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Delivery of a protein transduction domain-mediated Prdx6 protein ameliorates oxidative stress-induced injury in human and mouse neuronal cells

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Singh SP, Chhunchha B, Fatma N, Kubo E, Singh SP, Singh DP. Delivery of a protein transduction domain-mediated Prdx6 protein ameliorates oxidative stress-induced injury in human and mouse neuronal cells. Am J Physiol Cell Physiol 310: C1–C16, 2016. First published October 7, 2015; doi:10.1152/ajpcell.00229.2015.—Oxidative stress or reduced expression of naturally occurring antioxidants during aging has been identified as a major culprit in neuronal cell/tissue degeneration. Peroxiredoxin (Prdx) 6, a protective protein with GSH peroxidase and acidic calcium-independent phospholipase A2 activities, acts as a rheostat in regulating cellular physiology by clearing reactive oxygen species (ROS) and thereby optimizing gene regulation. We found that under stress, the neuronal cells displayed reduced expression of Prdx6 protein and mRNA with increased levels of ROS, and the cells subsequently underwent apoptosis. Using Prdx6 fused to TAT transduction domain, we showed evidence that Prdx6 was internalized in human brain cortical neuronal cells, HCN-2, and mouse hippocampal cells, HT22. The cells transduced with Prdx6 conferred resistance against the oxidative stress inducers paraquat, H2O2, and glutamate. Furthermore, Prdx6 delivery ameliorated damage to neuronal cells by optimizing ROS levels and overstimulation of NF-κB. Intriguingly, transduction of Prdx6 increased the expression of endogenous Prdx6, suggesting that protection against oxidative stress was mediated by both extrinsic and intrinsic Prdx6. The results demonstrate that Prdx6 expression is critical to protecting oxidative stress-evoked neuronal cell death. We propose that local or systemic application of Prdx6 can be an effective means of delaying/postponing neuronal degeneration.

Prdx6; hydrogen peroxide; reactive oxygen species; transduction domain; NF-κB

REACTIVE OXYGEN SPECIES (ROS)-driven oxidative stress has been implicated in initiation and progression of several age-associated neurodegenerative disorders, such as Alzheimer’s and Parkinson’s diseases (2, 6). Recent in vitro and in vivo studies support the notion that neuronal cell death and tissue degeneration are tightly linked to excessive oxidative load induced by cellular or environmental stimuli or reduced expression of antioxidants. Cells with lower expression of antioxidants lose their homeostasis and become vulnerable to oxidative damage (5). Furthermore, ROS are produced intracellularly as a byproduct of physiological processes including excess release of glutamate (4, 21) as well as external environmental stress. Moreover, glutamate plays an important role in neurological diseases (11, 35). Its excessive production causes neuronal cell death mediated by oxidative stress (31, 35). The overexpression of intracellular ROS is controlled by antioxidant defenses such as catalase, superoxide dismutases (SOD), glutathione peroxidase (GPx), and, most importantly, the newly discovered peroxiredoxin (Prdx) family. This family includes six members that are classified as having 1-cysteine (Cys) or 2-Cys. Prdx 1 through Prdx5 have 2-Cys; Prdx6 has 1-Cys at position 47 (Cys47). The antioxidant protein Prdx6 has GSH peroxidase as well as acidic Ca2+ -independent phospholipase activities (aiPLA2) (13, 25, 43). Prdx6 uses redox-active Cys47 to reduce peroxides and protect cells from membrane, DNA, and protein damage mediated by lipid peroxidation (12, 43). Prdx6, a cytosolic protein, is also present in lysosomes (59), endoplasmic reticulum (ER), plasma membrane, and mitochondria. Its localization pattern explains its biological importance in regulating ROS (20).

However, alterations in the cellular environment have been shown to be associated with modulation in the activity of several transcription factors including NF-κB (49, 51). At normal physiological condition, NF-κB is inactive; it is activated during cellular stresses (52). The proapoptotic and antiapoptotic roles of NF-κB depend on cell types and cellular microenvironment as well as levels of intracellular ROS (45). Several published reports have shown that NF-κB is a regulator of several genes, and by doing so it exerts its proapoptotic and antiapoptotic roles (13, 39). Thus it seems reasonable that modulation of ROS production at certain levels might alter the activity of NF-κB, leading to repression of Prdx6 in cells. Prdx6 is known to protect many cell types derived from both mouse and human against various oxidative stresses, such as H2O2, paraquat, UVB, heat stress, and ER stress (12, 25). Furthermore, Prdx6 is a potent cytoprotective enzyme in the epidermis (40) and is required for blood vessel integrity in wounded skin (41). Prdxs are distributed in the normal mammalian brain (29). Aon-Bertolino et al. (3) reported a relatively intense Prdx6 staining in the hippocampus of the rat brain (34). These qualities make Prdx6 an ideal molecule for therapeutic use. However, despite its recognized functions, little is known whether extrinsically delivered recombinant Prdx6 has a pro-
tective role for neuronal cells. Neuronal cells have been found more sensitive than others, possibly because of higher consumption of oxygen and lower capacity to regenerate than other cells or tissues (2, 36). Furthermore, markers of oxidative damage have been observed in the brain tissue of patients with neurodegenerative diseases, suggesting a pivotal role for oxidative stress (55). Recent evidence from genetically engineered mouse lines also shows the roles of oxidative stress and loss of antioxidant protein in neuronal cell degeneration (36).

Advances in protein/gene delivery and identification of several protein transduction domains have made possible the delivery of proteins to cells or organs (30, 38). HIV-transactivator of transcription (TAT) domain has 11 amino acids (aa; YGRKKRRQRRR) and has 100% potential for intracellular delivery of proteins across the plasma membrane and the blood-brain barrier (BBB) (8, 24, 38, 46). In the present study, we used Prdx6 for extrinsic delivery to neuronal cells HCN-2 and HT22 facing oxidative stress induced by internal and external oxidative stress inducers. We assessed the efficacy of a transduction protein-linked Prdx6 in protecting those neuronal cells, keeping in mind the possibility of developing a rationale for developing a therapeutic molecule for age-related degenerative diseases. We showed that the oxidative stressor—H₂O₂, paracetamol, or glutamate—caused a loss of Prdx6 in degenerative diseases. We showed that the oxidative stressor—rationale for developing a therapeutic molecule for age-related neurodegenerative diseases, suggesting a pivotal role for oxidative stress inducers. We assessed the efficacy of and HT22 facing oxidative stress induced by internal and we used Prdx6 for extrinsic delivery to neuronal cells HCN-2 blood-brain barrier (BBB) (8, 24, 38, 46). In the present study, lular delivery of proteins across the plasma membrane and the delivery of proteins to cells or organs (30, 38). HIV-trans
dication into cells, as described earlier (37). HCN-2 or HT22 cells (5 × 10⁵) were cultured in 96-well plates for 24 h with DMEM-10% FBS, after which cells were exposed to different concentrations of glutamate and/or paracetamol (in complete media) and/or H₂O₂ (in DMEM medium containing 0.2% BSA). After 8 h or 24 h, cells were washed and medium was replaced with HBSS and incubated in the same buffer containing 10 μM H₂O₂-DCF-DA dye. After 30 min of incubation at 37°C, intracellular fluorescence was recorded at excitation 485 nm and emission 530 nm (Ex485/Em530nm) by Spectra Max Gemini EM (Molecular Devices, Sunnyvale, CA). To examine the effect of TAT-HA-Prdx6 or its mutant at Cys47 (to isoleucine 47, I47), HCN-2 or HT22 cells were pretreated with TAT-HA-Prdx6-mut or TAT-HA-Prdx6 for 3 h followed by paracetamol and/or glutamate and/or H₂O₂ stress. After 8 h or 24 h, ROS levels were measured at Ex485/Em530nm as described above.

**Cell viability assay (MTS assay).** A colorimetric MTS assay (Promega, Madison, WI) was performed as described previously (37). Briefly, 5 × 10⁵ cells were cultured in 96-well plates for 24 h with DMEM-10% FBS and were exposed to different concentrations of glutamate and/or paracetamol (in complete media) and/or H₂O₂ (in DMEM medium containing 0.2% BSA) for 24 h. This assay of cellular proliferation/viability uses 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS). When added to medium containing viable cells, MTS is reduced to a water-soluble formazan salt. The A₄₉₀nm value was measured after 4–6 h with a microplate reader. Results were normalized with absorbance of the untreated control(s).

**Expression and purification of TAT-HA-Prdx6 recombinant protein.** A full-length cDNA of Prdx6 was isolated from a human LEC cDNA library using Prdx6-specific sense (5'-GTGCCGCAATGGCGGAGGAGCGTCGTGCTTC-3' contained in Ncol site) and antisense (5'-AATTGGGACGCTGACATCCTCTGGTC-3') primers. The PCR products were purified by preparative agarose gel electrophoresis. The purified products were ligated into a TA-cloning vector (Invitrogen) and then transformed into a competent cell, and the plasmids of selected colonies were purified. The purified TA vector containing Prdx6 cDNA was digested with Ncol and EcoRI and then subcloned into a pTAT-HA expression vector (a kind gift of Dr. S. F. Dowdy) that had been digested with the same restriction enzymes. Recombinant protein was purified using QIAexpress Ni-NTA Fast Start kit column (Qiagen, Valencia, CA). The host *Escherichia coli* BL21 (DE3) was transformed with pTAT-HA-Prdx6, and the transformants were selected on a Luria broth (LB) plate with ampicillin. The selected colonies were cultured in 10 ml LB medium containing ampicillin at 37°C with shaking at 200 rpm overnight. After incubation, 10 ml of the overnight cultures were combined with 250 ml of prewarmed media (with ampicillin) and were then grown at 37°C with vigorous shaking using an OD₆₀₀ = 0.6–0.8. Isopropylthiogalactoside (IPTG) was added to a concentration of 1 mM, and the incubation was continued for 4–5 h. Cells were harvested by centrifugation at 4,000 g for 20 min. Pellets were suspended in 10 ml of lysis buffer (50 mM NaH₄PO₄, 50 mM NaCl, and 10 mM imidazole, pH 8.0) containing lysozyme and benzamidase nuclease and incubated for 30 min on ice. The suspension was then centrifuged at 14,000 g for 30 min. Supernatant was added to the Ni-NTA fast start column and allowed to
Prdx6’s ROLE IN NEUROPROTECTION

C3

drain before being washed twice with 4 ml of wash buffer (50 mM NaH2PO4, 50 mM NaCl, and 20 mM imidazole, pH 8.0), followed by elution with an elution buffer (50 mM NaH2PO4, 50 mM NaCl, and 250 mM imidazole, pH 8.0). Finally, the eluent was dialyzed to remove imidazole. Furthermore, a batch of recombinant protein, TAT-HA-Prdx6 was passed through Detoxi-Gel Endotoxin Removing Gel column (product no. 20344, Pierce) to remove endotoxin contamination, if any. This purified protein can be either used to transduce HCN-2 and HT22 cells, or aliquoted and stored frozen in 10% glycerol at −80°C for further use. To monitor TAT-HA-Prdx6 internalization into cells, cultured neuronal cells were supplied with TAT-HA-Prdx6. At predefined time intervals, cell were washed and treated with mild trypsin exposure to remove TAT-HA-Prdx6 contamination on the cell wall, if any. Cellular extracts was prepared and immunoblotted using Prdx6-specific antibody.

Site-directed mutagenesis. PCR base site-directed mutagenesis was carried out using the QuikChange site-directed mutagenesis kit (Invitrogen), following the company’s protocol. Because cysteine (Cys) 47 of Prdx6 is responsible for its antioxidant property (GSH peroxi-
tatic, Indianapolis, IN) in a Roche LC480 Sequence detector System. PCR was performed with SYBR Green Master Mix (Roche Diagnos-
script II RNAase H-Reverse Transcriptase. Quantitative real-time PCR base site-directed mutagenesis was

Table 1. Primer sequences

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Primer Sequence</th>
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<tbody>
<tr>
<td>Prdx6 (human)</td>
<td>Forward: 5′-GCGATCCTGTTTCCACAGACT-3′</td>
</tr>
<tr>
<td>β-Actin (human)</td>
<td>Forward: 5′-CGACGACGAGAGATGAC-3′</td>
</tr>
<tr>
<td>Prdx6 (mouse)</td>
<td>Forward: 5′-TTTCTAATAGACAGTGGTAGAC-3′</td>
</tr>
<tr>
<td>β-Actin (mouse)</td>
<td>Forward: 5′-CTAAGGCACACGTTGGAAG-3′</td>
</tr>
</tbody>
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Prdx6, peroxiredoxin 6.

Determinant of NF-κB activation using HIV-1 LTR-CAT. To learn whether NF-κB is activated in H22 neuronal cells facing H2O2-induced oxidative stress, and the activation of the NF-κB signaling is functional, HIV-1 LTR-CAT constructs and its mutant at NF-κB site (a kind gift from Dr. Carole Kretz-Remy) were used to transfect HT22 cells. The HIV-1 promoter contains binding sites for many transcriptional factor NF-κB, and can be upregulated 12- to 150-fold following various stresses, including oxidative stress (23). Thus, we used pL-TR-CAT wild type and pL-TR-CAT PstI (where NF-κB sites are disrupted) to monitor the activation of NF-κB. HT22 cells were transfected with mutant and/or wild-type constructs and 24 later were transduced with TAT-HA-Prdx6-mut or TAT-HA-Prdx6 for 3 h followed by pararaquat and/or glutamate exposure. After 48 h, CAT-ELISA was performed to monitor CAT activity following the manufacturer’s protocol. Absorbance was measured at 405 nm using a microtiter plate ELISA reader. Transactivation activities were adjusted for transfection efficiencies using GFP/SEAP values (13, 23).

Statistical analysis. Data are presented as means ± SD of the indicated number of experiments. Data were analyzed by Student’s t-test when appropriate. P < 0.05 indicates a statistically significant difference.

RESULTS

Loss of Prdx6 in HCN-2 neuronal cells during oxidative stress resulted in ROS accumulation and cell death and was rescued by an extrinsic supply of Prdx6-linked protein transduction domain. Recent reports show ROS-driven oxidative stress as a cause of neuronal cell death that ultimately leads to age-related degenerative diseases (13, 44). To evaluate whether loss of Prdx6 in neuronal cells is associated with increased ROS expression and decreased cell viability during oxidative stress, we exposed primary cell culture of HCN-2 to different concentrations of pararquat, a superoxide generator (12, 19). We assessed cell viability and expression levels of ROS and Prdx6, as shown in Fig. 1. A–C. Viability of HCN-2 cells was decreased with an increase in pararquat concentration (Fig. 1A; black bar, 100 and 200 μM), and this was directly related to an increase of oxidative load as evidenced by levels of ROS quantified using H2DCF-DA dye method (Fig. 1B). We next monitored the expression level of Prdx6 in the pararquat-treated cells and found a reduction. Cellular extracts were resolved onto SDS-PAGE and immunoblotted with antibody specific to Prdx6. Prdx6 expression was dramatically decreased in pararquat-treated HCN-2 cells (Fig. 1C), and the reduction was correlated with a significant increase in ROS levels (Fig. 1B) and a decrease in cell viability (Fig. 1A). The experiment indicated that a reduction in level of Prdx6 could increase HCN-2 cell death caused by ROS-evoked oxidative stress. To evaluate whether delivery of extrinsic Prdx6 would lessen the rate of cell death, we prepared a protein transduction domain-linked recombinant Prdx6 and examined the transduction and protective efficacy of recombinant TAT-

HCN-2 cell death caused by ROS-evoked oxidative stress.
immunoblotted with anti-Prdx6 antibody to examine internalization of TAT-HA-Prdx6 in cells. Figure 2A illustrates that TAT-HA-Prdx6 internalized efficiently in the HCN-2 cells. This confirmed our as well as others’ previous reports (8, 37). We used different concentrations of TAT-HA-Prdx6 protein to assess its cytotoxicity against cells (data not shown). Thus, we chose 5 μg/ml of TAT-HA-Prdx6 to assess its protective potential against paraquat-induced cellular injuries as this concentration was nontoxic. We examined the efficiency and functionality of transduced Prdx6-linked to TAT transduction domain in attenuating paraquat-induced HCN-2 cell death. Cultured HCN-2 cells pretreated with 5 μg/ml TAT-HA-Prdx6 or its mutant TAT-HA-Prdx6 (where its redox-active functional site, Cys47, was destroyed by exchanging Cys47 with I47) as a control were exposed to paraquat for 24 h as depicted in Fig. 2. As expected, the cell survival assay revealed that delivery of Prdx6 in culture gave the cells significant protection from paraquat-induced death (Fig. 2B; black bar vs. gray bar). Quantitation by H2-DCF-DA dye (13, 25) demonstrated that Prdx6 acted to protect cells by reducing their oxidative load (Fig. 2C, black bar vs. gray bar). Figure 2D is representative of the photomicrographs of HCN-2 cells cultured without (Fig. 2D,a) and with (Fig. 2D,b) paraquat plus TAT-HA-Prdx6-mut at the Cys47 site or paraquat plus TAT-HA-Prdx6 (Fig. 2D,c). Careful microscopic examination showed that cells treated with wild-type TAT-HA-Prdx6 had increased growth and survival and plate wells with fewer floating/dead cells in comparison with cells treated with paraquat plus TAT-HA-Prdx6-mut only (Fig. 2D, b vs. c).

Prdx6 knockdown assay revealed that Prdx6 was a prerequisite for HCN-2 cell protection against stressors. We initially examined the expression of all members of the Prdx family in HCN-2 cells. Western analysis revealed the presence of all Prdx’s members (Prdx 1–6) in HCN-2 cells; however, Prdx6 was found to be relatively highly expressed (Fig. 3A). This finding indicates that the expression of Prdx6 may be cell/tissue-specific. Furthermore, from the results derived from the above experiments (Figs. 1 and 2), it was apparent that reduction in Prdx6 expression was associated with increased expression of ROS and reduced viability of cells against oxidative stress. However, from those experiments it was not clear whether Prdx6 is a prerequisite for cell survival during oxidative stress. To examine this, we utilized antisense of Prdx6 to knock down Prdx6 expression by transfecting the cells with antisense (As)-Prdx6 construct. Western analysis revealed that transfectants had significant reduced expression of Prdx6 (Fig. 3B1). Next, we performed experiments using these transfectants, and Prdx6-deficient cells were divided into four groups as shown in Fig. 3, B and C. Cells with scramble siRNA or As-Prdx6 (Prdx6-deficient) were supplied with TAT-linked Prdx6-mut or wild-type, TAT-linked Prdx6 and then exposed to paraquat-induced oxidative stress. Cell viability assay at 24 h revealed TAT-linked Prdx6’s ability to protect cells against paraquat-induced cell death (Fig. 3B). Quantitation of ROS expression in Prdx6 knockdown HCN-2 cells by the oxidative conversion of DCF-DA to fluorescent DCF showed that TAT-linked Prdx6-mut failed to lower ROS, while TAT-linked Prdx6 did lower ROS expression (Fig. 3C), and thereby enhanced the viability cells (Fig. 3B). Interestingly, cells containing reduced levels of Prdx6 (As-Prdx6) were highly susceptible to increased ROS-driven cell death (Fig. 3, B and C; scramble siRNA vs. As-Prdx6 groups), and cell death further accelerated following paraquat exposure and TAT-linked Prdx6-mut could not protect cells, suggesting that loss of naturally occurring Prdx6 is a cause of cell death, and extrinsic supply of TAT-linked Prdx6 had the ability to protect the cells against stress. This finding signifies the physiological importance of Prdx6 expression in HCN-2 cells. Furthermore, our current finding also indicates that the expression levels of Prdx 1–5 could not maintain HCN-2 integrity against stressors. Since other Prdxs could not counteract oxidative stress-induced injuries occurring in Prdx6-deficient HCN-2 cells, we consider the role of Prdx6 as major, at least in HCN-2 cells.
HT22 murine hippocampal neuronal cells showed reduced viability with increased ROS and reduced Prdx6 expression during oxidative stress by paraquat or H₂O₂. As oxidative stressors from different sources can differ in their effect on specific cell types, we selected mouse hippocampal cell line HT22 to see whether downregulation of Prdx6 is indeed associated with increased ROS levels and cell death. Because overproduction of ROS is associated with reduced expression or malfunction of antioxidants during aging or oxidative stress, we used the most abundant ROS generator in nature, H₂O₂, and the chemical paraquat to evaluate their effects on cell viability and its association with oxidative load and Prdx6 expression during oxidative stress. HT22 cells were exposed to variable concentrations of either paraquat or H₂O₂ as shown in Figs. 4 and 5, respectively. Applying increasing concentrations of either caused a dose-dependent decrease in cell survival (Figs. 4A and 5A; black bars). Examining the levels of ROS using H₂-DCF-DA assay, we found that the decrease in cell viability was directly related to increased levels of ROS (Figs. 4B and 5B; black bars), suggesting that ROS-driven oxidative stress caused HT22 cell death with exposure to either paraquat or H₂O₂. Figs. 4C and 5C show photomicrographs of cells taken at 24 h of oxidant(s) treatment. Microscopic analysis of well plates revealed that the cell death induced by paraquat or H₂O₂ was concentration-dependent, as increasing numbers of white rounded and floating dead cells were observed with an increase of oxidant concentration in well plates. Since downregulation of Prdx6 is associated with ROS-induced cell injury (13, 25), we tested whether Prdx6 expression was modulated during oxidative stress. Cells exposed to either paraquat or H₂O₂ as shown in Figs. 4, D and E, and 5, D and E, were subjected to expression analysis for Prdx6 protein and transcript as described in MATERIALS AND METHODS. Immunoblotting of cell lysates and real-time PCR analysis of Prdx6 transcript using Prdx6-specific probes showed an obvious decrease in band of 24 kDa of Prdx6 protein and mRNA (Figs. 4, D and E, and 5, D and E), respectively. This indicated that oxidants repressed Prdx6 transcription, probably by affecting transcriptional machinery.

A transduction domain linked-Prdx6 protein was efficacious in alleviating oxidative stress-induced cellular insult in mouse hippocampal cells, HT22. Because the experiments including Prdx6 knockdown experiment described above showed that loss of Prdx6 can be related to neuronal cell death, we next examined the protective effectiveness of TAT-linked recombinant protein Prdx6 in protecting HT22 neuronal cells against oxidative stress. Transactivator of transcription (TAT)-HA-Prdx6 internalized and enhanced cell viability by blunting oxidative stress evoked by paraquat. A: transduction of TAT-HA-Prdx6 in HCN-2 cells. TAT-HA-Prdx6 was added to culture media and transduction was assessed after 24 h. Cells were washed, and extracted lysate was immunoblotted using anti-Prdx6 antibody. B: MTS assay showing effect of TAT-HA-Prdx6 in protecting HCN-2 cells against paraquat-induced cytotoxicity. Cells were cultured in 96-well plates containing 5,000 cells/well in complete DMEM medium supplemented with either TAT-HA-Prdx6-mut or TAT-HA-Prdx6 for 3 h then exposed to paraquat for further 24 h. Cell viability was estimated using MTS assay as indicated (gray bar vs. black bar). Results are means ± SD of three individual experiments. *P < 0.001. C: intracellular ROS expression in cells was reduced by addition of TAT-HA-Prdx6. Cells were pretreated with either TAT-HA-Prdx6-mut or TAT-HA-Prdx6 (5 μg/ml). After 3 h cells were exposed to paraquat and ROS were measured using H₂-DCF-DA as described in MATERIALS AND METHODS. Results shown are means ± SD of three individual experiments (black bars vs. gray bar, *P < 0.001). D: photomicrograph of HCN-2 cells showing protective effects of transduced TAT-HA-Prdx6 against paraquat. a, control; b, paraquat (100 μM) plus TAT-HA-Prdx6-mut (5 μg/ml); c, paraquat (100 μM) plus TAT-HA-Prdx6 (5 μg/ml). Arrowhead indicates dead cells.
both paraquat and H₂O₂. As shown in Figs. 6 and 7, cultured HT22 cells in 35-mm plates were supplied with TAT-HA-Prdx6 or its mutant at Cys47 to I47 (wherein GSH peroxidase activity is destroyed) as described in MATERIALS AND METHODS (38). We found that 5 μg/ml of TAT-HA-Prdx6 was the optimum concentration for the HT22 cells, as it had no cytotoxic effect (data not shown). Thus, that concentration was chosen as the optimum effective dose. We exposed HT22 pretreated with TAT-HA-Prdx6-mut or wild-type TAT-HA-Prdx6 to the oxidants. Figures 6A and 7A illustrate enhanced viability of HT22 cells (gray bar vs. black bar). Quantitation of ROS in HT22 by H₂-DCF-DA dye demonstrated that Prdx6 acted by reducing the oxidative load (Figs. 6B and 7B, gray bar vs. black bar) exerted by paraquat or H₂O₂ exposure. Furthermore, to examine whether Prdx6-depleted HT22 cells (Prdx6 knockdown experiments) were more vulnerable to oxidants, we found indeed that loss of Prdx6 is causally related to HT22 cell death (data not shown) as observed in the knockdown experiments with HCN-2 cells (Fig. 3, B and C).

Next, we determined cell death caused by those oxidants by microscopic examination and Annexin V-FITC binding assay followed by FACS analysis as described previously (12). Photomicrographs of HT22 pretreated with Prdx6 were taken 24 h after oxidant exposure, revealing significantly fewer dead cells and increased growth (Figs. 6C and 7C, gray bar vs. black bars) compared with cells pretreated with TAT-HA-Prdx6-mut, suggesting that indeed, the extrinsic supply of Prdx6 was efficacious in protecting HT22 cells. Furthermore, cells pretreated with TAT-HA-Prdx6 and exposed to either of the oxidants showed fewer apoptotic cells. As shown in Figs. 6D and 7D, the percentage of apoptosis was significantly decreased compared with Prdx6-mut pretreated cells (Fig. 6D, gray bars vs. black bars). These data suggest that an extrinsic supply of TAT-linked Prdx6 had the ability to attenuate oxidative stress-induced cellular insults caused by different types of stressors. However, we also observed that cell death was not absolutely apoptotic, but it also was necrotic. We inferred...
that Prdx6 contains the ability to abate the processes of both kinds of cell death.

Glutamate induced cell death and increased ROS production with reduced expression of Prdx6 in HT22 neuronal cells. Glutamate-mediated neuronal cell death plays a major role in the etiology of neurodegenerative disorders (9). However, glutamate-induced cytotoxicity is associated not only with the excitotoxic pathway, but also with ROS-driven oxidative stress (15, 63). We examined whether glutamate-induced HT22 injuries are associated with reduced expression of Prdx6 and increased oxidative load. HT22 cells exposed to variable concentrations of glutamate showed a dose-dependent decline in cell viability at 24 h as evident from Fig. 8A. MTS analysis showed reduced cell viability of ~30% and ~60% in cells exposed to glutamate at 5 mM and 10 mM, respectively. Glutamate exposure at 20 mM was very toxic, causing >95% cell death. Based on these results, we decided to use 5 mM glutamate to induce toxicity and evaluate the protective efficacy of Prdx6 against these molecules. ROS levels were measured by H$_2$DCF-DA dye quantitation assay, which showed increased ROS expression in the exposed cells (Fig. 8B). The increase was directly related to cell death (Fig. 8, A–C). A representative photomicrograph (Fig. 8C) illustrates cell death, the rate of which was dependent on the concentration of glutamate. Cells expressing higher levels of ROS had significantly reduced expression of Prdx6 protein (Fig. 8D, lane 2) and mRNA (Fig. 8E, black bar vs. gray bar). The data revealed that oxidative stress driven by excess ROS production contributed to glutamate-induced HT22 cell death and that Prdx6 expression level played an important role in survival of the cells.

Extrinsic application of Prdx6 mitigated glutamate-mediated cytotoxicity in HT22 murine hippocampal neuronal cells by lowering intracellular ROS expression. Prdx6 expression has been shown to be essential in many types of cells for growth and protection against various stressors (12, 20, 22). Based on our current studies and reports by others (20, 37, 41, 43), we examined whether the moonlight protein Prdx6 would
protect cells directly exposed to the detrimental effects of glutamate, which exerts its cytotoxicity not only by producing oxidative stress, but also by other death pathways (15, 28, 54, 63). To test the protective ability and efficacy of Prdx6, HT22 cells were pretreated with TAT-HA-Prdx6 (5 μg/ml). After 3 h, cells were subjected to 5 mM glutamate for 24 h as shown in Fig. 9. Results showed that, indeed, addition of TAT-HA-Prdx6-mut or TAT-HA-Prdx6 in culture significantly protected the cells against glutamate-induced cell death. Quantification by H2-DCF-DA dye assay as described in MATERIALS AND METHODS established a lower prevalence of ROS in cells pre-treated with TAT-HA-Prdx6 (Fig. 9B, gray bar vs. black bar), arguing that Prdx6 protected cells by optimizing ROS. Possibly, glutamate produces its cytotoxicity by increasing production of ROS and reducing expression of antioxidants such as Prdx6. However, DCF fluorescence is not specific for H2O2 and other oxidants, such as O2 and NO, and may also oxidize H2-DCF in DCF. Thus, measured fluorescence reflected overall oxidative stress in cells. Next, we determined the type of cell death, performing microscopic analysis and Annexin V-FITC binding assay, followed by FACS analysis. Fig. 9C, a, b, and c is representative of the photomicrographs taken after 24 h of glutamate exposure. The figure demonstrates that, indeed, extrinsically supplied Prdx6 protected HT22 cells from glutamate-induced oxidative stress. H2O2-exposed or unexposed cells were processed for isolation of cell lysate and RNA as described in MATERIALS AND METHODS. Cell lysates containing equal amounts of protein or RNA were immunoblotted and subjected to real-time PCR with Prdx6-specific probe. D, immunoblot analysis, lane 1 vs. 2 and E, gray bar vs. black bar. *Statistically significant difference (P < 0.001 vs. control).

NF-κB was activated by ROS and was involved in repressing Prdx6 expression in HT22 cells exposed to oxidative stressors. ROS have been shown to induce activation of NF-κB. We surmised that NF-κB activation may be involved in modulation of Prdx6 expression depending on the cellular microenvironment and cell background (13, 24). The results shown in Figs. 4, 5, and 8 reveal downregulation of Prdx6 mRNA, suggesting
that Prdx6 repression can be at the transcriptional level as Prdx6 gene promoter has NF-κB-responsive elements. We postulated that repression of Prdx6 in HT22 may be associated with overactivation of NF-κB by overshooting of increased ROS, leading to a vicious feed-forward process and resulting in accumulation of ROS. Initially, we tested whether NF-κB is activated in HT22 cells during oxidative stress induced by paraquat (Fig. 10, A and B), and then we exposed to paraquat (0.5 mM). ROS levels were measured at 8 h and/or 24 h by replacing the medium with Hanks’ solution that had 10 μM H$_2$DCFH-DA dye. The histogram is representative of three experiments. C: HT22 cells were cultured and either untreated (a) or pretreated with TAT-HA-Prdx6-mut (b) or TAT-HA-Prdx6 (c) and were then exposed to paraquat (0.5 mM). After 24 h, cells were photomicrographed. Arrows point to white and rounded dead cells. D: cells were trypsinized, and the percentage of apoptotic cells was assessed by Annexin V-FITC staining, followed by fluorescence-activated cell sorter (FACS) analysis. A representative photomicrograph and FACS analysis of Annexin V-FITC and propidium iodide (PI) staining are provided. E: histograms reveal relative percentage of inhibition of apoptosis by Prdx6 against paraquat-induced oxidative stress. Results are expressed as means ± SD for three replicated determinations in each treatment group and were significant (*P < 0.001) compared with control.

Fig. 6. TAT-HA-Prdx6 attenuated paraquat-induced HT22 cell death by nullifying oxidative load and blocking the apoptotic process. A: enhanced viability of HT22 cells supplied with either TAT-HA-Prdx6-mut or TAT-HA-Prdx6 exposed to paraquat demonstrated by MTS assay. Cells were pretreated with 5 μg/ml of TAT-HA-Prdx6 or with mutant Prdx6 (inactive) and then subjected to paraquat stress (0.5 mM) for 24 h. The effect of TAT-HA-Prdx6 on cell viability was assessed by MTS assay. The results (histograms) are means ± SD of three experiments. B: effect of TAT-HA-Prdx6 on reducing ROS expression. Cells were pretreated with either TAT-HA-Prdx6-mut or TAT-HA-Prdx6 protein and then exposed to paraquat (0.5 mM). ROS levels were measured at 8 h and/or 24 h by replacing the medium with Hanks’ solution that had 10 μM H$_2$DCFH-DA dye. The histogram is representative of three experiments. C: HT22 cells were cultured and either untreated (a) or pretreated with TAT-HA-Prdx6-mut (b) or TAT-HA-Prdx6 (c) and were then exposed to paraquat (0.5 mM). After 24 h, cells were photomicrographed. Arrows point to white and rounded dead cells. D: cells were trypsinized, and the percentage of apoptotic cells was assessed by Annexin V-FITC staining, followed by fluorescence-activated cell sorter (FACS) analysis. A representative photomicrograph and FACS analysis of Annexin V-FITC and propidium iodide (PI) staining are provided. E: histograms reveal relative percentage of inhibition of apoptosis by Prdx6 against paraquat-induced oxidative stress. Results are expressed as means ± SD for three replicated determinations in each treatment group and were significant (*P < 0.001) compared with control.
that HT22 under oxidative stress had an activated form of NF-κB, and this activation could be optimized by extrinsic delivery of Prdx6. Because NF-κB can function as a repressor of Prdx6 in situations of cellular background (13, 23, 27), we examined the expression levels of Prdx6 protein and cellular activity of NF-κB in HT22 cells during acute oxidative stress. The HT22 cells were pretreated with SN50, a cell-permeable inhibitor peptide that blocks translocation of the NF-κB active complex into nucleus. The pretreated cells were exposed to H2O2-induced oxidative stress. Nuclear and cytosolic protein extracted from cells was immunoblotted and examined. Results disclosed that pretreatment of cells with SN50 inhibited ROS-induced migration of NF-κB in the nucleus (Fig. 11A, lane 3). In contrast, NF-κB translocated into the nucleus after H2O2 exposure (Fig. 11A, lane 2). SN50 treatment of cells resulted in an increase of Prdx6 expression (Fig. 11B, lane 3), with an increase in cell viability (data not shown), suggesting the involvement of NF-κB in suppressing Prdx6 expression during oxidative stress. Therefore, we surmised that, during oxidative stress, a decline in Prdx6 levels might be due to activation of NF-κB and a causal event for HT22 insult. This destructive
process might be prevented by administration of Prdx6 to optimize NF-κB activation.

DISCUSSION

Reduced antioxidant defense capacity along with an increase in oxidative stress is now believed to cause many age-related disorders, including some that are neurodegenerative (7, 13, 50). Injurious oxidative load can be remedied either by reducing intrinsic and/or extrinsic oxidative sources, by enhancing natural antioxidants, or by providing an extrinsic supply of antioxidant capable of clearing the oxidative load. During aging, sources of oxidative stress are impossible to control, and endogenous antioxidants are difficult to balance, so enhancement of antioxidant defense appears the most feasible, reasonable, and practical approach. In the current paper we studied whether extrinsic supply of an endogenous antioxidant, such as Prdx6, might block or delay oxidation-initiated disease. To facilitate the investigation, we 1) constructed TAT-HA-Prdx6, 2) isolated recombinant Prdx6 fused to TAT domain, and 3) isolated TAT-HA-Prdx6 and its mutant Cys47 as described previously (21, 37, 38). We exposed cells to the oxidative stress inducers paraquat, H$_2$O$_2$, and glutamate and tested the capacity of Prdx6 to blunt their injurious effects. At first, we examined variable concentrations of paraquat, H$_2$O$_2$, and glutamate and found that the optimum concentrations to assess protective ability of Prdx6 were as follows: For HCN-2 cells, 100 μM paraquat (suggesting that HCN-2 is more sensitive to oxidative stress), while for HT22 cells, 0.5 mM paraquat, 100 μM H$_2$O$_2$, and 5 mM glutamate (37, 38). In the present investigation, TAT-HA-Prdx6-pretreated cells showed significant resistance against all of the oxidants, and we observed a dramatic decrease in ROS levels (Figs. 2, 6, 7, and 9). Analysis of Prdx6 expression in cells facing oxidants clearly showed that all of the oxidants used in the study repressed the expression of Prdx6 protein and transcripts (Figs. 1C, 4, D and E; 5, D and E; 8, D and E) and induced cell death by both apoptosis and necrosis (Figs. 6, 7, and 9). Importantly, a supply of Prdx6 was able to counter oxidant-mediated injury...
Because cells exposed to oxidants display loss of Prdx6, we postulated the occurrence of a vicious feed-forward cycle as reduction in Prdx6 level raises ROS accumulation during oxidative stress. Thus Prdx6 expression appears to be a determinant of cellular fate.

Furthermore, evidence shows both direct and indirect links between acute or chronic oxidative stress and neuronal cell damage or degeneration. Neuronal cells are known to be more vulnerable to oxidative damage than other cells/tissues. Brain cells are metabolically highly active, consume more oxygen, and have less ability to proliferate with aging (17). Moreover, neuronal cells bear relatively weak antioxidant defense due to reduced expression of antioxidants with high levels of phospholipids, which are highly sensitive to oxidative modification (18, 50). The protective activity of Prdx6 has been documented in neuronal cells (3, 29). Most of the antioxidant proteins including Prdx6 reside in cytosol to remove ROS enzymatically. Importantly, Prdx6 expression, localization, and protective function have also been shown in mitochondria (20), endoplasmic reticulum (ER) and lysosomal organelles (58, 59), and plasma membrane (1). Loss of Prdx6 causes etiopathogenesis in many type of tissues and organisms, leading to such conditions as diabetes and neuronal cell degeneration, while introduction of Prdx6 reverses or delays the process (20, 43, 47, 57). In the current study, we demonstrated that human and mouse neuronal cells, HCN-2 and HT22, are susceptible to oxidative stress from paraquat, glutamate, or H2O2. When abundant ROS were induced by various stressors, the expression of Prdx6 protein and mRNA was reduced. The cells underwent apo-
ptosis as well as necrotic types of cell death. Intriguingly, we found that a transduction protein-linked Prdx6 internalized in neuronal cells and inhibited oxidative stress and cell death induced by any of the oxidants (Figs. 6, 7, and 9). Glutamate plays a crucial role in neuronal cell damage, which is not restricted to the excitotoxic pathway, but also includes ROS-driven oxidative stress (25, 54). We found that, indeed, glutamate is an inducer of oxidative stress (Fig. 8). Although it is not clear whether oxidative stress-induced cell death evoked by glutamate is a primary event, this injurious process was attenuated by delivery of Prdx6 (Fig. 9).

Moreover, paraquat is a contact dihydropyridine herbicide which is extremely toxic to mammals, causing multiorgan failure and finally death (32). Paraquat has been reported to cause brain damage via formation of oxygen-free radicals (60), leading to neuronal cell death that finally causes disease. Glutamate, an endogenous neurotransmitter, in high concentrations acts as a neurotoxicant by increasing intracellular ROS levels (62). Glutamate-induced oxidative stress plays an im-

Fig. 10. Redox-active transcription factor NF-κB was in active form in HT22 cells facing stress from paraquat or glutamate but was normalized by TAT-HA-Prdx6 delivery. A and B: TAT-HA-Prdx6 downregulated NF-κB-dependent transcriptional activation of HIV-1 LTR in HT22 cells exposed to paraquat (A) and/or glutamate (B). HT22 cells were transiently transfected with HIV-1 LTR-CAT or its mutant construct (top, a and b). Cells were transduced with TAT-HA-Prdx6 or its mutant protein as indicated. After 3 h, transfectants were treated with paraquat (0.5 mM; A) or glutamate (5 mM; B). Transactivation assay was performed after 48 h and NF-κB activity was as shown. Results indicated that NF-κB was activated following paraquat (A; black bar) and glutamate (B; black bar), but its activity was reduced when TAT-HA-Prdx6 was supplied to cells (A and B; gray bars). Results are means ± SD of three individual experiments. *P < 0.001.

Fig. 11. Suppression of Prdx6 expression was associated with NF-κB activation in HT22 cells under oxidative stress and was reversed by NF-κB blocker SN50. HT22 cells were treated with or without SN50, a NF-κB inhibitor, for 2 h followed by 100 μM H2O2. After 12 h, nuclear (A) and cytosolic (B) extracts were prepared, resolved on SDS-PAGE, and immunoblotted with anti-NF-κB, anti-IκB, or anti-Prdx6 antibody. An increase in Prdx6 levels demonstrated the repressive role of NF-κB for Prdx6 in HT22 cells with specific cellular background. Results are means ± SD of three individual experiments. *P < 0.001.
Portant role in neuronal cell injury and degeneration in many brain disorders (16, 33). We believe that the progressive loss of HCN-2 and HT22 cells in neurodegenerative disease is associated with long-term effects of oxidative damage induced by ROS that are locally generated by higher levels of oxidant stressors within the cellular microenvironment. HT22 cells have been successfully and widely used as an in vitro model to study glutamate-induced neurotoxicity (14, 48). HT22 cells lack the functional ionotrophic glutamate receptor; therefore glutamate-induced HT22 cell death can be investigated through exposure to oxidative stress. When cells were exposed to paraquat, glutamate, or H$_2$O$_2$ in vitro, they showed higher levels of ROS and reduced expression of Prdx6 and underwent spontaneous apoptosis (Figs. 6, 7, and 9). Prdx6 maintains the cellular homeostasis by optimizing ROS levels and also by maintaining Ca$^{2+}$ homeostasis. In neuronal cell death, increased ROS production leads to alterations in Ca$^{2+}$ homeostasis, which is required for glutamate oxidative toxicity in HT22 cells (35, 42).

Paraquat and/or H$_2$O$_2$-induced toxicity causes neuronal cell death by increasing ROS production and reducing the antioxidant defense system (Figs. 1, 4, and 5) (13, 48). Importantly, in HT22 and HCN-2 cells, activation of NF-$\kappa$B has been found to induce apoptotic signaling, while suppression of its activation significantly enhances cell viability (13). However, hyperactivation or inadequate activation of NF-$\kappa$B may be disastrous (10, 21). Overall, our results revealed that HCN-2 and HT22 cell death induced by paraquat and/or glutamate or H$_2$O$_2$ is likely associated with failure of cellular homeostasis due to higher ROS production as well as hyperactivation of NF-$\kappa$B during neurodegenerative disorders (Figs. 10 and 11) (26). NF-$\kappa$B fine-tunes Prdx6 expression at normal physiological conditions, while overactivation of NF-$\kappa$B during oxidative stress represses Prdx6 transcription in cell background (13, 27). NF-$\kappa$B acts in both proapoptotic and apoptotic roles, depending on the cellular microenvironment (53, 56). Prdx6-deficient cells display higher expression of ROS and overstimulation of NF-$\kappa$B, while Prdx6 optimizes the activity of NF-$\kappa$B, suggesting that loss of Prdx6 during oxidative stress leads to NF-$\kappa$B-mediated suppression of Prdx6. In the current study we found that HT22 exposed to any of the oxidants contains the activated form of NF-$\kappa$B, and the activation of NF-$\kappa$B was normalized by introducing Prdx6 to cells (Figs. 6, 7, and 9). Treating cells with SN50, an NF-$\kappa$B inhibitor, led to increased expression of Prdx6 (Fig. 11) and increased viability of cells (data not shown), clearly demonstrating the involvement of NF-$\kappa$B in suppressing Prdx6 expression. Furthermore, the protective effect of SN50 by regulating NF-$\kappa$B is well documented in many cell types. Herein, our study revealed the role of Prdx6 in maintaining survival signaling by maintaining appropriate levels of ROS expression. The recent identification of Prdx6 expression and activity in different organelles, specifically mitochondria and ER, which are largely responsible for ROS production, emphasizes the importance of physiological expression of Prdx6 and further argues for consideration of Prdx6 as a therapeutic protein. However, the current study demonstrated the neuroprotective effect of a transduction protein-linked Prdx6 delivered to primary human neuronal cells, HCN-2, as well as murine hippocampal cells, HT22. In conclusion, our study provides a proof of concept that TAT-HA-Prdx6 has therapeutic potential and should be supplied to neuronal cells. Finally, we propose that local, focal, or systemic application of a transduction protein-linked Prdx6 should be an ideal tool to reinforce natural Prdx6-mediated cellular defense to attenuate ROS-based oxidative damage and NF-$\kappa$B-mediated aberrant signaling. The findings also provide a background for future testing of Prdx6 as a therapeutic molecule in animal model systems.

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**Pxdx6’s ROLE IN NEUROPROTECTION**

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