

S-Nitrosylated protein-disulphide isomerase links protein misfolding to neurodegeneration

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Stress proteins located in the cytosol or endoplasmic reticulum (ER) maintain cell homeostasis and afford tolerance to severe insults^{1–3}. In neurodegenerative diseases, several chaperones ameliorate the accumulation of misfolded proteins triggered by oxidative or nitrosative stress, or of mutated gene products^{4,5}. Although severe ER stress can induce apoptosis^{2,6}, the ER withstands relatively mild insults through the expression of stress proteins or chaperones such as glucose-regulated protein (GRP) and protein-disulphide isomerase (PDI), which assist in the maturation and transport of unfolded secretory proteins. PDI catalyses thiol–disulphide exchange, thus facilitating disulphide bond formation and rearrangement reactions^{7–10}. PDI has two domains that function as independent active sites with homology to the small, redox-active protein thioredoxin^{7,8}. During neurodegenerative disorders and cerebral ischaemia, the accumulation of immature and denatured proteins results in ER dysfunction¹¹, but the upregulation of PDI represents an adaptive response to protect neuronal cells^{12–14}. Here we show, in brains manifesting sporadic Parkinson's or Alzheimer's disease, that PDI is S-nitrosylated, a reaction transferring a nitric oxide (NO) group to a critical cysteine thiol to affect protein function^{15–18}. NO-induced S-nitrosylation of PDI inhibits its enzymatic activity, leads to the accumulation of polyubiquitinated proteins, and activates the unfolded protein response. S-Nitrosylation also abrogates PDI-mediated attenuation of neuronal cell death triggered by ER stress, misfolded proteins or proteasome inhibition. Thus, PDI prevents neurotoxicity associated with ER stress and protein misfolding, but NO blocks this protective effect in neurodegenerative disorders through the S-nitrosylation of PDI.

Initially, we gathered two independent lines of chemical evidence to show that PDI was S-nitrosylated *in vitro* and *in vivo* to form an S-nitrosylated protein (SNO-P). First, in a specific fluorescence assay for SNO-P, we demonstrated the reaction of recombinant PDI with the physiological NO donor S-nitrosocysteine (SNOC; Fig. 1a). This assay detects the formation of SNO-P by the conversion of 2,3-diaminonaphthalene (DAN) to the fluorescent compound 2,3-naphthyltriazole (NAT)^{17,19,20}. SNOC-treated PDI resulted in significant SNO-P formation in a concentration-dependent manner.

Mammalian PDI has six cysteine residues in all, with four of them representing two thioredoxin-like domains (one near the amino terminus and the other near the carboxy terminus) that contain the Cys-Gly-His-Cys sequence at the active site. To determine the target site(s) of S-nitrosylation, we performed the DAN assay on immunoprecipitates from HEK-293T cell lysates transfected with wild-type or mutant PDI (cysteine-to-serine mutations in both of the active-site sequences). The fluorescence intensity in this assay of the N-terminal (C36S, C39S) and C-terminal (C383S, C386S) mutants

was decreased by about 50% compared with the wild type. The double mutant (N-terminal and C-terminal) was completely devoid of fluorescence, indicating that both thioredoxin-like domains are targets of S-nitrosylation (Fig. 1b). Next we showed that PDI in 293T cells was S-nitrosylated by the biotin-switch method. In this assay, a SNO-P is identified on western blots after replacing SNO with a more stable biotin group by chemical reduction with ascorbate, as described previously^{15,20,21}. SNOC markedly enhanced the level of S-nitrosylated PDI (SNO-PDI) in cell lysates or intact cells (Fig. 1c), whereas under the same conditions the NO[•] donor diethylamine-NO (DEA-NO) or hydrogen peroxide did not (Supplementary Fig. 1a). Additionally, in HEK-293 cells stably expressing neuronal NO synthase (nNOS), endogenous PDI was nitrosylated by endogenous NO, and this reaction was inhibited by a NOS inhibitor (Fig. 1d, e). Furthermore, by the biotin-switch assay we identified the cysteine residues that were S-nitrosylated. We found that endogenous nNOS activity led to the S-nitrosylation of cysteine residues in both thioredoxin-like domains of PDI (Fig. 1f and Supplementary Fig. 1b). Moreover, with mass spectrometry we found that one of the cysteine residues in the C-terminal thioredoxin-like domain was possibly further oxidized to sulphinic acid (–SO₂H) after exposure to NO (Supplementary Fig. 2). These data are consistent with our previous observation that reversible S-nitrosylation may facilitate further oxidation of the same cysteine thiol¹⁹.

Next, we sought to determine whether SNO-PDI was produced in neurodegenerative disorders associated with high levels of nitrosative stress and protein misfolding, such as Parkinson's disease (PD). First, we incubated dopaminergic SH-SY5Y cells with the mitochondrial complex I inhibitor rotenone, which is known to induce a parkinsonian phenotype, at least in part, in a NO-dependent fashion²². Exposure to rotenone led to the generation of SNO-PDI in these cells (Fig. 1g). To extend this finding to humans, we examined PD brains obtained shortly after death. We found evidence for SNO-PDI formation in each of four PD brains but not in controls obtained from patients who had died of disorders that were not of central nervous system origin (Fig. 1h and Supplementary Table 1). Additionally, brains from another major neurodegenerative disorder associated with protein aggregation and nitrosative stress, Alzheimer's disease (AD), also showed evidence of SNO-PDI (Fig. 1i, Supplementary Fig. 3 and Supplementary Table 1), consistent with the notion that this finding could represent a common denominator linking free-radical stress and protein misfolding.

To determine whether S-nitrosylation affects PDI function, we monitored PDI chaperone and isomerase activities. Chaperone activity was assessed in a standard assay by measuring the degree of aggregation of rhodanese induced by guanidinium, as previously described⁹. Rhodanese aggregation occurred in a time-dependent manner, and

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incubation with recombinant wild-type PDI, but not dominant-negative PDI (produced by the N-terminal and C-terminal mutant)¹³, suppressed this aggregation by about 80%. S-Nitrosylation of PDI also significantly inhibited this chaperone activity (Fig. 2a). Next we measured isomerase activity in a standard assay that uses as a substrate an inactive form of RNase A containing scrambled disulphide bonds¹⁰. PDI catalyses the renaturation (refolding) of this inactive RNase A. Recovery of RNase A activity by wild-type PDI was attenuated about 50% by S-nitrosylation (Fig. 2b). Thus, S-nitrosylation inhibited the functional activities of PDI. In addition, direct oxidation of PDI by hydrogen peroxide could also decrease its activity (Supplementary Fig. 4), indicating

that the sulphinated PDI derivative observed by mass spectrometry after exposure to NO (Supplementary Fig. 2) might also be pathophysiological relevant to the inhibition of PDI activity.

From these results we reasoned that PDI might function in attenuating protein misfolding and consequent aggregation in

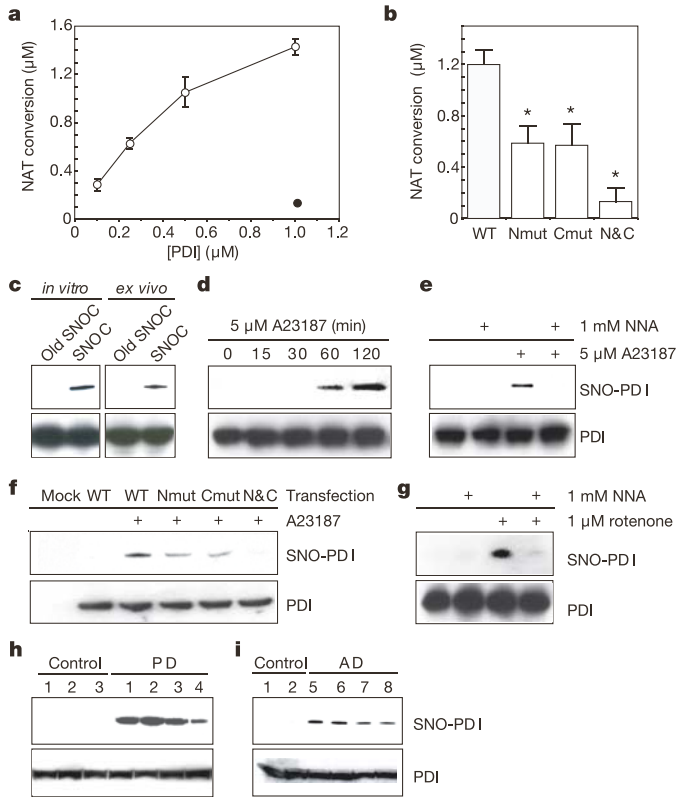


Figure 1 | S-Nitrosylation of PDI *in vitro* and *in vivo*. **a**, Recombinant PDI protein was incubated with SNOC (100 μ M) for 30 min at room temperature 21 $^{\circ}$ C. SNO-PDI thus generated was assessed by DAN assay ($n = 4$ experiments). Open symbols, with SNOC; filled symbol, without SNOC. Values are mean \pm s.e.m. **b**, Cell lysates transfected with wild-type (WT) or mutant (N-terminal (Nmut), C-terminal (Cmut), or N-terminal and C-terminal (N&C)) PDI were immunoprecipitated with anti-PDI antibody. Immunoprecipitates were then incubated in the presence or absence of SNOC and subjected to DAN assay. Values are mean \pm s.e.m., $n = 5$; asterisks, $P < 0.01$ by analysis of variance. **c**, Top: cell lysates from human 293T cells were incubated with SNOC at room temperature to assay for SNO-PDI. Control samples were subjected to decayed (old) SNOC. SNO-PDI was detected by biotin-switch assay 30 min after SNOC exposure. Bottom: total PDI in cell lysates by western analysis. **d–f**, Top panels: HEK-293 cells stably expressing nNOS were assayed for endogenous SNO-PDI. nNOS was activated by Ca^{2+} ionophore A23187 (5 μ M) in the presence or absence of NOS inhibitor (N-nitro-L-arginine; NNA). Bottom panels: Total PDI. Activation of nNOS increased endogenous SNO-PDI (**d**). NNA prevented this increase (**e**). Mutation of critical cysteine thiol groups of PDI also prevented its S-nitrosylation (**f**). **g**, SNO-PDI increased in cells exposed to rotenone in a NOS-dependent manner. Top: lysates from SH-SY5Y cells exposed to rotenone for 6 h in the presence or absence of NNA. Bottom: Total PDI. **h, i**, Brain tissues from controls, from PD patients with diffuse Lewy body disease (**h**), or AD patients (**i**) were subjected to biotin-switch assay to detect *in vivo* S-nitrosylation (see additional results in Supplementary Fig. 3 and Supplementary Table 1).

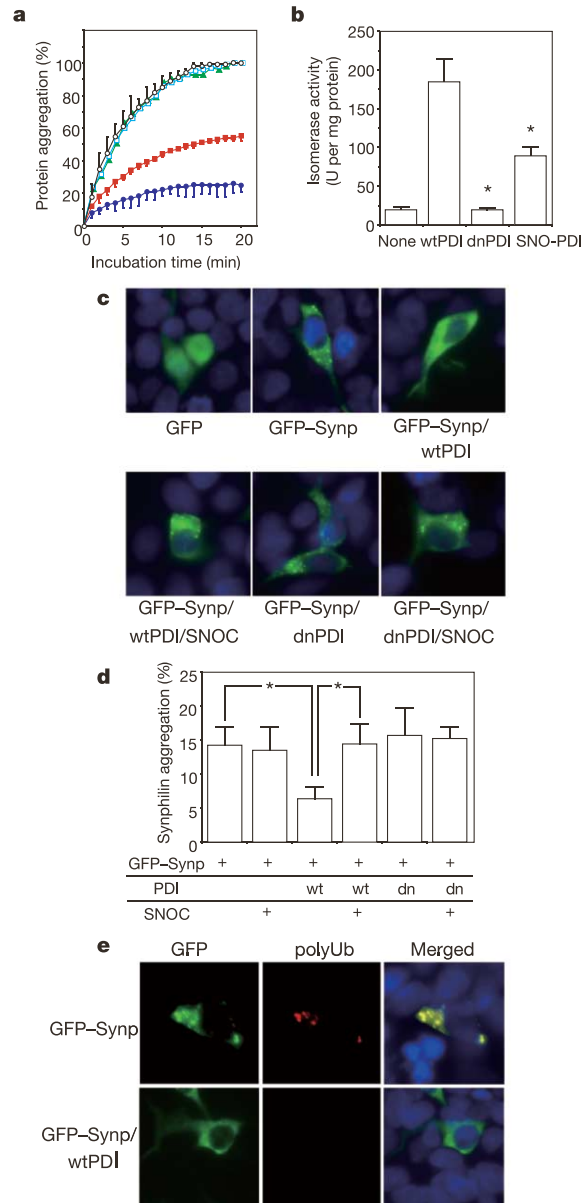


Figure 2 | S-Nitrosylation of PDI regulates its enzymatic activity. **a**, Effect of S-nitrosylation on PDI chaperone activity. Unfolded rhodanase was diluted in buffer containing wild-type PDI (blue), SNO-PDI (red), SNOC (green), dominant-negative PDI (N-terminal and C-terminal mutant; cyan) or buffer alone (black). Rhodanase aggregation was monitored ($n = 8$ experiments). Values are mean \pm s.e.m. **b**, S-Nitrosylation of PDI attenuates its isomerase activity. Scrambled RNase A from bovine pancreas was incubated with wild-type (wt) PDI, dominant-negative (dn) PDI or SNO-PDI. Asterisks, $P < 0.01$ for $n = 5$ experiments. Values are mean \pm s.e.m. **c**, PDI inhibits the aggregation of synphilin-1. SH-SY5Y cells were transfected with green fluorescent protein (GFP)–synphilin-1 (GFP–Synp) and wild-type or dominant-negative PDI. Inclusion body formation was monitored by deconvolution microscopy 24 h after exposure to SNOC or control solution. Images were deconvolved with SlideBook software (Intelligent Imaging Innovations, Inc.). **d**, Percentage of cells with GFP–Synp inclusions. Values are mean \pm s.e.m. for $n = 2,500$ transfectants counted in five experiments; asterisks, $P < 0.05$. **e**, Co-localization of synphilin-1 and polyubiquitin (polyUb) by immunofluorescence ($n = 3$).

neurodegenerative diseases, and that the formation of SNO-PDI might inhibit neuroprotective activity. Moreover, previous work had suggested that ER stress can directly or indirectly influence the aggregation of both ER and cytosolic proteins^{4,5,23}. We therefore attempted to show an inhibitory effect of PDI on the aggregation of synphilin-1, as observed in Lewy body inclusions in the brains of PD patients. Initially, we tested whether PDI could prevent the ubiquitinated, Lewy-body-like inclusions that are formed in the cytosol after synphilin-1 overexpression in cultured SH-SY5Y cells (Fig. 2c). When wild-type PDI was co-expressed with synphilin-1, discrete inclusions were greatly decreased, and instead ubiquitin-negative synphilin-1 was localized diffusely in the cytosol (Fig. 2c–e). As a control, immunofluorescent staining of transfected SH-SY5Y cells revealed that overexpression of PDI did not alter its predominant intracellular distribution in the ER (Supplementary Fig. 5). NO attenuated the protective effect of PDI on synphilin-1 inclusions (Fig. 2d). These findings suggest that PDI is involved in protein folding linked to PD in a NO-sensitive manner.

Excitotoxic damage is also thought to have a function in neurodegenerative disorders such as PD by triggering the production of free radicals, including NO, in part through the excessive stimulation of *N*-methyl-D-aspartate (NMDA)-type glutamate receptors; mild exposure to NMDA results in neuronal apoptosis^{24–27}. Here we found that exposure of cerebrocortical neurons to NMDA induced

SNO-PDI in a NOS-sensitive fashion (Fig. 3a). We surmised that abrogation of PDI chaperone and enzymatic activity by *S*-nitrosylation might contribute to the accumulation of unfolded and consequently polyubiquitinated proteins marked for degradation by the proteasome. We therefore next examined whether the accumulation of polyubiquitinated proteins occurred in response to NMDA by using a polyubiquitin-specific antibody. Within 12 h of exposure to NMDA, we detected polyubiquitin immunoreactivity in neurons, but the cells remained viable at this time point (Fig. 3b, c). By 24 h, many of the polyubiquitinated neurons had undergone apoptosis. Overexpression of wild-type PDI decreased the number of apoptotic, polyubiquitinated cells, indicating that PDI has a function in preventing the accumulation of unfolded, polyubiquitinated proteins in response to NMDA insult, and subsequent neuronal cell death. Next we evaluated the involvement of the unfolded protein response (UPR) signalling pathway that is activated by the accumulation of misfolded proteins or ER dysfunction. Representing this pathway we detected *CHOP* mRNA induction and *XBP-1* mRNA processing by activated IRE1- α after exposure of cortical cultures to

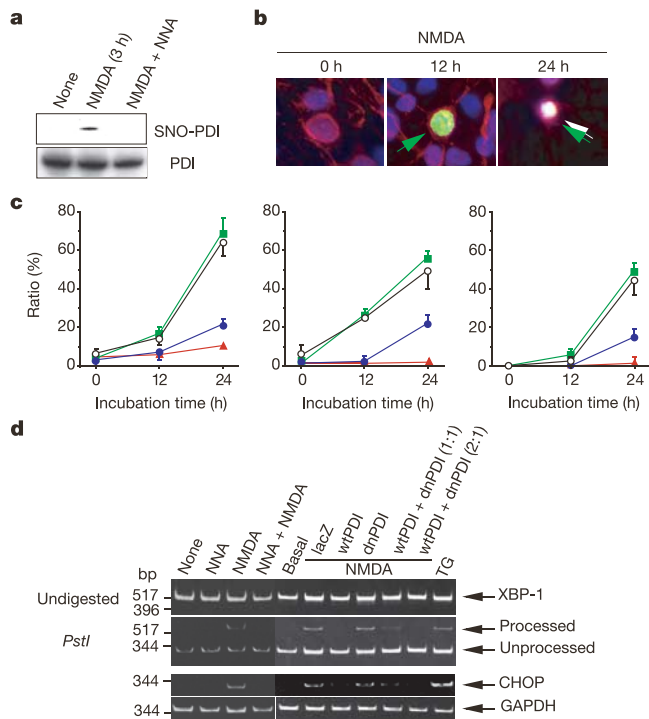


Figure 3 | NMDA stimulates the accumulation of polyubiquitinated proteins and UPR pathway. **a**, SNO-PDI was detected in a NOS-sensitive manner in primary cortical cultures exposed to NMDA. **b**, Primary cortical cells infected with adenoviral Ad-LacZ or Ad-wtPDI were exposed to NMDA and immunostained for polyubiquitinated protein (green) and neuron-specific MAP2 (red). Hoechst-stained DNA (blue) was used to assess condensed, apoptotic nuclei (white in merged image). **c**, Quantification of apoptotic and/or polyubiquitinated neurons similar to those shown in **b**. Left, apoptotic cells; middle, polyubiquitinated cells; right, apoptotic and polyubiquitinated cells. Values are ratio ($\times 100\%$) of affected to total neurons, expressed as mean \pm s.e.m. for $n = 7,000$ neurons counted in six experiments; asterisks, $P < 0.01$. Open circles, Ad-LacZ; blue circles, Ad-wtPDI; green squares, Ad-dnPDI; red triangles, Ad-wtPDI plus *N*-nitro-L-arginine. **d**, NMDA-stimulated processing of *XBP-1* mRNA and induction of *CHOP*. Processing of endogenous *XBP-1* mRNA was evaluated by *PstI* endonuclease digestion of *XBP-1* cDNA. TG, thapsigargin; bp, base pairs.

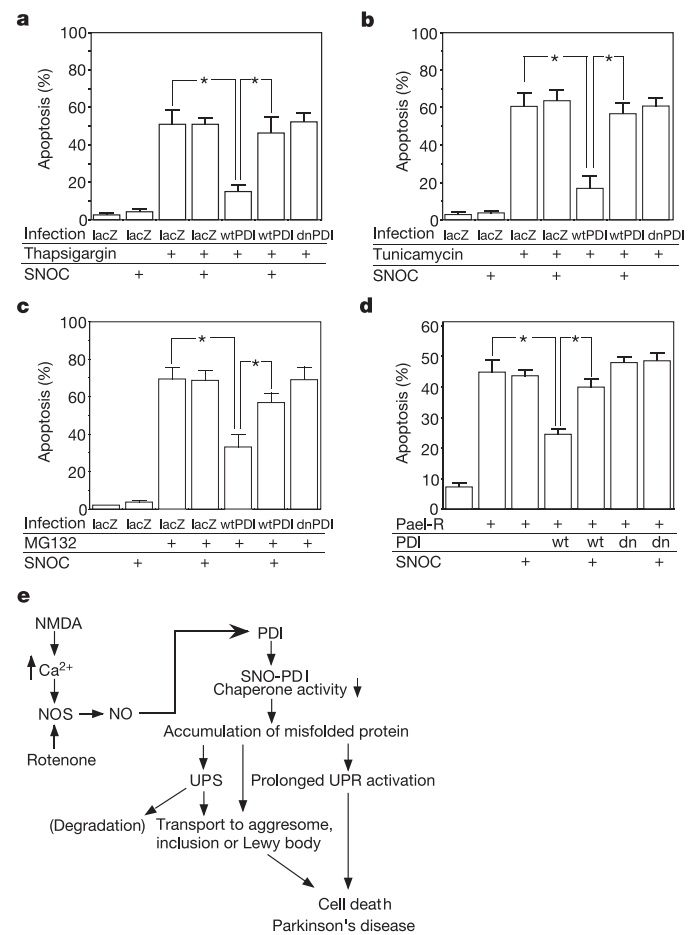


Figure 4 | Neuroprotection by PDI against ER stress, proteasome inhibition, or Pael receptor expression. **a–d**, SH-SY5Y cells were transfected for 24 h with expression vectors for control lacZ (**a–c**), PDI constructs (**a–d**), or Pael receptor (**d**). Cultures were then incubated for 15 h in the presence or absence of 100 μ M SNOC with 5 μ M thapsigargin (**a**), 10 μ g ml⁻¹ tunicamycin (**b**) or 0.1 μ M proteasome inhibitor MG132 (**c**). Exposure to SNOC abolished the protective effect of PDI on cell death induced by ER stress, proteasome inhibitor or Pael receptor. dn, dominant-negative; wt, wild type. For each panel, values are mean \pm s.e.m. for $n = 3,500$ cells counted in five experiments; asterisks, $P < 0.01$. **e**, Possible mechanism of SNO-PDI contributing to the accumulation of aberrant proteins and to cell death in human neurodegenerative disorders. UPS, ubiquitin-proteasome system.

NMDA^{1,2}. This UPR was attenuated by overexpression of wild-type PDI but not by dominant-negative PDI (Fig. 3d). Additionally, the NOS inhibitor *N*-nitro-*L*-arginine blocked NMDA-induced apoptosis and the UPR, indicating that a pathophysiologically relevant amount of NO was produced under these conditions (Fig. 3c, d and Supplementary Fig. 6). Taken together, these findings indicate that NMDA activates a NO-mediated UPR through ER dysfunction, but this dysfunction can be mitigated by PDI activity.

For further clarification of the relationship between the protective function of PDI and its *S*-nitrosylation, we investigated the effect of PDI on neuronal death after ER stress or proteasome inhibition (resulting in the accumulation of polyubiquitinated proteins that cannot be degraded by the proteasome). For this purpose we used SH-SY5Y cells because, unlike cortical neurons, they were resistant to direct NO-induced damage under conditions of SNO-PDI formation (Fig. 1), allowing us to tease apart the effect of NO on cell death and PDI *S*-nitrosylation. We found that cell death precipitated by thapsigargin and tunicamycin (to induce ER stress) or MG132 (to inhibit the proteasome) was largely abrogated by wild-type PDI; however, this protective effect was reversed by exposure to SNO (Fig. 4a–c and Supplementary Fig. 6). Similarly, wild-type PDI ameliorated cell death triggered by overexpression of the Pael receptor, a protein that abnormally accumulates in Parkinson's disease and serves as a potent inducer of the UPR and substrate of the E3 ubiquitin ligase parkin^{6,28}; exposure to SNO also reversed this protective effect (Fig. 4d). These results are consistent with the notion that NO impairs the protective role of PDI through *S*-nitrosylation. From these findings we conclude that cell death in response to proteasome inhibition or ER stress, which contributes to ER dysfunction, UPR activation and protein misfolding, can be attenuated by PDI.

These results show that SNO-PDI forms in brains of patients with PD and AD, neurodegenerative disorders that are characterized by abnormal protein accumulations. Cell models of neurodegeneration produced by exposure to the pesticide rotenone, NO or NMDA also result in the formation of SNO-PDI. *S*-Nitrosylation of PDI inhibits its activity, allows the accumulation of polyubiquitinated proteins and contributes to neuronal cell death (Fig. 4e). To determine whether the level of SNO-PDI in neurodegenerative human brain is of pathophysiological significance, we calculated the ratio of SNO-PDI (by biotin-switch assay) to total PDI (from western blotting) and found that this ratio was similar to that encountered in our neuronal cell models manifesting polyubiquitinated proteins and cell death (Supplementary Fig. 3). This finding indicates that pathophysiologically relevant amounts of SNO-PDI are present in human brains with PD and AD.

In the absence of nitrosylation, wild-type PDI attenuates abnormal protein accumulation and ubiquitination, including the parkinsonian-related protein synphilin-1, and affords neuroprotection. Previous reports have shown the development of ER stress and UPR activation in cellular models of PD²⁹. In addition, there is increasing evidence that the accumulation of aggregated or misfolded proteins links cellular stress to the pathogenesis of PD^{6,20,30}. Other reports have shown that NO can be involved in neurodegeneration by a variety of mechanisms^{15,19,20,24–27,30}. Our data demonstrate a previously unrecognized relationship between NO and protein misfolding in degenerative disorders, showing that PDI can be a target of NO after mitochondrial insult in cellular models of PD and in human neurodegenerative diseases. Nitrosative stress resulting in PDI dysfunction therefore provides a mechanistic link between deficits in molecular chaperones, accumulation of misfolded proteins, and neuronal demise in neurodegenerative disorders. The elucidation of this SNO-PDI-mediated pathway that contributes to neuronal injury and apoptosis might permit the development of new therapeutic approaches for neurodegenerative diseases and other disorders associated with abnormal protein accumulation and nitrosative stress.

METHODS

Fluorimetric detection of *S*-nitrosothiols. We measured *S*-nitrosothiols by the conversion of DAN to fluorescent NAT, as described^{17,19}. NAT was quantified with a FluoroMax-2 spectrofluorometer and DataMax software (Instruments S.A.). Serial NAT dilutions were used to construct a standard curve.

Cell injury/death assays. Cerebrocortical neurons or SH-SY5Y cells were transduced with a wild-type or mutated PDI gene or with a Pael receptor expression construct and incubated for 24 h (Supplementary Fig. 7). The cells were then treated for 15 h with 5 μ M thapsigargin, 10 μ g ml⁻¹ tunicamycin, 0.1 μ M MG-132 or diluent in the presence or absence of 100 μ M SNO. Neurotoxicity of the Pael receptor was analysed as described⁶. Cortical neurons exposed to NMDA were incubated as described previously²⁴. Hoechst staining was used to assess morphological changes of apoptotic nuclei. MAP2 staining was used to assess injury or retraction of neuronal processes. Additional details are described in Supplementary Information.

Expression and purification of recombinant PDI proteins, isolation of PDI complementary DNA and construction of adenoviral vectors, detection of *S*-nitrosylated proteins with the biotin-switch assay, liquid chromatography–mass spectrometry of PDI, cell culture and transduction by means of adenovirus, PDI enzymatic activity assays, detection of aggregated synphilin-1, immunocytochemistry, *XBP-1* mRNA splicing and *CHOP* mRNA induction, human subjects, and statistics are all described in the Supplementary Information.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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