ANTIOXIDANTS & REDOX SIGNALING
Volume 10, Number 1, 2008

DOI: 10.1089/ars.2007.1858

Forum Review

Emerging Roles of *S*-Nitrosylation in Protein Misfolding and Neurodegenerative Diseases

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ABSTRACT

Overactivation of *N*-methyl-D-aspartate (NMDA)-type glutamate receptors accounts, at least in part, for excitotoxic neuronal damage, potentially contributing to a wide range of acute and chronic neurologic disorders. Recent studies have suggested that generation of excessive nitric oxide (NO) and reactive oxygen species (ROS) can mediate excitotoxicity, in part by triggering protein misfolding. *S*-Nitrosylation, which is a covalent reaction of a NO group with a cysteine thiol, represents one such mechanism that can contribute to NO-induced neurotoxicity. The ubiquitin-proteasome system (UPS), in conjunction with molecular chaperones, can prevent accumulation of aberrantly-folded proteins. For example, protein disulfide isomerase (PDI) can provide neuroprotection from misfolded proteins or endoplasmic reticulum stress through its molecular chaperone and thiol-disulfide oxidoreductase activities. Here, the authors present recent evidence suggesting that NO contributes to degenerative conditions by *S*-nitrosylating PDI (forming SNO-PDI) and the ubiquitin protein ligase, parkin (forming SNO-parkin). Moreover, it is demonstrated for the first time that inhibition of excessive NMDA receptor activity by memantine, via a mechanism of uncompetitive open-channel block with a relatively rapid off-rate, can ameliorate excessive production of NO, protein misfolding, and neurodegeneration. *Antioxid. Redox Signal.* 10, 87–101.

INTRODUCTION

Overproduction of reactive nitrogen species (RNS) and reactive oxygen species (ROS), which lead to neuronal cell injury and death, is a potential mediator of neurodegenerative disorders, including Parkinson's disease (PD), Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), polyglutamine (polyQ) diseases such as Huntington's disease, glaucoma, human immunodeficiency virus-associated dementia, multiple sclerosis, and ischemic brain injury, to name but a few (10, 11, 40, 85, 101). While many intra- and extracellular molecules may participate in neuronal injury, accumulation of nitrosative stress due to excessive generation of nitric oxide (NO) appears to be a potential factor contributing to neuronal cell damage and death (86, 90). A well-established model for NO production entails a central role of the *N*-methyl-D-aspartate (NMDA)-type glutamate receptors in the nervous system. Excessive activation of NMDA receptors drives Ca^{2+} influx, which in turn activates neuronal NO synthase (nNOS), as well as the generation of ROS (17, 45). Accumulating evidence suggests that NO can mediate both protective and neurotoxic effects by reacting with cysteine residues of target proteins to form *S*-nitrosothiols (SNOs), a process termed *S*-nitrosylation because of its effects on the chemical biology of protein function. Importantly, normal mitochondrial respiration may also generate free radicals, principally ROS, and one such molecule, superoxide anion ($O_2^{\cdot -}$), reacts rapidly with free radical NO to form the very toxic product peroxynitrite ($ONOO^-$) (12, 87).

An additional feature of most neurodegenerative diseases is accumulation of misfolded and/or aggregated proteins (13, 22,

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29, 102). These protein aggregates can be cytosolic, nuclear, or extracellular. Importantly, protein aggregation can result from either (a) a mutation in the disease-related gene encoding the protein, or (b) post-translational changes to the protein engendered by nitrosative/oxidative stress (150). A key theme of this article, therefore, is the hypothesis that nitrosative or oxidative stress contributes to protein misfolding in the brains of neurodegenerative patients. In this review, we discuss specific examples showing that *S*-nitrosylation of (a) ubiquitin E3 ligases such as parkin, or (b) endoplasmic reticulum chaperones such as protein-disulfide isomerase (PDI) is critical for the accumulation of misfolded proteins in neurodegenerative diseases such as PD and other conditions (27, 89, 140, 147).

PROTEIN MISFOLDING IN NEURODEGENERATIVE DISEASES

A shared histological feature of many neurodegenerative diseases is the accumulation of misfolded proteins that adversely affect neuronal connectivity and plasticity, and trigger cell death signaling pathways (13, 102). For example, degenerating brain contains aberrant accumulations of misfolded aggregated proteins, such as α-synuclein and synphilin-1 in PD, and amyloid-β (Aβ) and tau in AD. The inclusions observed in PD are called Lewy bodies and are mostly found in the cytoplasm. AD brains show intracellular neurofibrillary tangles, which contain tau, and extracellular plaques, which contain Aβ. Other disorders manifesting protein aggregation include Huntington's disease (polyQ), ALS, and prion disease (29). The above-mentioned aggregates may consist of oligomeric complexes of non-native secondary structures, and demonstrate poor solubility in aqueous or detergent solvent. It has been suggested that either genetic mutations or an increase in nitrosative/oxidative stress can facilitate protein aggregation.

In general, protein aggregates do not accumulate in unstressed healthy neurons, due in part to the existence of cellular 'quality control machineries.' For example, molecular chaperones are believed to provide a defense mechanism against the toxicity of misfolded proteins because chaperones can prevent inappropriate interactions within and between polypeptides, and can promote refolding of proteins that have been misfolded because of cell stress. In addition to the quality control of proteins provided by molecular chaperones, the ubiquitin-proteasome system (UPS) is involved in the clearance of abnormal or aberrant proteins. When chaperones cannot repair misfolded proteins, they may be tagged via addition of polyubiquitin chains for degradation by the proteasome. In neurodegenerative conditions, intra- or extracellular protein aggregates are thought to accumulate in the brain as a result of a decrease in molecular chaperone or proteasome activities. In fact, several mutations that disturb the activity of molecular chaperones or UPS-associated enzymes can cause neurodegeneration (31, 102, 152). Along these lines, postmortem samples from the substantia nigra of PD patients (versus non-PD controls) manifest a significant reduction in proteasome activity (98). Moreover, overexpression of the molecular chaperone HSP70 can prevent neurodegeneration *in vivo* in models of PD (9).

Historically, lesions that contain aggregated proteins were considered to be pathogenic. Recently, several lines of evidence have suggested that aggregates are formed through a complex multistep process by which misfolded proteins assemble into inclusion bodies; currently, soluble oligomers of these aberrant proteins are thought to be the most toxic forms via interference with normal cell activities, while frank aggregates may be an attempt by the cell to wall off potentially toxic material (7, 17).

GENERATION OF RNS/ROS

Glutamate is the major excitatory neurotransmitter in the brain and is important for normal functioning of the nervous system; however, excessive activation of glutamate receptors is implicated in neuronal damage in many neurological disorders ranging from acute hypoxic–ischemic brain injury to chronic neurodegenerative diseases. John Olney coined the term "excitotoxicity" to describe this phenomenon (105, 106). This form of toxicity is mediated at least in part by excessive activation of NMDA-type receptors (23, 86, 90), resulting in excessive Ca^{2+} influx through a receptor associated ion channel. Excessive Ca^{2+} leads to the production of damaging free radicals (*e.g.*, NO and ROS) and other enzymatic processes, contributing to cell death (15, 19, 33, 77, 87, 90). Intracellular Ca^{2+} triggers the generation of NO by activating neuronal NO synthase (nNOS) in a Ca^{2+}/calmodulin (CaM)-dependent manner (17, 45) (Fig. 1). It is currently thought that overstimulation of extrasynaptic NMDA receptors mediates this neuronal damage, while, in contrast, synaptic activity may activate survival pathways (55, 109). Intense hyperstimulation of excitatory receptors leads to necrotic cell death, but more mild or chronic overstimulation can result in apoptotic or other forms of cell death (4, 15, 19).

Increased levels of neuronal Ca^{2+}, in conjunction with the Ca^{2+}-binding protein CaM, trigger the activation of nNOS and subsequent generation of NO from the amino acid L-arginine (2, 17). NO is a gaseous free radical (thus highly diffusible) and a key molecule that plays a vital role in normal signal transduction but in excess can lead to neuronal cell damage and death. The discrepancy of NO effects on neuronal survival can also be caused by the formation of different NO species or intermediates: NO radical (NO·), nitrosonium cation (NO^+), nitroxyl anion (NO^-, with high energy singlet and lower energy triplet forms) (87). Three subtypes of NOS have been identified; two constitutive forms of NOS—nNOS and endothelial NOS (eNOS)—take their names from the cell type in which they were first found. The name of the third subtype—inducible NOS (iNOS)—indicates that expression of the enzyme is induced by acute inflammatory stimuli. All three isoforms are widely distributed in the brain.

Recent studies further pointed out the potential connection between ROS/RNS and mitochondrial dysfunction in neurodegenerative diseases, especially in PD (11, 14). Pesticide and other environmental toxins that inhibit mitochondrial complex I result in oxidative and nitrosative stress, and consequent aberrant protein accumulation (1, 27, 56, 140, 147). Administration to animal models of complex I inhibitors, such as MPTP, 6-hydroxydopamine, rotenone, and paraquat, which result in over-

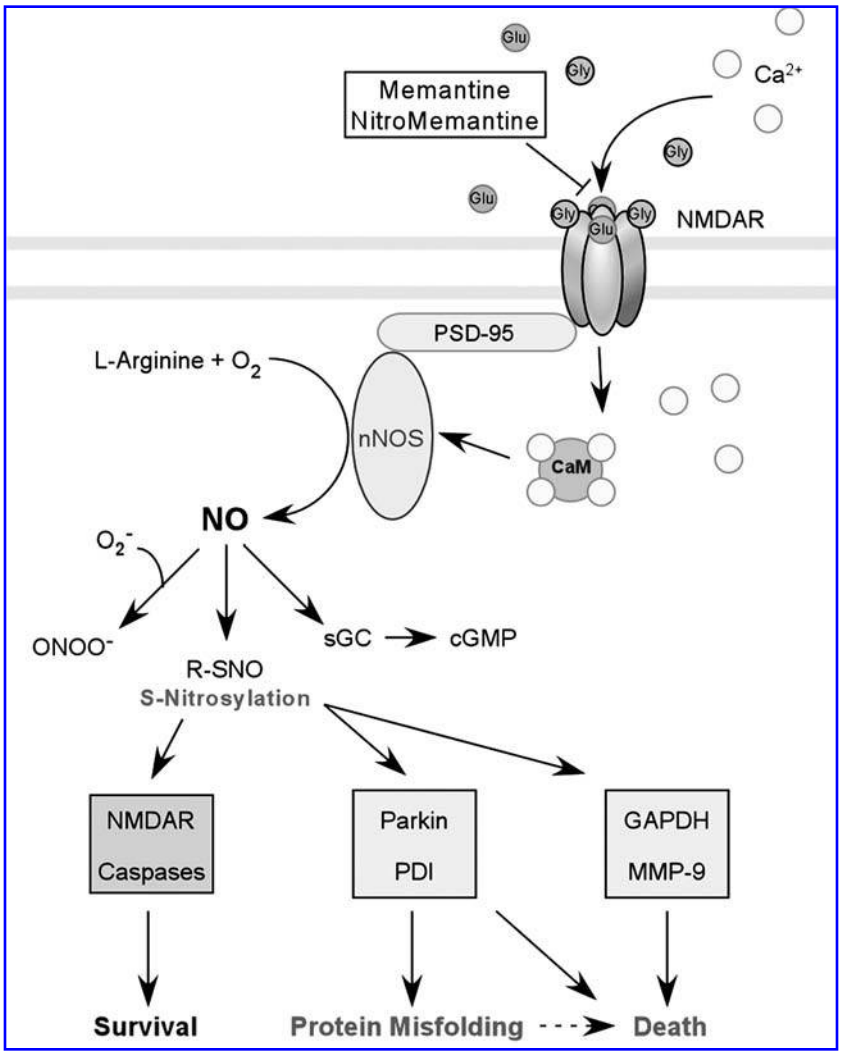

FIG. 1. Activation of the NMDA receptor (NMDAR) by glutamate (Glu) and glycine (Gly) induces Ca^{2+} influx and consequent NO production via activation of nNOS. nNOS is part of a protein complex attached to the NR1 subunit of the NMDA receptor via binding of its PDZ domain to postsynaptic density protein (PSD-95). Many subsequent effects of NO are mediated by chemical, enzymatic, and redox reactions within neurons. NO activates soluble guanylate cyclase (sGC) to produce cyclic guanosine monophosphate (cGMP), and cGMP can activate cGMP-dependent protein kinase. Excessive NMDA receptor activity, leading to the overproduction of NO can be neurotoxic. For example, *S*-nitrosylation of proteins such as parkin, PDI, GAPDH, and MMP-9 can contribute to neuronal cell damage and death. *S*-Nitrosylation of parkin and PDI can also trigger accumulation of misfolded proteins. Neurotoxic effects of NO are also mediated by peroxynitrite ($ONOO^-$), a reaction product of NO and superoxide anion (O_2^-). In contrast, *S*-nitrosylation can mediate neuroprotective effects, for example, by inhibiting caspase activity and by preventing overactivation of NMDA receptors.

production of ROS/RNS, reproduces many of the features of sporadic PD, such as dopaminergic neuron degeneration, upregulation and aggregation of α-synuclein, Lewy body-like intraneuronal inclusions, and behavioral impairment (11, 14). In addition, it has recently been proposed that mitochondrial cytochrome oxidase can produce NO in a nitrite (NO_2^-)- and pH-dependent but non-Ca^{2+}-dependent manner (20).

Increased nitrosative and oxidative stress are associated with chaperone and proteasomal dysfunction, resulting in accumulation of misfolded aggregates (67, 150). However, until recently little was known regarding the molecular and pathogenic mechanisms underlying contribution of NO to the formation of inclusion bodies such as amyloid plaques in AD or Lewy bodies in PD.

PROTEIN *S*-NITROSYLATION AND NEURONAL CELL DEATH

Early investigations indicated that the NO group mediates cellular signaling pathways, which regulate broad aspects of brain function, including synaptic plasticity, normal development, and neuronal cell death (18, 33, 104, 120). In general, NO exerts physiological and some pathophysiological effects via stimulation of guanylate cyclase to form cyclic guanosine-3′, 5′-monophosphate (cGMP) or through *S*-nitros(yl)ation of regulatory protein thiol groups (45, 67, 71, 80, 87, 129). *S*-Nitrosylation is the covalent addition of an NO group to a critical cysteine thiol/sulfhydryl (RSH or, more properly, thiolate anion, RS^-) to form an *S*-nitrosothiol derivative (R-SNO). Such modification modulates the function of a broad spectrum of mammalian, plant, and microbial proteins. In general, a consensus motif of amino acids comprised of nucleophilic residues (generally an acid and a base) surround a critical cysteine, which increases the cysteine sulfhydryl's susceptibility to *S*-nitrosylation (57, 131). Our group first identified the physiological relevance of *S*-nitrosylation by showing that NO and related RNS exert paradoxical effects via redox-based mechanisms—NO is neuroprotective via *S*-nitrosylation of NMDA receptors (as well as other subsequently discovered targets, including caspases), and yet can also be neurodestructive by formation of peroxynitrite (or, as later discovered, reaction with additional molecules such as MMP-9 and GAPDH) (25, 34, 49, 54, 74, 87, 94,

99, 137) (Fig. 1). Over the past decade, accumulating evidence has suggested that *S*-nitrosylation can regulate the biological activity of a great variety of proteins, in some ways akin to phosphorylation (27, 49, 51, 54, 57, 68, 87, 88, 124, 126, 128, 130, 140, 147). Chemically, NO is often a good "leaving group," facilitating further oxidation of critical thiol to disulfide bonds among neighboring (vicinal) cysteine residues or, via reaction with ROS, to sulfenic (—SOH), sulfinic ($—SO_2H$), or sulfonic ($—SO_3H$) acid derivatization of the protein (49, 127, 140, 147). Alternatively, *S*-nitrosylation may possibly produce a nitroxyl disulfide, in which the NO group is shared by close cysteine thiols (60).

Analyses of mice deficient in either nNOS or iNOS confirmed that NO is an important mediator of cell injury and death after excitotoxic stimulation; NO generated from nNOS or iNOS is detrimental to neuronal survival (62, 64). In addition, inhibition of NOS activity ameliorates the progression of disease pathology in animal models of PD, AD, and ALS, suggesting that excess generation of NO plays a pivotal role in the pathogenesis of several neurodegenerative diseases (21, 53, 82, 113). Although the involvement of NO in neurodegeneration has been widely accepted, the chemical relationship between nitrosative stress and accumulation of misfolded proteins has remained obscure. Recent findings, however, have shed light on molecular events underlying this relationship. Specifically, we recently mounted physiological and chemical evidence that *S*-nitrosylation modulates the (a) ubiquitin E3 ligase activity of parkin (27, 89, 147), and (b) chaperone and isomerase activities of PDI (140), contributing to protein misfolding and neurotoxicity in models of neurodegenerative disorders.

PARKIN AND THE UPS

Recent studies on rare genetic forms of PD have found that mutations in the genes encoding parkin (*PARK2*), PINK1 (*PARK6*), α-synuclein (*PARK1/4*), DJ-1 (*PARK7*), ubiquitin C-terminal hydrolase L1 (UCH-L1) (*PARK5*), leucine-rich repeat kinase-2 (LRRK2) (*PARK8*), or ATP13A2 (*PARK9*) can cause PD pathology (16, 75, 81, 108, 112, 115, 141, 153). The discovery that mutations in these genes predispose patients to very rare familial forms of PD have allowed us to begin to understand the mechanism of protein aggregation and neuronal loss in the more common sporadic form of PD. For instance, the identification of α-synuclein as a familial PD gene led to the recognition that one of the major constituents of Lewy bodies in sporadic PD brains is α-synuclein. In addition, identification of errors in the genes encoding parkin (an ubiquitin E3 ligase) and UCH-L1 in rare familial forms of PD has implicated possible dysfunction of the UPS in the pathogenesis of sporadic PD as well. The UPS represents an important mechanism for proteolysis in mammalian cells. Formation of polyubiquitin chains constitutes the signal for proteasomal attack and degradation. An isopeptide bond covalently attaches the C-terminus of the first ubiquitin in a polyubiqutin chain to a lysine residue in the target protein. The cascade of activating (E1), conjugating (E2), and ubiquitin-ligating (E3) type enzymes catalyzes the conjugation of the ubiquitin chain to proteins. In addition, individual E3 ubiquitin ligases play a key role in the recognition of specific substrates (118).

Mutations in the parkin gene can cause autosomal recessive juvenile Parkinsonism (ARJP), accounting for some cases of hereditary PD manifest in young patients with onset beginning anywhere from the teenage years through the 40s (31, 75, 121). Parkin is a member of a large family of E3 ubiquitin ligases that are related to one another by the presence of RING finger domains. Parkin contains a total of 35 cysteine residues, the majority of which reside within its RING domains, which coordinate a structurally important zinc atom often involved in catalysis (95). Parkin has two RING finger domains separated by an "in between RING" (IBR) domain (Fig. 2). This motif allows parkin to recruit substrate proteins as well as an E2 enzyme (*e.g.*, UbcH7, UbcH8, or UbcH13). Point mutations, stop mutations, truncations, and deletions in both alleles of the parkin gene will eventually cause dysfunction in its activity and are responsible for many cases of ARJP, as well as rare adult forms of PD. Parkin mutations usually do not facilitate the formation of Lewy bodies, although there is at least one exception: familial PD patients with the R275W parkin mutant manifest Lewy bodies (42). Biochemical characterization of parkin mutants show that not all parkin mutations result in loss of parkin E3 ligase activity; some of the familial-associated parkin mutants (*e.g.*, the R275W mutant) have increased ubiquitination activity compared to wild-type (52, 97, 125). Additionally, parkin can mediate the formation of nonclassical and "nondegradative" lysine 63-linked polyubiquitin chains (83, 84). This finding may explain how some parkin mutations induce formation of Lewy bodies and why proteins are stabilized within the inclusions.

Several putative target substrates have been identified for parkin E3 ligase activity. One group has reported that mutant parkin failed to bind glycosylated α-synuclein for ubiquitination, leading to α-synuclein accumulation (122), but most authorities do not feel that α-synuclein is a direct substrate of parkin. Synphilin-1 (α-synuclein interacting protein), on the other hand, is considered to be a substrate for parkin ubiquitination, and it is included in Lewy body-like inclusions in cultured cells when co-expressed with α-synuclein (28). Other substrates for parkin include parkin-associated endothelin receptor-like receptor (Pael-R) (66), cell division control related protein (CDCrel-1) (151), cyclin E (132), p38 tRNA synthase (32), and synaptotagmin XI (63), α/β tubulin heterodimers (117), as well as possibly parkin itself (auto-ubiquitination). It is generally accepted that accumulation of these substrates can lead to disastrous consequences for the survival of dopaminergic neurons in familial PD and possibly also in sporadic PD. Therefore, characterization of potential regulators that affect parkin E3 ligase activity may reveal important molecular mechanisms for the pathogenesis of PD. Heretofore, two cellular components have been shown to regulate the substrate specificity and ubiquitin E3 ligase activity of parkin. The first represents post-translational modification of parkin through *S*-nitrosylation (see below for details) or phosphorylation (146), and the second, binding partners of parkin, such as CHIP (65) and BAG5 (70). CHIP enhances the ability of parkin to inhibit cell death through upregulation of parkin-mediated ubiquitination, while BAG5-mediated inhibition of parkin E3 ligase activity facilitates neuronal cell death. In addition, Fallon *et al*. (41) re-

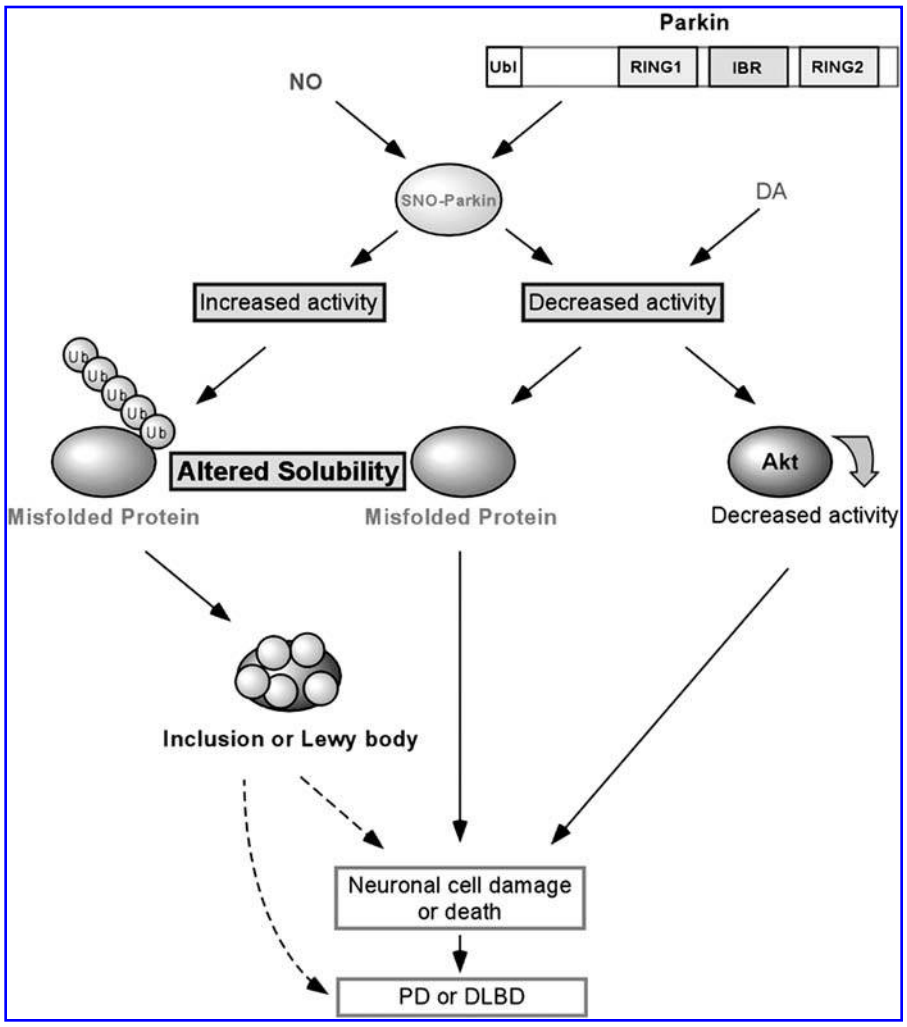

FIG. 2. Possible mechanism of *S*-nitrosylated parkin (SNO-Parkin) contributing to the accumulation of aberrant proteins and damage or death of dopaminergic neurons. Nitrosative stress leads to *S*-nitrosylation of parkin, and, initially, to a dramatic increase followed by a decrease in its E3 ubiquitin ligase activity (27, 89, 147). The initial increase in this E3 ubiquitin ligase activity leads to enhanced ubiquitination of parkin substrates (*e.g.*, synphilin-1, Pael-R, and parkin itself). Increased parkin E3 ubiquitin ligase activity may contribute to Lewy body formation and impair parkin function, as also suggested by Sriram *et al.* (125). The subsequent decrease in parkin activity may allow misfolded proteins to accumulate. Downregulation of parkin may also result in decreased Akt neuroprotective activity because of enhanced EGFR internalization (41). Dopamine quinone can also modify cysteine thiols of parkin and reduce its activity (79). DLBD, diffuse Lewy body disease; PD, Parkinson's disease; Ub, ubiquitin.

cently reported another mechanism for parkin-mediated neuronal survival via a proteasome-independent pathway (41). In this model, parkin mono-ubiquitinates the epidermal growth factor receptor (EGFR)-associated protein, Eps15, leading to inhibition of EGFR endocytosis. The resulting prolongation of EGFR signaling via the phosphoinositide-3 kinase /Akt (PKB) signaling pathway is postulated to enhance neuronal survival.

Another important molecule that links aberrant UPS activity and PD is the ubiquitin hydrolase Uch-L1, a deubiquitinating enzyme that recycles ubiquitin. Autosomal dominant mutations of Uch-L1 have been identified in two siblings with PD (81). Interestingly, a recent study suggested that a novel ubiquitin-ubiquitin ligase activity of Uch-L1 might also be important in the pathogenesis of PD (92). Additional mutations in α-synuclein, DJ-1, PINK1, and LRRK2 may contribute to UPS dysfunction and subsequently lead to PD.

S-NITROSYLATION AND PARKIN

PD is the second most prevalent neurodegenerative disease and is characterized by the progressive loss of dopamine neurons in the substantia nigra pars compacta. Appearance of Lewy bodies that contain misfolded and ubiquitinated proteins generally accompanies the loss of dopaminergic neurons in the PD brain. Such ubiquitinated inclusion bodies are the hallmark of many neurodegenerative disorders. Age-associated defects in intracellular proteolysis of misfolded or aberrant proteins might lead to accumulation and ultimately deposition of aggregates within neurons or glial cells. Although such aberrant protein accumulation had been observed in patients with genetically-encoded mutant proteins, recent evidence from our laboratory suggests that nitrosative and oxidative stress are potential causal factors for protein accumulation in the much more common sporadic form of PD. As illustrated below, nitrosative/oxidative stress, commonly found during normal aging, can mimic rare genetic causes of disorders, such as PD, by promoting protein misfolding in the absence of a genetic mutation (27, 89, 147). For example, *S*-nitrosylation and further oxidation of parkin or Uch-L1 result in dysfunction of these enzymes and thus of the UPS (24, 26, 27, 50, 103, 147). We and others recently discovered that nitrosative stress triggers *S*-nitrosylation of parkin (forming SNO-parkin) not only in rodent models of PD but also in the brains of human patients with PD and the related α-synucleinopathy, DLBD (diffuse Lewy body disease). SNO-parkin

initially stimulates ubiquitin E3 ligase activity, resulting in enhanced ubiquitination as observed in Lewy bodies, followed by a decrease in enzyme activity, producing a futile cycle of dysfunctional UPS (83, 89, 147) (Fig. 2). We also found that rotenone led to the generation of SNO-parkin and thus dysfunctional ubiquitin E3 ligase activity. Moreover, *S*-nitrosylation appears to compromise the neuroprotective effect of parkin (27). These mechanisms involve *S*-nitrosylation of critical cysteine residues in the first RING domain of parkin (147). Nitrosative and oxidative stress can also alter the solubility of parkin via post-translational modification of cysteine residues, which may concomitantly compromise its protective function (143, 144). Additionally, it is likely that other ubiquitin E3 ligases with RING-finger thiol motifs are *S*-nitrosylated in a similar manner to parkin to affect their enzymatic function; hence, *S*-nitrosylation of E3 ligases may be involved in a number of degenerative conditions.

The neurotransmitter dopamine (DA) may also impair parkin activity and contribute to neuronal demise via the modification of cysteine residue(s) (79). DA can be oxidized to DA quinone, which can react with and inactivate proteins through covalent modification of cysteine sulfhydryl groups; peroxynitrite has been reported to promote oxidation of DA to form dopamine quinone (78). DA quinone can preferentially attack cysteine residues (C268 and C323) in the RING1 and IBR domains of parkin, forming a covalent adduct that abrogates its E3 uibiquitin ligase activity (79, 144). DA quinone also reduces the solubility of parkin, possibly inducing parkin misfolding after disruption of the RING–IBR–RING motif. Therefore, oxidative/nitrosative species may either directly or indirectly contribute to altered parkin activity within the brain, and subsequent loss of parkin-dependent neuroprotection results in increased cell death.

THE UNFOLDED PROTEIN RESPONSE (UPR) AND PROTEIN-DISULFIDE ISOMERASE (PDI)

The ER normally participates in protein processing and folding but undergoes a stress response when immature or misfolded proteins accumulate (3, 38, 123, 133). ER stress stimulates two critical intracellular responses (Fig. 3). The first represents expression of chaperones that prevent protein aggregation via the unfolded protein response (UPR), and is implicated in protein refolding, post-translational assembly of protein complexes, and protein degradation. This response is believed to contribute to adaptation during altered environmental conditions, promoting maintenance of cellular homeostasis. At least three ER trans-

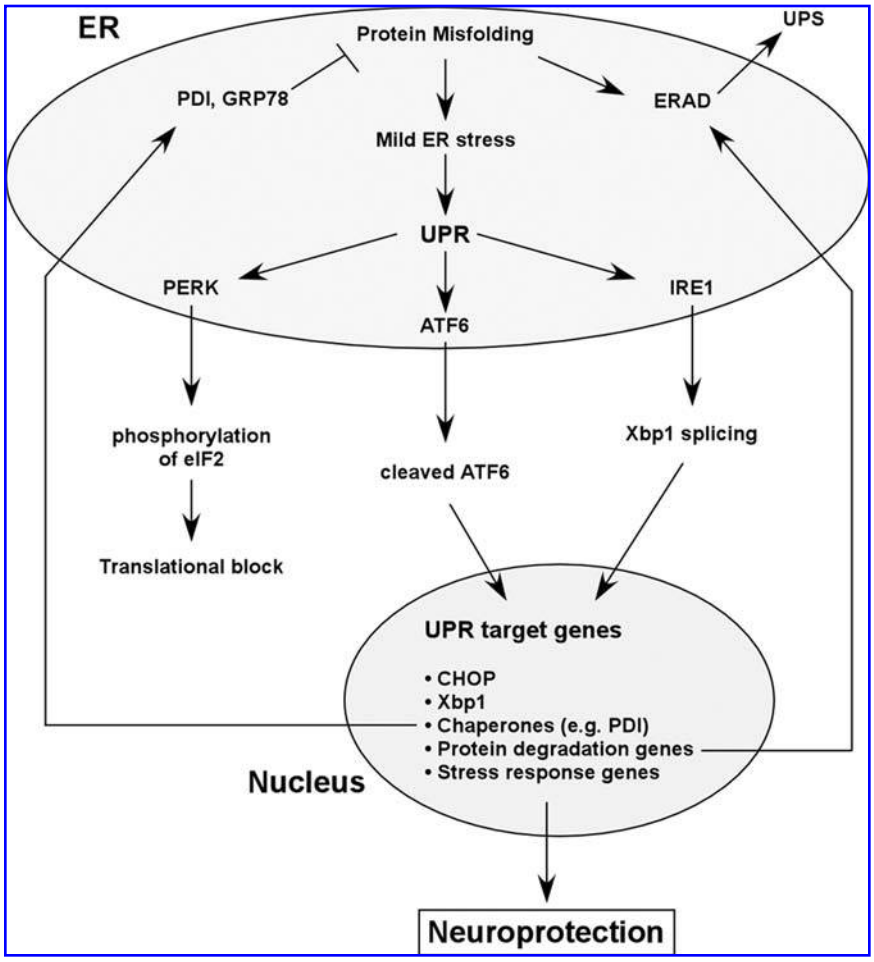

FIG. 3. ER stress and UPR. ER stress is triggered when misfolded proteins accumulate within the ER lumen, inducing the unfolded protein response (UPR). The UPR is usually a transient homeostatic mechanism for cell survival, while prolonged UPR elicits neuronal cell death. ER chaperones, such as PDI and GRP78, modulate the activity of UPR sensors by mediating proper protein folding in the ER. The UPR includes activation of pancreatic ER kinase (PKR)-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1). Activated PERK phosphorylates eukaryotic initiation factor 2 (eIF2) to inhibit protein synthesis, thus decreasing the arrival of nascent proteins in the ER to promote cell survival. Proteolysis of ATF6 and IRE1-mediated mRNA splicing of X box-binding protein 1 (Xbp1) enhance their transcriptional activities, thus upregulating transcription of UPR target genes. Additionally, proteins that fail to attain their native folded state are eventually retro-translocated across the ER membrane to be disposed of by cytosolic proteasomes. This process, known as ER-associated degradation (ERAD), is essential in preventing protein accumulation and aggregation in the ER (3, 38, 123, 133).

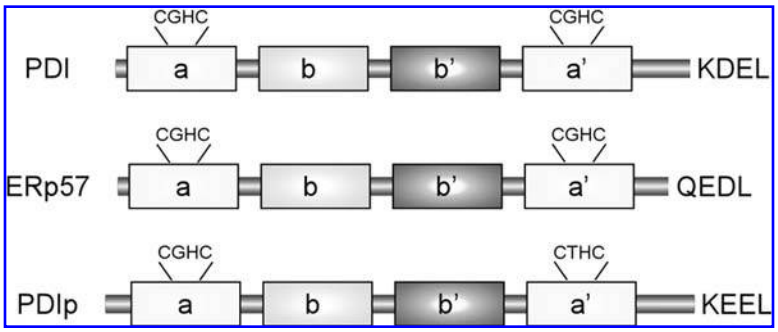

FIG. 4. Domain organization of PDI family. Each *rectangle* represents a thioredoxin (TRX)-like domain, and the catalytic a and a' domains include a CXXC active-site motif. The signal sequence is not shown. PDI family members usually contain a typical C-terminal ER-retention signal, such as KDEL, QEDL, or KEEL. There are 14 PDI family members in the ER [see Ellgaard and Ruddock (39) for a review]. Previous evidence has suggested that at least three of the PDI family members (PDI, ERp57, and PDIp) are involved in protection of neurons against protein misfolding and cell death.

membrane sensor proteins are involved in the UPR: PKR-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1). The activation of all three proximal sensors results in the attenuation of protein synthesis via eukaryotic initiation factor-2 (eIF2) kinase and increased protein folding capacity of the ER (72, 100, 110, 149, 150). The second ER stress response, termed ER-associated degradation (ERAD), specifically recognizes terminally misfolded proteins for retrotranslocation across the ER membrane to the cystosol, where they can be degraded by the UPS. Additionally, although severe ER stress can induce apoptosis, the ER withstands relatively mild insults via expression of stress proteins such as glucose-regulated protein (GRP) and PDI. These proteins behave as molecular chaperones that assist in the maturation, transport, and folding of secretory proteins.

During protein folding in the ER, PDI can introduce disulfide bonds into proteins (oxidation), break disulfide bonds (reduction), and catalyze thiol/disulfide exchange (isomerization), thus facilitating disulfide bond formation, rearrangement reactions, and structural stability (93). PDI has four domains that are homologous to thioredoxin (TRX) (termed a, b, b', and a'). Only two of the four TRX-like domains (a and a') contain a characteristic redox-active CXXC motif, and these two-thiol/disulfide centers function as independent active sites (37, 39, 48, 142) (Fig. 4). These active-site cysteines can be found in two different redox states: oxidized (disulfide) or reduced (free sulfhydryls or thiols). During oxidation of a target protein, oxidized PDI catalyzes disulfide formation in the substrate protein, resulting in the reduction of PDI. In contrast, the reduced form of the active-site cysteines can initiate isomerization by attacking the disulfide of a substrate protein and forming a transient intermolecular disulfide bond. As a consequence, an intramolecular disulfide rearrangement occurs within the substrate itself, resulting in the generation of reduced PDI (Fig. 5). The recently determined structure of yeast PDI revealed that the four TRX-like domains form a twisted "U" shape with the two active sites facing each other on opposite sides of the "U" (138). Hydrophobic residues line the inside surface of the "U," facilitating interactions between PDI and misfolded proteins. Several mammalian PDI homologues, such as ERp57 and PDIp,

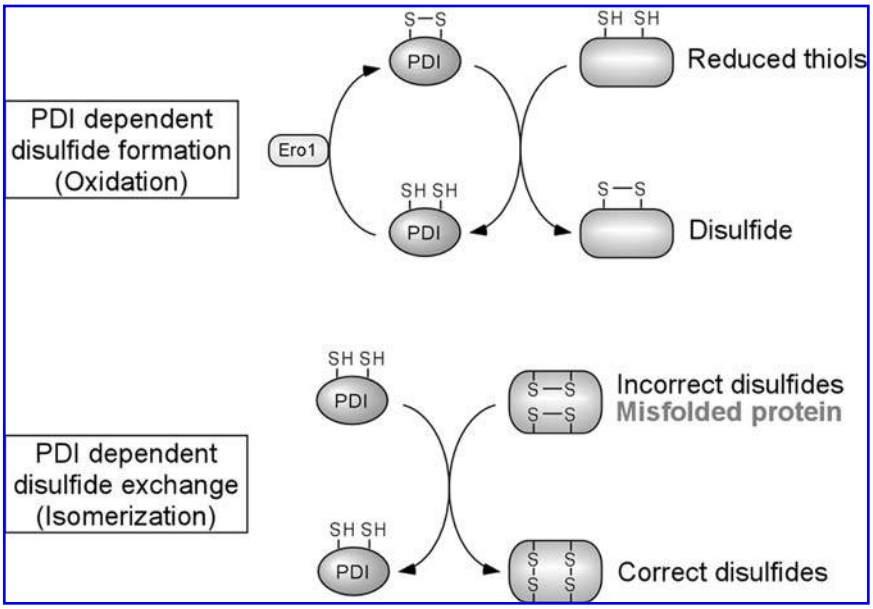

FIG. 5. Molecular mechanisms of PDI-dependent oxidative protein folding in the ER. *Top:* Oxidized PDI (*left*), which contains a disulfide bond at its active site, catalyzes the formation of a disulfide bond in a substrate protein (*right*), resulting in the reduction of PDI. Conversely, the ER oxidoreduction protein, Ero1, can reoxidize and regenerate the PDI active site. By repeating this cycle, PDI can continuously insert disulfide bonds into different substrate proteins. *Bottom:* In the early process of protein folding in the ER, cysteine residues often form inaccurate disulfide bonds (resulting in a misfolded protein). The isomerase activity of PDI (*left*) converts these incorrect disulfide bonds to their correct ative form. This reaction occurs through breakage of substrate disulfide, formation of intramolecular disulfide, and reformation of intermolecular disulfide bonds with different thiols in the target substrate protein (*right*). For simplicity, the redox state of only one PDI active site is shown.

also localize to the ER and may manifest similar functions (30, 59) (Fig. 4). Increased expression of PDIp in neuronal cells under conditions mimicking PD suggest the possible contribution of PDIp to neuronal survival (30).

In many neurodegenerative disorders and cerebral ischemia, the accumulation of immature and denatured proteins results in ER dysfunction (8, 30, 61, 116), but upregulation of PDI represents an adaptive response promoting protein refolding and may offer neuronal cell protection (30, 59, 76, 135). In a recent study, we reported that the *S*-nitrosylation of PDI (to form SNO-PDI) disrupts its neuroprotective role (140).

S-NITROSYLATION OF PDI MEDIATES PROTEIN MISFOLDING AND NEUROTOXICITY IN CELL MODELS OF PD OR AD

Disturbance of Ca^{2+} homeostasis within the ER plays a critical role in the accumulation of misfolded proteins and ER stress because the function of several ER chaperones requires high concentrations of Ca^{2+}. In addition, it is generally accepted that excessive generation of NO can contribute to activation of the ER stress pathway, at least in some cell types (46, 107). Molecular mechanisms by which NO induces protein misfolding and ER stress, however, have remained enigmatic until recently. The ER normally manifests a relatively positive redox potential in contrast to the highly reducing environment of the cytosol and mitochondria. This redox environment can influence the stability of protein *S*-nitrosylation and oxidation reactions (43). *S*-Nitrosylation can enhance the activity of the ER Ca^{2+} channel-ryanodine receptor (5, 145), which may provide a clue to how NO disrupts Ca^{2+} homeostasis in the ER and activates the cell death pathway. Interestingly, we have recently reported that excessive NO can also lead to *S*-nitrosylation of the active-site thiol groups of PDI, and this reaction inhibits both its isomerase and chaperone activities (140). Mitochondrial complex I insult by rotenone can also result in *S*-nitrosylation of PDI in cell culture models. Moreover, we found that PDI is *S*-nitrosylated in the brains of virtually all cases examined of sporadic AD and PD. Under pathological conditions, it is possible that both cysteine sulfhydryl groups in the TRX-like domains of PDI form *S*-nitrosothiols. Unlike formation of a single *S*-nitrosothiol, which is commonly seen after denitrosylation reactions catalyzed by PDI (124), dual nitrosylation may be relatively more stable and prevent subsequent disulfide formation on PDI. Therefore, we speculate that these pathological *S*-nitrosylation reactions on PDI are more easily detected during neurodegenerative conditions. Additionally, it is possible that vicinal (nearby) cysteine thiols reacting with NO can form nitroxyl disulfide (60), and such reaction may potentially occur in the catalytic side of PDI to inhibit enzymatic activity. In order to determine the consequences of *S*-nitrosylated PDI (SNO-PDI) formation in neurons, we exposed cultured cerebrocortical neurons to neurotoxic concentrations of NMDA, thus inducing excessive Ca^{2+} influx and consequent NO production from nNOS. Under these conditions, we found that PDI was *S*-nitrosylated in a NOS-dependent manner. SNO-PDI formation led to the accumulation of polyubiquitinated/misfolded proteins and activation of the UPR. Moreover, *S*-nitrosylation abrogated the inhibitory effect of PDI on aggregation of proteins observed in Lewy body inclusions (28, 140). *S*-Nitrosylation of PDI also prevented its attenuation of neuronal cell death triggered by ER stress, misfolded proteins, or proteasome inhibition (Fig. 6). Further evidence suggested that SNO-PDI may in effect transport NO to the extracellular space, where it could conceivably exert additional adverse effects (124). Additionally, NO can possibly mediate cell death or injury via *S*-nitrosylation or nitration reactions on other TRX-like proteins, such as TRX itself and glutaredoxin (5, 51, 136).

In addition to PDI, *S*-nitrosylation is likely to affect critical thiol groups on other chaperones, such as HSP90 in the cytoplasm (96) and possibly GRP in the ER. Normally, HSP90 stabilizes misfolded proteins and modulates the activity of cell signaling proteins including NOS and calreticulin (102). In AD brains, levels of HSP90 are increased in both the cytosolic and membranous fractions, where HSP90 is thought to maintain tau and Aβ in a soluble conformation, thereby averting their aggregation (35, 69). Martínez-Ruiz *et al*. (96) recently demonstrated that *S*-nitrosylation of HSP90 can occur in endothelial cells, and this modification abolishes its ATPase activity, which is required for its function as a molecular chaperone. These studies imply that *S*-nitrosylation of HSP90 in neurons of AD brains may contribute to the accumulation of tau and Aβ aggregates.

The UPS is apparently impaired in the aging brain. Additionally, inclusion bodies similar to those found in neurodegenerative disorders can appear in brains of normal aged individuals or those with subclinical manifestations of disease (47). These findings suggest that the activity of the UPS and molecular chaperones may decline in an age-dependent manner (111). Given that we have not found detectable quantities of SNO-parkin and SNO-PDI in normal aged brain (27, 140, 147) we speculate that *S*-nitrosylation of these and similar proteins may represent a key event that contributes to susceptibility of the aging brain to neurodegenerative conditions.

PDI ACTIVITY IN ALS AND PRION DISEASE

Recently, PDI has been implicated in the pathophysiology of familial ALS (8). Mutations in Cu/Zn superoxide dismutase (SOD1) are known to be involved in motor neuron death in some forms of familial ALS. SOD1 is an intracellular homodimeric metalloprotein that forms a stable intra-subunit disulfide bond. Biochemical evidence suggests that the disulfide-reduced monomer of mutant SOD1 (mtSOD1) forms inclusion bodies (6, 36, 44, 114, 139), and aggregates of misfolded mt SOD1 are commonly associated with the disease, as seen at post-mortem examination. In addition, although wild-type (wt)SOD1 is found predominantly in the cytoplasm, mtSOD1 forms monomers or insoluble high molecular weight multimers within the ER (73). Atkin *et al*. (8) recently showed that inhibition of PDI activity with bacitracin can increase aggregation of mt-SOD1 in neuronal cells. Moreover, PDI co-localized and bound

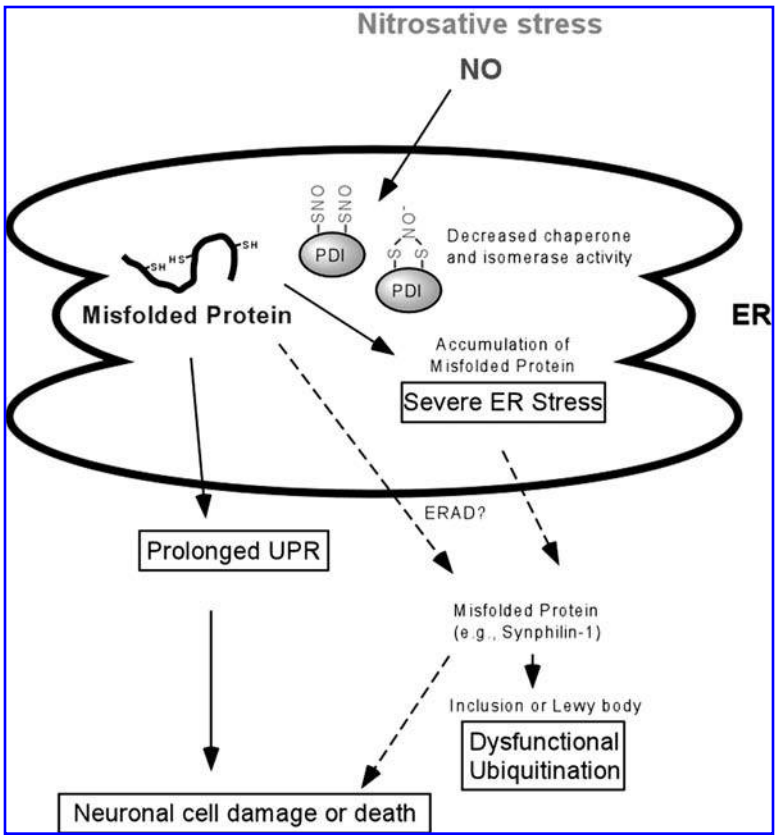

FIG. 6. Possible mechanism of *S*-nitrosylated PDI (SNO-PDI) contributing to the accumulation of aberrant proteins and neuronal cell damage or death. Under conditions of severe nitrosative stress, *S*-nitrosylation of neuronal PDI inhibits normal protein folding in the ER, activates ER stress, and induces a prolonged UPR, thus contributing to protein aggregation and cell damage or death. For simplicity, *S*-nitrosylation of only one (of two) thioredoxin domains of PDI is shown, resulting in formation of SNO-PDI or possibly nitroxyl-PDI, as described in Uehara *et al*. (140) and Forrester *et al*. (43).

to intracellular aggregates of mtSOD1. Upregulation of the UPR was also observed in mtSOD1 mice. These findings suggest that ER stress may contribute to the pathophysiology of familial ALS, and PDI could potentially reduce mtSOD1 aggregation and affect neuronal survival. Interestingly, *S*-nitrosothiol levels have also been found to be abnormal in the spinal cords of mtSOD1 transgenic mice (119). Whether SNO-PDI is involved in SOD1 aggregation and motor neuron injury in ALS remains to be studied.

Finally, transmissible spongiform encephalopathies (TSE), also known as prion diseases, are transmissible neurodegenerative disorders and include Creutzfeldt–Jacob disease, bovine spongiform encephalopathy, and scrapie. Cerebral accumulation of misfolded prion protein (PrP) and extensive neuronal apoptosis represent pathological hallmarks of these prion diseases. Recent reports have suggested that a prolonged UPR due to PrP misfolding in the ER may contribute to neuronal dysfunction (58, 59, 148). This ER stress response is mainly associated with upregulation of ERp57, an ER chaperone with PDI-like activity, suggesting that this chaperone may represent a cellular response to prion infection (59). In fact, *in vitro* studies on Erp57, either overexpressing (via transfection) or downregulating (via RNAi), demonstrated that this ER chaperone protects cells against PrP misfolding and toxicity. Collectively, these studies raise the possibility that SNO-PDI and *S*-nitrosylation of other chaperone molecules may represent potential therapeutic targets to prevent protein aggregation in several neurodegenerative diseases.

POTENTIAL TREATMENT OF EXCESSIVE NMDA-INDUCED Ca^{2+} INFLUX AND FREE RADICAL GENERATION

One mechanism that could potentially curtail excessive Ca^{2+} influx and resultant overstimulation of nNOS activity would be inhibition of NMDA receptors. Until recently, however, drugs in this class blocked virtually all NMDA receptor activity, including physiological activity, and therefore manifest unacceptable side effects by inhibiting normal functions of the receptor. For this reason, many previous NMDA receptor antagonists have disappointingly failed in advanced clinical trials conducted for a number of neurodegenerative disorders. In contrast, studies in our laboratory first showed that the adamantane derivative, memantine, preferentially blocks excessive (pathological) NMDA receptor activity while relatively sparing normal (physiological) activity. Memantine does this in a surprising fashion because of its low (micromolar) affinity, even though its actions are quite selective for the NMDA receptor at that concentration (Fig. 7). "Apparent" affinity of a drug is determined by the ratio of its "on-rate" to its "off-rate" for the target. The on-rate is not only a property of drug diffusion and interaction with the target, but also the drug's concentration. In contrast, the off-rate is an intrinsic property of the drug–receptor complex, unaffected by drug concentration. A relatively fast off-rate is a major contributor to memantine's low affinity for the NMDA receptor. The inhibitory activity of memantine involves

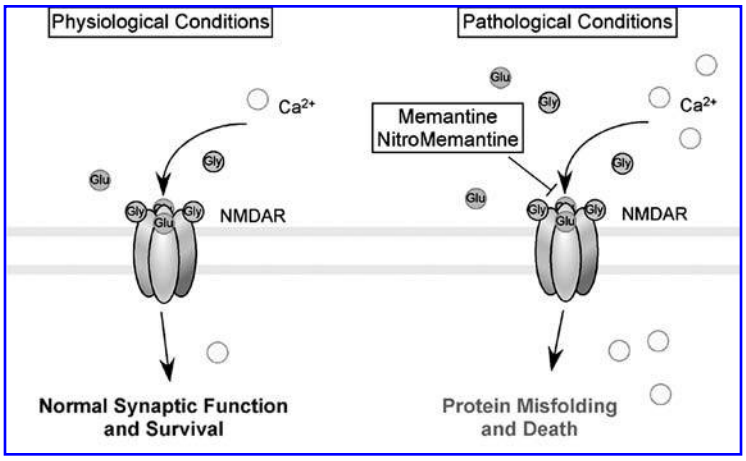

FIG. 7. Memantine/NitroMemantine preferentially block excessive NMDA receptor activity. Memantine preferentially blocks excessive (pathological/extrasynaptic) NMDA receptor activity while relatively sparing normal (physiological/synaptic) activity. This unique inhibitory action of memantine is achieved by (a) blockade of NMDA receptor-associated ion channels predominantly when they are excessively open in the face of increasing agonist concentrations (uncompetitive inhibition by open-channel block), and (b) relatively low affinity for the target ion channels (predominantly because of a relatively fast off-rate), thus avoiding accumulation in the channels. NitroMemantine drugs target NO to the NMDA receptor to add to memantine neuroprotective action because *S*-nitrosylation of the receptor has also been shown to limit its excessive activity.

blockade of the NMDA receptor-associated ion channel when it is excessively open (termed open-channel block). The unique and subtle difference of the memantine blocking sites in the channel pore may explain the advantageous properties of memantine action.

Also critical for the clinical tolerability of memantine is its uncompetitive mechanism of action. An uncompetitive antagonist can be distinguished from a noncompetitive antagonist, which acts allosterically at a noncompetitive site (*i.e.*, at a site other than the agonist-binding site). An uncompetitive antagonist is defined as an inhibitor whose action is contingent upon prior activation of the receptor by the agonist. Hence, the same amount of antagonist blocks higher concentrations of agonist relatively better than lower concentrations of agonist. Some open-channel blockers function as pure uncompetitive antagonists, depending on their exact properties of interaction with the ion channel. This uncompetitive mechanism of action coupled with a relatively fast off-rate from the channel yields a drug that preferentially blocks NMDA receptor-operated channels when they are excessively open while relatively sparing normal neurotransmission. In fact, the relatively fast off-rate is a major contributor to a drug like memantine's low affinity for the channel pore. While many factors determine the clinical efficacy and tolerability of a drug, it appears that the relatively rapid off rate is a predominant factor in the tolerability of memantine, in contrast to other NMDA-type receptor antagonists.(23, 86) Thus, the critical features of memantine's mode of action are its Uncompetitive mechanism and Fast Off-rate, or what we call a UFO drug—a drug that is present at its site of inhibitory action only when needed and then quickly disappears. Memantine has been used for many years in Europe to treat PD, and regulatory agencies in both Europe and the United States recently voted its approval as the first treatment for moderate-to-severe AD. It is currently under study for a number of other neurodegenerative disorders.

As promising as the results with memantine are, we are continuing to pursue ways to use additional modulatory sites on the NMDA receptor to block excitotoxicity even more effectively and safely than memantine alone. New approaches in this regard are explored below.

FUTURE THERAPEUTICS: NITROMEMANTINES

NitroMemantines are second-generation memantine derivatives that are designed to have enhanced neuroprotective efficacy without sacrificing clinical tolerability. *S*-Nitrosylation site(s) is located on the extracellular domain of the NMDA receptor, and *S*-nitrosylation of this site (*i.e.*, NO reaction with the sulfhydryl group of a critical cysteine residue), downregulates (but does not completely shut off) receptor activity (Fig. 7) (86). The drug nitroglycerin, which generates NO-related species, can act at this site to limit excessive NMDA receptor activity. In fact, in rodent models, nitroglycerin can limit ischemic damage (91), and there is some evidence that patients taking nitroglycerin for other medical reasons may be resistant to glaucomatous visual field loss (154). Consequently, we carefully characterized the *S*-nitrosylation sites on the NMDA receptor in order to determine if we could design a nitroglycerin-like drug that could be more specifically targeted to the receptor. In brief, we found that five different cysteine residues on the NMDA receptor could interact with NO. One of these, located at cysteine residue #399 (Cys399) on the NR2A subunit of the NMDA receptor, mediates ≥90% of the effect of NO under our experimental conditions (25). From crystal structure models and electrophysiological experiments, we further found that NO binding to the NMDA receptor at Cys399 may induce a conformational change in the receptor protein that makes glutamate and Zn^{2+} bind more tightly to the receptor. The enhanced binding of glutamate and Zn^{2+} in turn causes the receptor to desensitize and, consequently, the ion channel to close (88). Electrophysiological studies have demonstrated this inhibitory effect of NO on the NMDA receptor-associated channel (25, 80, 87). Moreover, as the oxygen tension is lowered (a pO_2 of 10–20 torr is found in normal brain, and even lower levels under hypoxic/ischemic conditions), the NMDA receptor becomes more sensitive to inhibition by *S*-nitrosylation (134).

Unfortunately, nitroglycerin itself is not very attractive as a neuroprotective agent. The same cardiovascular vasodilator effect that makes it useful in the treatment of angina could cause dangerously large drops in blood pressure in patients with de-

mentia, stroke, traumatic injury, or glaucoma. However, the open-channel block mechanism of memantine not only leads to a higher degree of channel blockade in the presence of excessive levels of glutamate but also can be used as a homing signal for targeting drugs (*e.g.*, the NO group) to hyperactivated, open NMDA-gated channels. We have therefore been developing combinatorial drugs (NitroMemantines) that theoretically should be able to use memantine to target NO to the nitrosylation sites of the NMDAR in order to avoid the systemic side effects of NO. Two sites of modulation would be analogous to having two volume controls on your television set for fine-tuning the audio signal.

Preliminary studies have shown NitroMemantines to be highly neuroprotective in both *in vitro* and *in vivo* animal models (86). In fact, they appear to be more effective than memantine at lower dosage. Moreover, because of the targeting effect of the memantine moiety, NitroMemantines appear to lack the blood pressure lowering effects typical of nitroglycerin. More research still needs to be performed on NitroMemantine drugs, but by combining two clinically tolerated drugs (memantine and nitroglycerin), we have created a new, improved class of UFO drugs that should be both clinically tolerated and neuroprotective.

CONCLUSIONS

Excessive nitrosative and oxidative stress triggered by excessive NMDA receptor activation and/or mitochondrial dysfunction may result in malfunction of the UPS or molecular chaperones, thus contributing to abnormal protein accumulation and neuronal damage in sporadic forms of neurodegenerative diseases. Our elucidation of an NO-mediated pathway to dysfunction of parkin and PDI by *S*-nitrosylation provides a mechanistic link between free radical production, abnormal protein accumulation, and neuronal cell injury in neurodegenerative disorders such as PD. Elucidation of this new pathway may lead to the development of additional new therapeutic approaches to prevent aberrant protein misfolding by targeted disruption or prevention of nitrosylation of specific proteins such as parkin and PDI. Moreover, enhancing the action of PDI may represent a novel strategy for the treatment of other neurodegenerative disorders, such as ALS and TSE.

ACKNOWLEDGMENTS

This work was supported in part by a JSPS Postdoctoral Fellowship for Research Abroad (TN), NIH grants P01 HD29587, R01 EY05477, R01 EY09024, the American Parkinson's Disease Association, San Diego Chapter, and an Ellison Senior Scholars Award in Aging (SAL).

ABBREVIATIONS

Aβ, amyloid-β; AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; ARJP, autosomal recessive juvenile parkinsonism, ATF6, activating transcription factor 6; CaM, Ca^{2+}/calmodulin; cGMP, cyclic guanosine-3',5'-monophosphate; DLBD, diffuse Lewy body disease; EGFR, epidermal growth factor receptor; eIF2, eukaryotic initation factor-2; ER, endoplasmic reticulum; ERAD, ER-associated degradation; GRP, glucose-regulated protein; IBR, in between RING; IRE1, inositol-requiring enzyme 1; NMDA, *N*-methyl-D-aspartate; NO, nitric oxide; NOS, NO synthase; PERK, PKR-like ER kinase; PD, Parkinson disease; PDI, protein disulfide isomerase, RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; SNO, *S*-nitrosothiol; TRX, thioredoxin; TSE, transmissible spongiform encephalopathies; UPR, unfolded protein response; UPS, ubiquitin-proteasome system.

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Date of first submission to ARS Central, July 27, 2007; date of acceptance, July 31, 2007.

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