Up-regulation of Protein-disulfide Isomerase in Response to Hypoxia/Brain Ischemia and Its Protective Effect against Apoptotic Cell Death*

Shinji Tanaka, Takashi Uehara, and Yasuyuki Nomura‡
From the Department of Pharmacology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan

We isolated and identified a stress protein that is up-regulated in response to hypoxia in primary-cultured glial cells. Protein-disulfide isomerase (PDI) was up-regulated not only by hypoxia in glia in vitro, but also by transient forebrain ischemia in rats in vivo. To determine whether newly synthesized PDI is involved in tolerance to ischemic stress, we carried out two procedures to induce PDI gene expression in human neuroblastoma SK-N-MC cells, as well as intrahippocampal injection following electroporation of an expression vector capable of overexpressing PDI in rats. Overexpression of this gene resulted in attenuation of the loss of cell viability induced by hypoxia in neuroblastoma SK-N-MC cells and a reduction in the number of DNA-fragmented cells in the CA1 area of the hippocampus in brain ischemic rats, respectively. These findings suggest that up-regulated PDI may play a critical role in resistance to ischemic damage, and that the elevation of levels of this protein in the brain may have beneficial effects against brain stroke.

We isolated and identified a stress protein that is up-regulated in response to hypoxia in primary-cultured glial cells. Protein-disulfide isomerase (PDI) was up-regulated not only by hypoxia in glia in vitro, but also by transient forebrain ischemia in rats in vivo. To determine whether newly synthesized PDI is involved in tolerance to ischemic stress, we carried out two procedures to induce PDI gene expression in human neuroblastoma SK-N-MC cells, as well as intrahippocampal injection following electroporation of an expression vector capable of overexpressing PDI in rats. Overexpression of this gene resulted in attenuation of the loss of cell viability induced by hypoxia in neuroblastoma SK-N-MC cells and a reduction in the number of DNA-fragmented cells in the CA1 area of the hippocampus in brain ischemic rats, respectively. These findings suggest that up-regulated PDI may play a critical role in resistance to ischemic damage, and that the elevation of levels of this protein in the brain may have beneficial effects against brain stroke.

Two distinct phenomena are observed in the CA1 subfield of the hippocampus after transient forebrain ischemia in rodents: the death of neurons, which is referred to as “delayed neuronal death,” and the proliferation of glial cells, which is termed “gliosis” (1). Neurons are thus thought to be fragile and very sensitive to such stress, and, as a consequence, apoptotic cell death occurs (2, 3). Several studies have shown that caspases are involved in this neuronal apoptosis triggered by brain ischemia. Transgenic mice expressing dominant-negative mutants of caspase-1 and caspase-1-deficient mice show a reduced neuronal cell death induced by ischemic brain injury (4–6). Caspase inhibitors such as Z-VAD-fmk,1 YVAD-fmk, and DEVD-fmk also significantly reduce the neuronal cell death induced by ischemia (7–9). Furthermore, overexpression of Bcl-2 in transgenic mice as well as of neuronal apoptosis inhibitory protein, a member of the inhibitor of apoptosis protein family, by injection of adenovirus expression vectors has a protective effect against the neuronal cell death induced by focal cerebral or transient forebrain ischemia (10, 11). These results suggest that caspases are involved in ischemia-induced neuronal death in an anti-apoptotic protein-dependent manner.

On the other hand, glial cells show tolerance to ischemic stress, and their numbers are increased in areas originally containing neurons. Due to their abundance and ability to sustain environmental perturbations, glia play a critical role in maintaining neuronal function under both physiological and pathological conditions (12). Glial cells subjected to hypoxia express stress proteins such as a 70-kDa heat shock protein (HSP70), a 78-kDa glucose-regulated protein (GRP78), a 150-kDa oxygen-regulated protein (ORP150), a 36-kDa putative RNA-binding protein (RA301), and a 70-kDa putative vesicle transport-related protein (RA410) (13–16). Hori et al. (17) have reported that the inhibition of protein synthesis during early reoxygenation prevents effective astrocyte adaptation to hypoxia/reoxygenation, resulting in eventual cell death, indicating that several critical gene products are expressed in response to hypoxia/reoxygenation. Indeed, the inhibition of ORP150 using antisense results in reduced cell viability in hypoxia-treated cells, suggesting that ORP150 has a protective effect against hypoxia-stimulated cellular stress (18). These observations indicate the possibility that the expression of several stress proteins in glial cells is partly implicated in the acquisition of tolerance against hypoxic or ischemic stress.

In the present study, we attempted to isolate important molecules as protective factors against hypoxia in rat primary astrocytes. We found that protein-disulfide isomerase (PDI, EC 5.3.4.1) is up-regulated by hypoxia in rat primary astrocytes. Therefore, we assessed the protective potential of PDI by determining whether overexpression of this gene in vitro and in vivo renders neuronal cells more resistant to the damaging effects of hypoxic treatment and transient forebrain ischemia, respectively. Moreover, we attempted to characterize the possible critical sites in each thioredoxin-like domain.

**EXPERIMENTAL PROCEDURES**

Preparation of Rat Primary Astrocytes—Primary astrocytes were prepared from the whole brain of neonatal Wistar rats as described previously (19) with some modifications (20). Briefly, the cells were allowed to grow to confluence (12 days) in Dulbecco’s modified Eagle’s medium supplemented with heat-inactivated 10% fetal calf serum, 50 μg/ml penicillin, and 100 μg/ml streptomycin. Cultured cells were grown at 37 °C under an atmosphere of 5% CO2, 95% air. Subsequently, the cells were shaken at 120 rpm for 1 h at room temperature, and the adherent cells were cultured again for 6 days. The cells were then shaken to separate the astrocytes from the remaining microglia and oligodendrocytes, and they were then identified by immunohistochemical analysis with anti-glial fibrillary acidic protein (GFAP). The cell cultures were 95% positive for GFAP.
Role of PDI Up-regulated by Hypoxic Stress

Cell Culture—Human neuroblastoma SK-N-MC cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal calf serum at 37 °C in humidified 5% CO2, 95% air.

Treatment with Hypoxic Stress—When the cells (astrocytes or SK-N-MC) became subconfluent, they were cultured in a mixture of 5% CO2 and the balance N2 in a humidified incubator (ANX-1, Hiratsawa, Tokyo, Japan) at 37 °C within a sealed, anaerobic, gloved cabinet containing a catalyst to scavenge free oxygen as described previously (20, 21). The oxygen levels in the cabinet were measured with a monitor sensitive to oxygen concentrations of <10 ppm.

Two-dimensional Electrophoresis—Two-dimensional separation of proteins on small format gels was carried out as described (22, 23). Briefly, cytosolic proteins (10 μg) were first separated by isoelectric focusing in 1.5-mm × 7-cm rod gels containing 1.6% (w/v) amphoteries, pH 5–8 (Amersham Pharmacia Biotech) and 0.4% amphoteries, pH 3.5–10. First-dimension gels were attached to the top of 8 × 7 × 0.015-cm, 12% polyacrylamide gels. After electrophoresis, the gels were stained with silver.

Protein Sequencing—Cytosolic proteins from cells exposed to hypoxia were separated by two-dimensional electrophoresis and blotted onto polyvinylidene difluoride membranes. The protein bands on the membranes were stained in a solution of 0.2% Coomassie Brilliant Blue R250 in 7.5% methanol, 5% acetic acid in water and then rinsed in water. The protein was then extracted. The protein was combined with its counterpart from three gels transferred to 1.5 ml tubes, and then analyzed by Edman degradation using an automated protein sequence (model 492, Applied Biosystems, CA). The sequences obtained were compared with those in the GenBank data base.

Isolation of PDI cDNA and Site-directed Mutagenesis—Full-length PDI cDNA was isolated from rat astrocyte RNA by RT-PCR (24). The primers used were: rat PDI (upstream), 5′-CGA CTT CCG ACA TGC TGA GCC-3′; rat PDI (downstream), 5′-GGC TTC TGC ACT ACA GTT CAT′-3′. The three mutants of PDI were generated by the overlapping PCR method.

Transient Forebrain Ischemia Model—Adult male Wistar rats (250–300 g; Charles River Laboratories) were used for all of the experiments. All animal procedures conformed to the Guide for the Care and Use of Experimental Animals of the Medical Research Council of Japan. Transient forebrain ischemia was produced by the four-vessel occlusion (4VO) method. Animals that showed convulsions during 4VO for 15 min, and after reperfusion, along with those that did not fully develop loss of righting reflex during ischemia, were omitted from the study. In the case of sham-treated animals, the carotid arteries were exposed but not cut.

Assessment of Viability in SK-N-MC Cells—Cell viability was estimated by two methods. Initially, viability was measured by the counting of stained viable cells for β-galactosidase activity derived from transfection of that gene. Briefly, 0.3 μg of lacZ plus 1.25 μg of each PDI gene were co-transfected into SK-N-MC cells using SuperFect transfection reagent (Qiagen) and incubated for 24 h. After hypoxic challenge, cells were fixed with 1% glutaraldehyde for 10 min, rinsed three times with phosphate-buffered saline, and stained in 5-bromo-4-chloro-3-indolyly-β-D-galactopyranoside (X-gal) buffer (0.5 mg/ml X-gal, 4 mM K4[Fe(CN)6], 4 mM K3[Fe(CN)6], 1 mM MgCl2, 10 mM KC1, 0.1% Triton X-100 in 0.1 M sodium phosphate buffer, pH 7.5) at 37 °C for 2 h. The number of stained blue cells in cultures challenged by hypoxia versus that in normoxic cultures was counted by two investigators blind to the experimental treatments, and the results are expressed as the percentage of hypoxia/normoxia.

As another strategy, the viability was estimated by the lactate dehydrogenase (LDH) leakage method using a Cytotoxicity Detection Kit (Roche Molecular Biochemicals) according to the manufacturer's protocol. LDH activity was measured as the optimal density at 492 nm, and LDH leakage (%) was defined as the ratio of LDH activity in the culture medium to the total activity (%) = (extracellular activity/extracellular activity + remaining cellular activity).

In Vivo Transfection by Electroporation—Rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally). For intrahippocampal injection, 2 μg of plasmid DNA (pcDNA3-PDI) was dissolved in 0.2 μl of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5). The head of the rat was placed in a stereotaxic operating table. The skin was cut and retracted, and a burr hole was made. The DNA was injected into the right hippocampal CA1 region (anterior: −3.2 mm, lateral: 1.5 mm, height: 2.4 mm from bregma) in a final volume of 0.2 μl (2 μg) using a cannula (27-gauge needle) connected by polyethylene tubing to a 1-μl Hamilton syringe. The injection rate was 0.1 μl/min, and the cannula was kept in place for an additional 10 min after injection.

Subsequently, a pair of stainless steel electrodes (1.0 mm gap), 10 mm in length and 0.5 mm in diameter, was inserted into the CA1 region through the burr hole. Electric pulses were generated with a Square Electroporator (CUTY21, TR Tech, Tokyo, Japan) and were administered at 10 pulses/s (10 Hz). Electric pulses were 2 ms in duration at 45 V and less than 10 mA, and the electric field strength was 450 V/cm.

Immunohistochemical Analysis and Deoxynucleotidyltransferase-mediated DUTP-biotin nick end labeling (TUNEL) Staining—PDI and GFAP were detected using a monoclonal antibody (RL90, Affinity Bioreagents) and a polyclonal antibody (Dako), respectively. Immunohistochemical analyses of PDI (dilution 1:500) and GFAP (dilution 1:50) were performed on free-floating coronal sections (10 μm thick). The apoptotic cells were detected by TUNEL of nucleosomal fragments generated by endonuclease cleavage using a MEBSTAIN Apoptosis Kit II (MBL). All images were taken on a laser-scanning confocal microscope (LSM510; Carl Zeiss).

RESULTS

Identification of the 55-kDa Protein Induced by Hypoxia or Reoxygenation—We performed SDS-polyacrylamide gel electrophoresis and Coomassie Brilliant Blue R250 staining to detect proteins newly synthesized or up-regulated in response to hypoxia (2% O2, 48 h) and subsequent reoxygenation (20% O2, −48 h) in rat primary astrocytes. During treatment with hypoxia/reoxygenation, the levels of several proteins were significantly enhanced after hypoxia (48 h)/reoxygenation (6 h) (data not shown). Therefore, we further carried out two-dimensional electrophoresis and amino acid sequencing methods using these samples. On silver-stained two-dimensional polyacrylamide gels of total cell lysates from astrocytes treated with hypoxia/reoxygenation, the densities of protein bands with molecular masses of ~150, 95, 75, 60, and 55 kDa were increased in stress-treated cells (Fig. 1A). Since no protein of ~55 kDa has been reported to be up-regulated in response to hypoxia/reoxygenation in astrocytes at date, we determined its N-terminal amino acid sequence using the automated Edman sequencing technique. The sequence of the N-terminal 20 amino acids of the ~55-kDa protein was completely identical to N-terminal residues 20–39 of rat PDI (Fig. 1B). Since PDI is
specifically localized in the ER, it has a signal sequence from N-terminal residues 1–19. Hence, we believe that the coincidence between these sequences is sufficient to identify the ~55-kDa protein as PDI.

Kinetic Analysis of PDI Induction and Effects of Other Stressors—Northern blot analysis of RNA from cultured astrocytes using a 32P-labeled PDI cDNA probe showed that the PDI mRNA levels increased 24 h after hypoxic treatment; the levels of PDI mRNA reached a plateau at 48 h after hypoxia and 6 h after reoxygenation, decreasing gradually thereafter (Fig. 2A). Western blot analysis using anti-PDI monoclonal antibody (RL90) revealed a significant enhancement of PDI protein expression 24 h after hypoxia, which reached a maximum by 12 h after reoxygenation, decreasing gradually thereafter (Fig. 2A). Western blot analysis using anti-PDI monoclonal antibody (RL90) revealed a significant enhancement of PDI protein expression 24 h after hypoxia, which reached a maximum by 12 h after reoxygenation (Fig. 2B). We next investigated whether other stresses can induce PDI. The levels of PDI were slightly enhanced by treatment with deferoxamine, hydrogen peroxide (H2O2), or tunicamycin (Fig. 2C). Treatment with cobalt chloride (CoCl2) and heat shock, which increased levels of HSP70, had little effect on the expression of PDI. On the other hand, calnexin and calreticulin, which are closely related to PDI and are localized in the ER, were also up-regulated by treatment with hypoxia/reoxygenation (Fig. 2D).

Induction of PDI by Transient Forebrain Ischemia—We next investigated the changes in PDI levels following transient forebrain ischemia in rats using the 4VO model. Initially, we investigated whether PDI mRNA levels are increased by 15-min brain ischemia in rats and characterized the localization of this increase in vivo. Fig. 3A shows the results of the PDI mRNA induction in each area of the ischemic brain by the RT-PCR method. Under these analysis conditions (20 cycles of PCR), PDI mRNA was detectable in the cerebral cortex, but not in the hippocampus, cerebellum, or other areas. Kinetic analysis indicated that PDI mRNA expression begins to increase 1 day after ischemic treatment (15-min occlusion followed by reperfusion), peaks 3 days after ischemia, and returns to basal levels 5 days after ischemia. To ascertain whether the up-regulation of PDI protein expression induced by brain ischemia in vivo correlates with the induction of its mRNA, we subsequently performed immunohistochemical analysis in brain slices using anti-PDI antibody. PDI antigen was weakly expressed in all areas of the non-ischemic brain (Fig. 3B, left panel). In contrast, the immunoreactivity was markedly enhanced in the cerebral cortex of the ischemic brain, as shown in Fig. 3A (Fig. 3B, right panel). Moreover, double staining with anti-PDI and anti-GFAP antibodies showed that the PDI antigen induced by brain ischemia is primarily localized in GFAP-positive cells (Fig. 3C). In addition, PDI-positive cells overlap slightly with those positive for Ricinus communis agglutinin 1 (RCA1).

Effects of the Overexpression of PDI in Vitro and in Vivo—To confirm the roles of PDI in neuronal cell death in vitro and in vivo, we investigated the effects of this molecule on hypoxia-induced cell death in human neuroblastoma SK-N-MC cells. Because PDI contains two thioredoxin-like domains (one near the N terminus and another near the C terminus), we constructed mutants with cysteine-to-serine mutations in both of the CGHC active site sequences to determine the domain critical to the cytoprotective effect. To address the ability of wild-type or mutated PDI to protect against hypoxia-induced cell death, we carried out two independent experiments, one involving the counting of β-galactosidase-positive cells and the other a LDH leakage assay on cell viability. In both assays, the transient expression of wild-type PDI resulted in a significant recovery of cell viability by hypoxia compared with mock transfected (Fig. 4A and B). However, none of the mutants (N-terminal, C-terminal, both N- and C-terminal double mutants) showed alterations in the loss of viability.

As another strategy, we performed an intrahippocampal injection of a PDI expression vector followed by electroporation to overexpress this molecule in specific areas of the rat brain in vivo.

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Fig. 2. Up-regulation of PDI in response to hypoxia. A and B, changes in PDI mRNA and protein levels during hypoxia/reoxygenation in astrocytes. Total RNA and the cytosolic fraction were prepared from cells exposed to hypoxia/reoxygenation for the indicated periods. The mRNA (A) and protein (B) levels were analyzed by Northern blotting using full-length PDI cDNA and Western blotting using anti-PDI mAb (RL90, Affinity Bioreagents Inc., respectively. Under these conditions (10 μg of total RNA/lane), PDI mRNA was undetectable in the quiescent state but was detected 12 h after hypoxia. The levels peaked 24 h after hypoxia and were sustained until 12 h after reoxygenation, decreasing gradually thereafter. On the other hand, PDI protein was detected at low levels in the quiescent state, and the levels increased 24 h after hypoxia. The levels peaked 6 h after reoxygenation and remained constant for 48 h. C, effects of several stresses on PDI up-regulation. Astrocytes were exposed to stressors capable of inducing HSP70 or GRP78, and cytosolic fractions were then prepared, followed by Western blotting with anti-HSP70 mAb (Santa Cruz) and anti-GRP78 pAb (Santa Cruz). Cells were treated for 48 h under hypoxia, for 12 h with 250 μM deferoxamine (DFX), for 24 h with 1 mM CoCl2 (Co), for 48 h under glucose deprivation (Glu−), for 12 h with 10 μM H2O2, for 3 h at 43 °C (heat), or for 6 h under UV (40 J/m2). D, effects of hypoxia on other ER proteins. The same samples as described in B were subjected to Western blot analysis using anti-calnexin mAb (Transduction Laboratories) or anti-calreticulin antiserum (Stress Gen).
ischemia in vivo. A, RT-PCR analysis to determine PDI expression in the ischemic brain. RT-PCR was performed on mRNA from brain sections of sham-operated rats (s) and rats 12 h, 1 day (1d), 3 days (3d), and 5 days (5d) following 15 min of forebrain ischemia. Twenty cycles of PCR produced a linear relationship between the amounts of input RNA and resulting PCR product. B, immunohistochemical detection of PDI antigen with a monoclonal antibody against PDI in 15-μm sections of the cerebral cortex of sham-operated rats or 3 days after reperfusion. Asterisks, anti-PDI antibody-positive cells. Scale bar, 100 μm. C, confocal microscopic images indicating the localizations of PDI and GFAP immunoreactivities in single tissue sections (10 μm) from the cerebral cortex 3 days after reperfusion. GFAP and PDI immunoreactivities were visualized with rhodamine and fluorescein, respectively.

To ascertain the efficiency of in vivo transfection, we initially transfected green fluorescent protein (GFP) or β-galactosidase gene into the CA1 area. A number of GFP- or electroporation itself, we first examined the effects of transfection procedures. The PDI variants are shown with both cysteine-to-serine mutations (-CGHC- to -SGHS-) in the N-terminal and/or C-terminal thioredoxin-like active sites. PDI WT represents the wild-type PDI with both catalytic sites intact. The PDI N variant had both cysteines in the N-terminal thioredoxin-like active site mutated to serines, and the PDI C variant had both cysteines in the C-terminal thioredoxin-like active site mutated to serines. The PDI NC variant was a double mutant with cysteines in both N and C termini replaced with serines. B, cells were transfected with 1.25 μg of the vector (pcDNA3), PDI WT, PDI N, PDI C, or PDI NC plus 0.3 μg of pACTβ-gal using SuperFect reagent (QIAGEN). Twenty-four hours after transfection, each transfected cell was transferred to a low oxygen chamber and incubated for another 20 h. After fixation, the cells were incubated with X-gal buffer, and the stained cells were then counted. Viability is represented as the ratio of stained cells under normoxia and hypoxic challenge. Values represent the means ± S.E. of triplicate cultures run in parallel.

A schematic representation of the structures of PDI and each mutant. PDI consists of domains a, b, b′, a′, and c. The a and a′ domains are similar to thioredoxin, and both contain the -CGHC- sequence. The c domain represents a putative Ca2+-binding region. The PDI variants are shown with both cysteine-to-serine mutations (-CGHC- to -SGHS-) in the N-terminal and/or C-terminal thioredoxin-like active sites. PDI WT represents the wild-type PDI with both catalytic sites intact. The PDI N variant had both cysteines in the N-terminal thioredoxin-like active site mutated to serines, and the PDI C variant had both cysteines in the C-terminal thioredoxin-like active site mutated to serines. The PDI NC variant was a double mutant with cysteines in both N and C termini replaced with serines. B, cells were transfected with 1.25 μg of the vector (pcDNA3), PDI WT, PDI N, PDI C, or PDI NC plus 0.3 μg of pACTβ-gal using SuperFect reagent (QIAGEN). Twenty-four hours after transfection, each transfected cell was transferred to a low oxygen chamber and incubated for another 20 h. After fixation, the cells were incubated with X-gal buffer, and the stained cells were then counted. Viability is represented as the ratio of stained cells under normoxia and hypoxic challenge. Values represent the means ± S.E. of triplicate cultures run in parallel.
Role of PDI Up-regulated by Hypoxic Stress

PDI Up-regulation and Its Localization in the Ischemic Brain—We examined whether PDI expression is enhanced in response to brain ischemia in vivo. Surprisingly, transient forebrain ischemia markedly elevated PDI mRNA levels in the cerebral cortex, but not in the hippocampus, cerebellum, or ischemia in the PDI-transfected hippocampus, the numbers were significantly decreased compared with those in the mock-transfected hippocampus (5 ± 2/100 µm and 8 ± 3/100 µm, respectively). Scale bars: 100 µm. C, quantitative comparison of ischemia-induced DNA fragmentation in the CA1 region in mock- or PDI-transfected rats. The numbers of TUNEL-positive cells per 100-µm length of the CA1 area in the hippocampus are counted. Histograms and bars represent means and S.E. for five animals. Star represents significantly different values from those of PDI-transfected rats (*, p < 0.05; **, p < 0.01; Student’s t test).

**DISCUSSION**

Isolation and Identification of PDI from Hypoxia-treated Astrocytes—The aim of this study was to isolate and identify proteins capable of protecting cells against ischemic stress using rat primary astrocytes. We found some candidates in the hypoxia-treated astrocytes by two-dimensional electrophoresis and amino acid sequencing. Several of these molecules have already been reported, including a 94-kDa glucose-regulated protein (GRP94). Since the protein of approximately ~55-kDa had not been reported previously, we analyzed this protein further. The sequence of the N-terminal 20 amino acids of the ~55-kDa protein was completely homologous to the N-terminal residues 20–39 of rat PDI (Fig. 1B). PDI, a major protein present within the lumen of the ER, is known to catalyze the isomerization of both intramolecular and intermolecular disulfide bonds, yielding apparently native structures (25, 26), and has a signal sequence comprising its N-terminal residues 1–19 (27). The ~55-kDa protein identified here lacked this sequence, and we therefore believe that this sequence identity is sufficient to identify the 55-kDa protein as PDI. In the quiescent state, PDI is slightly expressed in primary astrocytes. However, exposure to hypoxia results in a significant enhancement of PDI mRNA and protein levels (Fig. 2, A and B). Deferoxamine and hydrogen peroxide (H₂O₂) also caused the up-regulation of PDI in the present study, whereas the levels were very low compared with those induced by hypoxia. Neither cobalt chloride (CoCl₂) nor heat shock, which can induce 70-kDa heat shock protein (HSP70) expression, altered PDI levels. These results suggest that the induction mechanisms of PDI and HSP70 are essentially different. In contrast, tunicamycin, which is known to be an ER stressor, enhances the levels of PDI and GRP78. GRP78, GRP94, and ORP150 induced by hypoxia/reoxygenation are localized specifically in the ER (13, 14, 17), suggesting that several proteins localized in the ER may be up-regulated in response to hypoxia in astrocytes. In agreement with this hypothesis, both calnexin and calreticulin, which are localized in the ER and act as chaperones, were up-regulated by hypoxia (Fig. 2B). Based on these observations, we speculated that hypoxic stress induces the up-regulation of several proteins localized in the ER such as PDI, calnexin, and calreticulin.

PDI Up-regulation and Its Localization in the Ischemic Brain—We examined whether PDI expression is enhanced in response to brain ischemia in vivo. Surprisingly, transient forebrain ischemia markedly elevated PDI mRNA levels in the cerebral cortex, but not in the hippocampus, cerebellum, or...
other areas (Fig. 3A). PDI was detected in the ischemic cortex from 1 to 3 days after 15 min of 4VO. Consistent with these RT-PCR results, PDI-like immunoreactivity was observed only in the cerebral cortex following brain ischemia. To address the localization of PDI-like immunoreactivity, we performed double staining of brain slices with anti-PDI and anti-GFAP antibodies. Most PDI-reactive cells were detected in GFAP-positive cells (Fig. 3C). However, a small proportion of cells were double-positive for PDI and RCA1 (data not shown), suggesting that PDI expression is enhanced primarily in astrocytes and only slightly in microglia.

Transient forebrain ischemia results in damage to neurons in the cerebral cortex, the CA1 area of the hippocampus, the reticular thalamus, and the striatum. At present, it is still unclear why PDI is up-regulated by ischemic stress in the cerebral cortex, but not the hippocampus and other areas. PDI up-regulation in the ischemic cortex may be involved at least partially in the acquisition of tolerance against ischemic stress by an as yet unidentified mechanism.

PDI May Have a Neuroprotective Effect against Hypoxia and Ischemia—To determine whether PDI plays a critical role in tolerance against ischemic stress, we investigated its effects on hypoxia-induced cell death and its possible active site. PDI is a modular protein that consists of domains α, β, a’, a, and c (Fig. 4A; Refs. 27–29). The a and a’ domains are similar to thioredoxin, and both contain the sequence -CGHC-, representing two independently acting catalytic sites for PDI activity (30–35). To determine the protective roles of these catalytic active sites in cell death, constructs were made with cysteine-to-serine mutations in both the CGHC active site sequences.

Transfection of the wild-type PDI (WT) gene into human neuroblastoma SK-N-MC cells significantly reversed the loss of cell viability triggered by hypoxia (Fig. 4, A and B). In contrast, none of the mutants reversed the loss of cell viability on hypoxic challenge. These results indicated that overexpressed PDI has a neuroprotective effect against hypoxia in neuronal cells, and that both thioredoxin-like domains are critical for this protective function against hypoxic stress.

Finally, the overexpression of PDI in vivo was used to determine whether increased levels of this protein could reduce the damaging effects in the CA1 region of the hippocampus by transient brain ischemia. As a control, mock transfection with an empty vector was performed followed by 4VO, since electroporation itself may have been responsible for the observed neuroprotective effect against ischemic stress. However, the numbers of DNA-fragmented cells in the CA1 region after 4VO in rats were almost same between untransfected and mock-transfected brains, suggesting that in vivo electroporation had no neuroprotective effect. On the other hand, the numbers of TUNEL-positive CA1 neurons were significantly reduced in the PDI-overexpressing ischemic brain, but not in mock transfectants (Fig. 5). These observations suggest that overexpression of the PDI gene significantly suppresses ischemia-induced apoptosis in the CA1 area of the hippocampus.

In summary, we have demonstrated here that PDI is up-regulated in response to hypoxia or transient forebrain ischemia in astrocytes. Furthermore, the overexpression of PDI in vitro and in vivo could confer resistance to hypoxic or ischemic injury. Because there have been some reports that PDI may act as a chaperone (36–38), this neuroprotective effect may be partially based on its chaperone activity. Iscemic stress results in the denaturation or unfolding of many proteins and also simultaneously induces several chaperones such as GRPs. Furthermore, ORP150 localized in the ER is induced by hypoxia and ischemia in astrocytes (14, 18). These findings suggest that hypoxia or brain ischemia induces the expression of several stress proteins or chaperones localized in the ER, which may result in an acquisition of tolerance against these stresses. Hence, agents that up-regulate PDI expression in neurons may be therapeutically useful in brain ischemia. Finally, we constructed a new technique that enables researchers to efficiently transfect a particular gene in vivo using a square electroporator apparatus. This method has led us to the hypothesis that the overexpression of the PDI gene in the CA1 area could protect against the apoptotic cell death induced by brain ischemia. Therefore, the PDI possibly located in the ER may play a critical role in the protection against stresses.
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