ANTIOXIDANTS & REDOX SIGNALING
Volume 9, Numbers 5, 2007

DOI: 10.1089/ars.2006.1517

Forum News & Views

Accumulation of Misfolded Protein Through Nitrosative Stress Linked to Neurodegenerative Disorders

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ABSTRACT

Protein quality control is a critical feature of intracellular homeostasis. In particular, unfolded or misfolded proteins resulting from environmental stresses or free radicals are rapidly degraded via the ubiquitin–proteasome pathway. Nitric oxide (NO), a free radical gas, has been reported to be involved in such processes as vasorelaxation and neurotransmission. Conversely, NO also is implicated in neuronal cell death or neurodegeneration. Recent reports suggest that *S*-nitrosylation of proteins is a significant cause of neural dysfunction leading to neurodegenerative disorders. Specifically, *S*-nitrosylation of parkin eventually leads to the accumulation of unfolded proteins and subsequent neuronal death. The focus of this review is the identity of the target of NO. Nitrosative stress prevents normal functioning of the endoplasmic reticulum (ER) via *S*-nitrosylation of protein-disulfide isomerase (PDI), which is located in the ER lumen. This may contribute to the accumulation of misfolded proteins, as well as sustained activation of the unfolded protein response (UPR) pathway. These phenomena may be linked to the development of sporadic neurodegenerative diseases. *Antioxid. Redox Signal.* 9, 597–601.

SYNTHESIS OF NITRIC OXIDE AND ITS PHYSIOLOGIC FUNCTION

Nitric oxide (NO) is a short-lived, free radical gas synthesized from L-arginine by the enzyme NO synthase (NOS). NO mediates cell–cell interactions in a variety of tissues. For example, the activation of guanylyl cyclase by NO is important in vasorelaxation and platelet inhibition (31). Although NO has been shown to have both physiologic and pathologic activities in neuronal tissues, the active form of the nitrogen oxide molecule, the target site of nitrogen oxide in cells, and the mechanisms of nitrogen oxide action have not been fully determined. NO includes an array of redox species with distinctive properties and reactivities: NO^+ (nitrosonium ion), nitric oxide (free radical, $NO^\bullet$), and NO^- (nitroxyl anion). NO and NO^+ also react rapidly with O_2 and H_2O_2 to yield higher oxides of nitrogen, such as nitrogen dioxide free radicals ($^\bullet NO_2$) and peroxynitrite ($ONOO^-$). The form in which NO is released from cells and is active in cells has been actively debated. The main candidate is nitric oxide, followed by a short-lived nitroso-compound, such as *S*-nitroso-cysteine (SNOC) (32). The predominant NO species in human plasma are *S*-nitrosothiols, mostly as *S*-nitrosoproteins (39). Covalent modification of targets by SNOC is one likely mechanism of NO action. Thiol groups in various proteins have been shown to be nitrosylated nitrogen oxides, and metal ions in several enzymes are subject to regulation by nitrogen oxide (41). Moreover, nitrogen oxide regulates the physiologic functions of several neurons by reacting with membrane-bound thiol groups on the *N*-methyl-D-aspartate (NMDA) receptor-channel complex (23). Although several mechanisms have been to explain the cytotoxicity of NO in neurons, the crucial mechanism remains unclear.

NO is a highly diffusible gas that acts as an intra- and intercellular messenger. It is implicated in a wide range of adaptive structural and functional changes in the nervous system, including synaptic plasticity (1). NO is produced in response to Ca^{2+} influx or release from intracellular stores; Ca^{2+} binds

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to calmodulin, which then activates NOS. Transient moderate levels of NO production regulate a variety of physiologic processes in neurons by directly or indirectly inducing posttranslational modifications of proteins. NO reacts with sulfhydryl groups via nitrosylation; such a modification of NMDA (glutamate) receptors provides a feedback mechanism to reduce Ca^{2+} influx through the NMDA-receptor channel (6).

Alternatively, NO activates soluble guanylate cyclase to produce cyclic guanosine monophosphate (cGMP), which then activates a kinase (cGMP-dependent protein kinase) that phosphorylates various protein substrates, including those involved in synaptic plasticity and cell survival. However, prolonged production of large amounts of NO can wreak havoc on cellular macromolecules and is believed to contribute to neuronal death in various disorders, including stroke and Parkinson disease (11). NO damages cells by interacting with a superoxide anion radical to produce the nitrogen radical peroxynitrite. Peroxynitrite promotes membrane lipid peroxidation and can also damage proteins by nitrating tyrosine residues.

Increasing evidence also points to a role for NO in the accumulation of garbage proteins and in the degeneration of dopaminergic neurons in Parkinson disease. For example, parkin, an E3 ubiquitin ligase that is associated with early-onset Parkinson disease, is *S*-nitrosylated in animal models of the disease, leading to accumulation of its substrates, including synuclein (7, 8).

S-NITROSYLATION REACTIONS

The formation and biologic properties of *S*-nitrosothiol compounds (RSNOs) play an important part in the biology of NO (41). Ignarro *et al*. (14) first revealed that RSNOs stimulate guanylyl cyclase, providing a mechanistic explanation of vasodilation by nitroglycerin and sodium nitroprusside. Myers *et al*. (32) suggested that because of their vasodilatory properties, low-molecular-weight *S*-nitrosothiol complexes, rather than NO itself, may be the endothelium-derived relaxation factor (EDRF). Later, Loscalzo *et al*. (39) demonstrated that protein *S*-nitrosothiol adducts also have vasodilatory properties. *S*-Nitrosothiol compounds such as SNOC, *S*-nitrosogluthathione (GSNO), and *S*-nitroso-*N*-acetylpenicillamine (SNAP) are common NO donors used to study various aspects of NO biology. Studies have proposed that *S*-nitrosohemoglobin adducts are involved in homeostasis of vascular tone and oxygen delivery (*e.g.*, 20). NO is reactive under reducing conditions and modifies the thiol group of cysteine by *S*-nitrosylation (40).

RELATION BETWEEN *S*-NITROSYLATION AND SPORADIC NEURODEGENERATION

Glutamate is the major excitatory neurotransmitter in the central nervous system. Excessive activation of its receptor, NMDA receptor, is associated with a wide range of neurologic disorders and neurodegenerative maladies, including hypoxic–ischemic brain injury, trauma, epilepsy, Parkinson disease, Huntington disease, amyotrophic lateral sclerosis, and AIDS-related dementia.

TABLE 1. KNOWN *S*-NITROSYLATED PROTEINS

Signal Transduction	
NMDA receptor, $GABA_A$ receptor, ATP-sensitive K channel, Na channel, Ca channel, p21Ras, Src, IKK, MEKK1, protein tyrosine phosphatase 1B, ryanodine receptor, adenylate cyclase, guanylate cyclase	
Apoptosis	
Caspase, ASK1, JNK	
Transcriptional factor	
NF-κB, HIF-1, OxyR, AP-1, c-Jun, p53	
Endothelial Regulation and Arginine Metabolism	
eNOS, argininosuccinate synthetase, hemoglobin, dimethylarginine dimethylaminohydrolase, dimethylarginine dimethylaminohydrolase, NADP(+)-dependent isocitrate dehydrogenase, ornithine decarboxylase	
Redox signal	
Thioredoxin, catalase, glutathione peroxidase	

Stimulation of NMDAR initiates several biochemical events that are associated with neuronal toxicity via Ca^{2+} influx. In particular, excessive NMDAR activation increases the intracellular Ca^{2+} concentration, and the subsequent production of free radicals and activation of proteolytic processes contributes to cell injury or death (5, 25, 26, 29, 37). In chronic neurodegenerative disorders, overactivation of the NMDAR causes an excessive influx of Ca^{2+} into the nerve cell, stimulating various signaling cascades that ultimately result in synaptic damage and apoptosis-like cell death. The injurious pathways induced by the influx of Ca^{2+} include Ca^{2+} overload of mitochondria, which results in oxygen free-radical formation, activation of caspases, and the release of apoptosis-inducing factor.

In addition, Ca^{2+}-dependent activation of neuronal NOS leads to increased NO production and the formation of toxic $ONOO^-$, as well as *S*-nitrosylated GAPDH, and stimulation of p38 mitogen-activated protein kinase (p38 MAPK) activates transcription factors that regulate genes involved in neuronal injury and apoptosis (3, 4, 9, 10, 17, 24, 34). Similar to oxidation, *S*-nitrosylation is a reversible posttranslational modification whereby NO, generated by NOS, attacks the thiol group of a Cys residue (40). It has been revealed that the biologic activities of a wide variety of proteins, including thioredoxin (16) and matrix metalloprotease-9 (15), are modulated by *S*-nitrosylation, and *S*-nitrosylation of proteins is facilitated under conditions of oxidative stress (8, 46) (Table 1). Notably, it has recently been shown that parkin is *S*-nitrosylated in the brains of patients with sporadic Parkinson disease, which decreases its activity as a ubiquitin ligase (8, 27, 46). Parkin participates in the ubiquitination of several substrates, such as synphilin and Pael receptor (19).

FUNCTIONS OF ER CHAPERONES AND ER-SPECIFIC SIGNALING

Protein misfolding is causative in ER stress that stimulates two critical intracellular responses (Fig. 1). One is the expression of chaperones (*i.e.*, proteins that prevent the aggregation

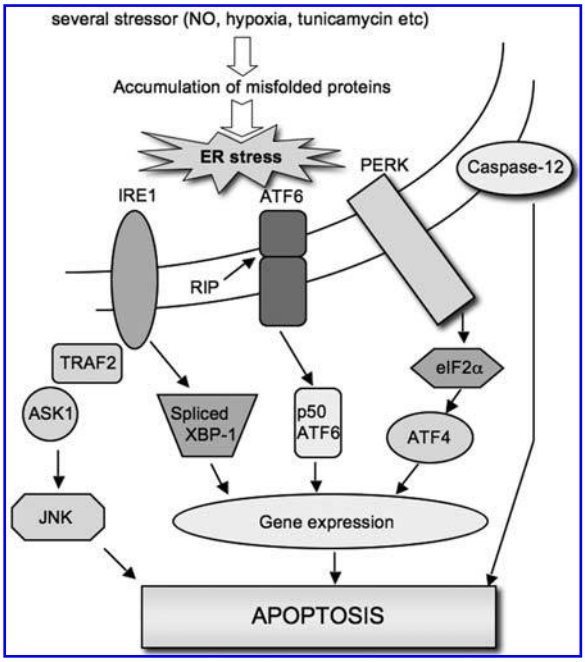

FIG. 1. Signal transduction evoked by endoplasmic reticulum (ER) stress. Some reagents such as thapsigargin and tunicamycin, environmental stress, and oxidative or nitrosative stress induce ER stress via accumulation of unfolded proteins in ER. At least four sensors are found in response to ER stress linked to cell death.

of other proteins and that are implicated in protein folding, posttranslational assembly of protein complexes, and protein degradation. This pathway is believed to contribute to the maintenance of cellular homeostasis during adaptation to altered environmental conditions (21, 30, 36, 44). Another is the attenuation of protein synthesis via a signaling pathway involving eukaryotic initiation factor (eIF2α) kinase. Through these responses, cellular adaptations to environmental change constitute critical protective pathways. The ER is an organelle in which secretory proteins are folded and processed before export from the cell (2), and it initiates stress responses when unfolded immature proteins accumulate (13, 38). Although severe ER stress can induce apoptosis through an ER-specific caspase (33), the ER withstands relatively mild stress by regulating the expression of stress proteins such as glucose-regulated protein (GRP) and PDI. These stress proteins behave as molecular chaperones (*i.e.,* functional proteins that assist in the maturation and transport of unfolded secretory proteins). Conversely, during protein folding in the ER, PDI catalyzes a thiol/disulfide exchange, including disulfide bond formation and rearrangement reactions (28). PDI has two domains with homology to the small redox-active protein thioredoxin (TRX) (12) that function as independent active sites (45). In particular, brain ischemia causes an accumulation of immature and denatured proteins, with consequent ER dysfunction (18, 35). It has been suggested that upregulated GRP, PDI, and other chaperones play essential roles in cell survival under conditions of severe stress (22, 42). Thus, PDI may contribute to adaptive responses to ischemic stress, thereby ultimately contributing to enhanced survival of neurons.

ABROGATION OF PDI ACTIVITY VIA *S*-NITROSYLATION

To determine the effects of *S*-nitrosylation of PDI in neurons, rat primary cultured cerebrocortical neurons were challenged with NMDA to stimulate Ca^{2+} influx and NO production. This treatment caused *S*-nitrosylation of PDI in a NOS-dependent fashion. Moreover, exposure of cultured neurons to both the mitochondrial complex I inhibitor rotenone and a calcium ionophore induced *S*-nitrosylation of PDI (43). Interestingly, it was observed that most neurons are positive for polyubiquitin antibody, consistent with an increased accumulation of misfolded proteins. PDI exists in the ER lumen and is upregulated by several stresses (22, 42). In addition, overexpression of wild-type PDI can protect cells against hypoxic and ischemic stresses *in vivo* or treatment with NMDA and ER stressors such as tunicamycin (a glycosylation inhibitor), thapsigargin (an inhibitor of the ER Ca^{2+}-ATPase), or overexpression of Pael receptor. It also diminishes the number of polyubiquitin-positive neurons (42, 43). It is noteworthy that PDI is significantly *S*-nitrosylated in brain tissue samples prepared from patients with sporadic Parkinson disease and Alzheimer disease compared with controls from patients with other peripheral diseases. Particularly, cysteine residues located in the thioredoxin-like domains of PDI are potentially *S*-nitrosylated, and one of the cysteine residues is further oxidized to sulfinic or sulfonic acid. *S*-nitrosylation of endogenous PDI inhibits isomerase and chaperone-like activity, and this impairment might lead to the formation of Lewy bodies (*i.e.*, intracellular accumulations of misfolded ubiquitinated proteins, like synphilin-1, that are characteristic of Parkinson disease). Overexpression of PDI prevents the formation of polyubiquitinated intracellular aggregates of synphilin-1 in human neuronal cells, and NO can reverse this effect.

These results strongly suggest a specific molecular mechanism by which NO may cause the accumulation of unfolded proteins and neuronal death in Parkinson disease. Recently, it was demonstrated that the accumulation of unfolded proteins in the ER initiates an ER-specific signal-transduction pathway called the UPR (21). The UPR is believed to contribute to the maintenance of cellular homeostasis during the adaptation to altered environmental conditions; however, severe ER stress can induce apoptosis through prolonged UPR activation (see Fig. 1). In this case, loss of PDI function via *S*-nitrosylation induces both the accumulation of unfolded proteins and UPR-related events, including the IRE1–XBP1 pathway and chop mRNA induction. However, the contribution of PDI to the accumulation of unfolded proteins and neuronal death in neurodegenerative diseases is unsolved. Nevertheless, it has been strongly suggested that the accumulation of aggregated or misfolded proteins in the ER is linked to Parkinson disease (7, 19). PDI may be a target for the NO derived from excessive glutamate release during brain ischemia or mitochondrial injury in Parkinson disease. Hence, nitrosative stress resulting in PDI dysfunction provides a mechanistic link between the deficits of molecular chaperones,

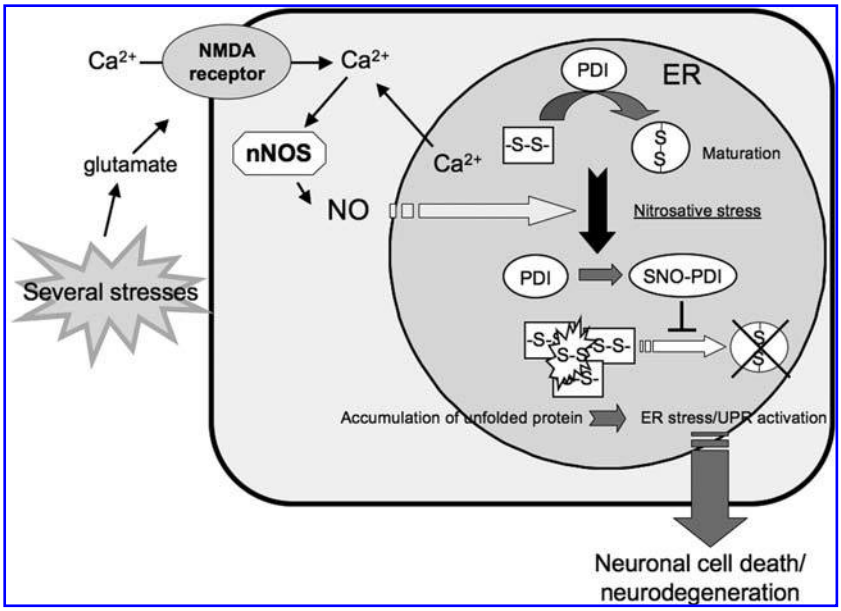

FIG. 2. Possible mechanism of SNO-PDI contributing to the accumulation of misfolded proteins and to neuronal cell death in human neurodegenerative diseases.

accumulation of misfolded proteins, and neuronal death that are characteristic of some neurodegenerative diseases (Fig. 2). Therefore, PDI may provide a novel target for therapeutic intervention in Parkinson disease and other protein aggregation–related disorders. Thus, the elucidation of an NO-mediated pathway to neuronal apoptosis may contribute to the development of new therapeutic approaches for neurodegenerative disease and other disorders associated with protein accumulation due to nitrosative and oxidative stress. These approaches may include the induction of PDI expression, enhancement of its enzymatic activity, abrogation of *S*-nitrosylation of PDI, and suppression of NO production. Because NO plays a vital role in other neurodegenerative disorders, including Alzheimer disease, Huntington disease, amyotrophic lateral sclerosis, and stroke, it is important to determine whether NO-mediated impairment of PDI function is also critical for the abnormal accumulation of garbage proteins and neuronal death in these disorders.

In conclusion, NO-mediated modification of PDI is critical for proper protein folding, and this modification is pivotal in the pathogenesis of Parkinson disease.

ABBREVIATIONS

cGMP, cyclic guanosine monophosphate; ER, endoplasmic reticulum; GRP, glucose-regulated protein; GSNO, *S*-nitrosoglutathione; NMDA, *N*-methyl-D-aspartate, NO, nitric oxide; NOS, NO synthase; p38 MAPK, p38 mitogen-activated protein kinase; PDI, protein-disulfide isomerase; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SNOC, *S*-nitroso-cysteine; TRX, thioredoxin; UPR, unfolded protein response.

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Date of first submission to ARS Central, November 26; date of final revision, November 27, 2006; date of acceptance, December 5, 2006.

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