDa isoform with a pI of ~5.7. Aβ-treated cells exhibited increased expression of the two 26-kDa Prx3 isoforms in addition to a 26-kDa isoform with a pI of 5.9. In contrast, Prx3 expression was decreased in the PC12r7 line, and only two 26-kDa isoforms (pI 6 and 6.1) were detected. In the PC12 parental cell line two 25-kDa Prx6 isoforms (pI 5.8 and 5.9) and three 22-kDa Prx6 isoforms (pI of 5.7, 6.2, and 6.4) were detected. All Prx6 isoforms were increased in Aβ-treated PC12 cells, and an additional 25-kDa isoform with a pI of 5.75 was also present. In the PC12r7 cell line increased expression of the 25 kDa (pI 5.9) and 22 kDa (pI 6.4) Prx6 isoforms were observed.
The oxidation of the catalytic cysteine residue of typical 2-Cys Prxs to sulfenic, sulfonic, and sulfonic derivatives results in the appearance of more acidic satellite spots after separation by two-dimensional gel electrophoresis (11, 28, 29). To determine whether the increase in acidic isoforms of Prx in Aβ-treated cells was due to increased sulfinylation/sulfonylation, we stripped the blots and then reprobed them with an antibody that specifically recognizes the sulfinic and sulfonic acid derivatives (Prx-SO₂⁻³) of Prx1, Prx2, and Prx3 (29). As expected Aβ treatment resulted in the increased oxidation of three 26-kDa Prx isoforms with a pl of roughly 5.65 and 6.4 in Aβ-treated cells. In contrast, oxidized Prxs were markedly decreased in the PC12r7 clone.

Because the decreased Prx sulfonylation in the Aβ-resistant cells may be due to their enhanced reductive and anti-ROS activity (5), we asked if these cells exhibit decreased sulfonylation even in the presence of oxidants such as hydrogen peroxide (H₂O₂). Aβ-sensitive and -resistant PC12 cell lines were exposed to low (20 μM) and high (100 μM) levels of H₂O₂ for 5 min, harvested, and analyzed by immunoblotting with Prx-SO₂⁻³-specific antibodies (Fig. 4B). H₂O₂ induced a concentration-dependent increase of Prx sulfinylation/sulfonylation in the parental cell line. In contrast, no Prx oxidation was detected in the resistant cell lines after 20 μM H₂O₂ exposure. The level of Prx oxidation in both resistant cell lines was significantly lower than the parental cell line after exposure to 100 μM H₂O₂ (Fig. 4C). To verify that the decrease in H₂O₂-induced sulfonylation in Aβ-resistant cells was not due to decreased Prx expression, the blots were reprobed with several antibodies that specifically recognize Prx isoforms that are also known to be detected with the Prx-SO₂⁻³-specific antibody after oxidation. Both PC12-resistant clones expressed increased levels of Prx1 and Prx2 and similar levels of Prx3 compared with the parental line. Therefore, the decrease in oxidized Prx in Aβ-resistant cells is due to a mechanism that maintains peroxiredoxins in a reduced state and not to an overall decrease in Prx levels.

**Increased Prx Expression Levels and Selective Prx Oxidation in Human AD Cortical Samples**—Previous studies have shown that certain Prx isoforms are elevated in AD brains (17, 30). We, therefore, examined Prx oxidation levels in extracts from postmortem control and AD frontal cortex tissue by immunoblotting with a Prx-SO₂⁻³ antibody (Fig. 6A). Although several AD brains displayed increased Prx-oxidation (AD patients 1, 4, and 7) compared with their age-matched controls, other AD samples exhibited either the same or decreased Prx oxidation levels. Averaging of the densitometric values for the Prx-SO₂⁻³-specific bands revealed no significant difference between control and AD brain sets (Fig. 6B). However, immunoblotting with Prx SO₂⁻³-specific antibodies revealed that Prx1 is significantly elevated (p < 0.05) in all the AD cortex extracts (Fig. 6B).

Although the overall level of Prx oxidation is not significantly elevated in AD cortex samples, we could not discern if a specific subset of Prxs are more oxidized in AD samples because the Prx-SO₂⁻³ antibody recognizes multiple Prx isoforms. We, therefore, analyzed control and AD brains by two-dimensional gel electrophoresis and MS to detect increased acidic (oxidized)
Increased Expression and Resistance to Oxidation of Prxs

In contrast, Western blot analysis of control and AD patient cortical extracts revealed no significant differences in either Trx or TrxR levels between control and AD patients (Fig. 7C). In the absence of an increase in Trx/TrxR expression or NAPDH levels, two rate-limiting redox factors, the increase in Prx1 expression in human AD patient brain tissue may not translate into an overall increase in Prx activity in affected brain regions.

**DISCUSSION**

Initial studies of PC12 clonal cell lines selected for resistance to Aβ revealed that these clones were also resistant to H₂O₂ and t-butyl H₂O₂ compared with the parental cells (2). Further examination demonstrated that the Aβ-resistant clones have higher expression and activity levels of glutathione peroxidase and catalase (5). Although the catalytic efficiencies of Prxs are somewhat lower than those of glutathione peroxidase and catalase, their high abundance in a wide range of cells suggests that they are equally important players in peroxide detoxification (9). In our initial two-dimensional gel electrophoresis proteomic analysis of Aβ-resistant PC12 cells we detected increased expression of Prx6 (Figs. 1 and 2, Table 2). Interestingly, Prx6 appeared on two-dimensional gels as multiple spots with different isoelectric points (pI 5.7, 5.75, 5.8, 5.9, 6.2, and 6.4) and at two different molecular masses (25 and 22 kDa) (Fig. 4).

Prx activity is regulated by post-translational modifications including oxidation (sulfinylation/sulfonylation), phosphorylation, and proteolytic cleavage (11, 32, 33). Both control and Aβ-treated PC12 cells expressed increased levels of both high and low molecular weight acidic Prx3 and Prx6 isoforms as compared with the Aβ-resistant line (Fig. 4). Immunoblot analysis indicated that the relative increase in acidic spots in Aβ-sensitive cells is due to increased oxidation since some of the Prx3-specific spots overlapped with the same spots detected with the anti-PrxSO₂–3 antibody (Fig. 4). Both Prx3 and Prx6 are inactivated after over-oxidation of the catalytic cysteine residue and are the slowest Prx isoforms to be reductively reactivated (34, 35). In addition, Prx3 has been shown to be cleaved and inactivated by a calpain-like cysteine protease after oxidant treatment in lymphoblasts (33). Importantly, Prxs are more

**FIGURE 4. Prxs are less oxidized in the Aβ-resistant cells.** Protein extracts from untreated and Aβ-treated (10 μM, overnight) PC12 cells and the Aβ-resistant clone PC12r7 were resolved by two-dimensional gel electrophoresis, electroblotted, and hybridized with antibodies against Prx3, Prx6, and an antibody that recognizes the sulfonlated/sulfonylated ([Prx-SO₂–3] form of Prx3 (A). Prx3 and Prx-SO₂–3, immunoreactive spots that overlap are indicated by arrows. Immunoblot analysis of PC12 cells and Aβ-resistant clones were treated with indicated concentrations of H₂O₂ for 5 min (B). Low concentration H₂O₂ exposure (20 μM) induced detectable levels of Prx oxidation ([Prx-SO₂–3]) in the parental cell line but not in the resistant clones. Higher concentrations of H₂O₂ (100 μM) induced a lower level of Prx oxidation in the resistant clones. Blots were reprobed with the indicated Prx antibodies and antibodies to actin to determine the relative levels of Prx isoforms that are also detectible by the Prx-SO₂–3 antibody. Densitometric analysis of Prx-SO₂–3 bands in the indicated cell lines treated with 100 μM H₂O₂ (standardized against actin) revealed that Prx oxidation levels are significantly lower (***, p < 0.001, ANOVA) in the Aβ-resistant clones than the parental cell lines (C). Data represent the average ± S.D. of three separate experiments.
resistant to H₂O₂-induced sulfinylation/sulfonylation (Fig. 4B) and H₂O₂-induced cleavage (data not shown) in Aβ/H9252-resistant cells. Therefore, the Aβ-resistant cells appear to have up-regulated mechanisms to maintain Prxs in a reduced state and also to prevent inactivating cleavage events.

Several studies have shown that overexpression of Prxs can protect neurons from various forms of toxic stimuli. Overexpression of Prx1 can prevent neurotrophic factor withdrawal-induced apoptosis and H₂O₂-induced cell death in PC12 cells and mouse hippocampal HT22 cells, respectively (26, 27). Adenoviral transfer of Prx3 can protect mouse hippocampal neurons from excitotoxic injury in vivo (36). Here we show that elevated expression of Prx1 can protect nerve cells from Aβ toxicity (Fig. 3). Interestingly, Prx1, the major Prx isoform that was increased in AD patient brains in our study is primarily expressed in astrocytes and microglia (16, 37). Activated microglia are found in association with plaques in AD brain tissue (38). The increase in Prx1 expression in AD brains may reflect the increased number of microglial cells or may occur as a reaction to the increased ROS production associated with microglial activation and Aβ accumulation (39).

Although Prx oxidation in some AD brains was elevated compared with age-matched control brains, the averaging of overall Prx oxidation levels revealed no significant differences between the two classes (Fig. 6B). However, there was a significant increase in Prx1 expression in all the AD brains relative to control brains (Fig. 6). Previous studies have shown an increase in Prx2 and Prx6 expression in AD post-mortem cortical tissue relative to controls (17, 30). In addition, increased Prx1 expression has been detected in brain tissue from a Huntington disease and prion mouse model (40). Proteomic analysis of cortical tissue from an AD mouse model revealed increased Prx6 expression in older transgenic (tg) animals compared with non-tg littermates (41). Collectively, these findings indicate that increased Prx expression is associated with amyloidogenic diseases.

Although increased Prx expression may be neuroprotective, if the level of ROS exceeds the detoxifying abilities of the vari-
Increased antioxidant systems in the cells, then Prxs become oxidatively inactivated. This appears to be the case for Prx2 in AD brains. In control brains both an acidic and basic spot (pI 5.4 and 5.5) are present, whereas only the acidic spot (pI 5.4) appears in AD brains (Fig. 6C). This acidic isoform in AD brains is most likely an inactive version of Prx2 with an oxidized (acidic) catalytic cysteine residue. Prxs depend on an active catalytic cysteine for antioxidant activity as mutation of this residue in Prx1 results in loss of Aβ-protection (Fig. 3). In an earlier proteomic study of post-mortem cortex samples, Prx2 was significantly increased in frontal cortex from Down syndrome, AD, and Parkinson disease patients (17). In addition, an acidic spot appeared beside the main Prx2 spot in the AD, Down syndrome, and Parkinson disease samples but not in the controls samples, although the authors did not identify this acidic satellite spot (17). A separate proteomic study of hippocampal tissue from a tg AD mouse model revealed increased expression of both basic and acidic Prx2 spots in tg but not wild type mice, suggesting that the two up-regulated spots in tg animals were both the active and inactive form of the enzyme (42). The increase in Prx2 may appear paradoxical as Prx2 is expressed exclusively in neurons, yet this is the same subset of cells that decline during AD progression. One possible explanation for this paradox could be that a compensatory mechanism of increased Prx expression, similar to that observed in Aβ-resistant PC12 cells, might be initiated by surviving neurons in AD brain tissue to protect themselves from Aβ-mediated neurotoxicity.

The catalytic function of Prxs is completely dependent on maintaining the active-site cysteine in a reduced state via the Trx/TrxR system with the ultimate electron donor being NADPH (9). Both Trx and TrxR were elevated in Aβ-resistant PC12 cells relative to controls (Fig. 7). This finding is in accordance with the observation that Prx3 and Prx6 isoforms are found predominately in a reduced state, and the overall degree of Prx oxidation is lower in Aβ-resistant PC12 cells compared with the parental cell line (Fig. 4). Exogenous addition of Trx or TrxR has been shown to protect primary hippocampal cultures from Aβ toxicity (31). Human neuroblastoma SH-SY5Y cells transfected with the Trx1 gene are also resistant to Aβ toxicity (43). In addition, stimulation of PC12 cells with sublethal concentrations of the pro-oxidant 4-hydroxynonenal induces an adaptive response, including up-regulation of TrxR, resulting in protection against subsequent oxidative stress (44). Collectively, these findings suggest that up-regulation of both the Trx/TrxR reductive system and Prx antioxidant enzymes confers resistance to oxidative stress elicited by Aβ exposure.

Although Prx1 expression was elevated in AD patient cortical extracts (Fig. 6), both Trx and TrxR protein levels were not significantly different between control and AD brain samples (Fig. 7). In a previous study, decreased Trx and increased TrxR levels were found in the amygdala and hippocampus regions of AD patients compared with control patients (31). More recently, immunohis-

![Figure 6. Increased Prx1 expression and Prx2 oxidation in brain extracts from AD patients.](image-url)
tochemical analysis of frontal cortex samples from AD patients revealed an overall decrease in Trx1 levels in neurons but an increase in Trx1 immunoreactivity in astrocytes when compared with age-matched control brains (43). In support of our findings, immunoblot analysis of cortex homogenates revealed no significant difference in Trx protein levels between AD and control brains, suggesting that the increased number of glial cells in AD brains masks the reduction in neuronal Trx1 when brain homogenates are analyzed (43). Therefore, in the absence of increased reductive capacity via the rate-limiting Trx/TrxR system and NAPDH levels, the increase in Prx1 levels observed in AD brain tissue is unlikely to result in increased Prx activity.

The major mechanism of producing reducing equivalents in the form of NAPDH is through the PPP (45). In the classical (F-type) PPP, glucose 6-phosphate is converted to ribulose 5-phosphate, leading to the generation of two molecules of NADPH and one of CO₂. The PPP consists of two branches, an irreversible oxidative branch that produces NADPH and ribulose 5-phosphate and a reversible, nonoxidative branch that permits the interconversion of glycolytic intermediates with pentose phosphates (45). Ribulose 5-phosphate can be converted to fructose 6-phosphate and glyceraldehyde 3-phosphate 3-phosphate and reenter the glycolytic pathway through the nonoxidative branch of the PPP (46). During conditions of oxidative stress, requiring the maximal amount of NADPH, glucose 6-phosphate is completely oxidized to CO₂ by six complete cycles through the PPP. Previously, we showed that Aβ-resistant PC12 cells have an enhanced flux of glucose through both the glycolytic pathway and PPP (7). This enhanced glucose metabolism in the resistant cells is mediated in part by activation of the transcription factor hypoxia-inducible factor 1 (7). As a result of an enhanced PPP, including glucose-6-phosphate dehydrogenase activity, the Aβ-resistant cells produce higher levels of NADPH (7, 46). Among the elevated glycolytic enzymes that were detected in our proteomic analysis of Aβ-resistant PC12 cells were transketolase, triosephosphate isomerase, and aldolase (Table 2). Transketolase catalyzes the reversible transfer of a ketol group from fructose 6-phosphate to glyceraldehyde 3-phosphate to generate erythro-4-phosphate and xylulose 5-phosphate in the classical PPP. Aldolase catalyzes the cleavage of fructose 1,6-diphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (both triose phosphates), and triosephosphate isomerase catalyzes the isomerization of dihydroxyacetone phosphate to glyceraldehyde 3-phosphate during glycolysis. However, both enzymes can also function in an alternate (L-type) PPP to facilitate the interconversion of triose phosphates and glycolytic hexose and thereby allow reentry of glycolytic intermediates back into either the oxidative or nonoxidative branch of the PPP (45). Therefore, one possible mechanism of Aβ resistance is through an up-regulation of glycolytic enzymes that can also recycle pentose phosphates through either the L-type or F-type PPP, leading to increased NAPDH production. Increased NAPDH production is vital for maintaining the activity of glutathione peroxidase, Trx/TrxR and the Prxs, thereby ensuring a robust antioxidant defense. Our findings suggest that augmentation of NAPDH or the Trx/TrxR system either directly with thiol-based antioxidants or indirectly through agents that increase the PPP may be of therapeutic relevance to AD and possibly other neurodegenerative disorders.

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Increased Expression and Resistance to Oxidation of Prxs

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