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FORUM REVIEW ARTICLE

S-Nitrosylation of Critical Protein Thiols Mediates Protein Misfolding and Mitochondrial Dysfunction in Neurodegenerative Diseases

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Abstract

Excessive nitrosative and oxidative stress is thought to trigger cellular signaling pathways leading to neurodegenerative conditions. Such redox dysregulation can result from many cellular events, including hyperactivation of the *N*-methyl-D-aspartate-type glutamate receptor, mitochondrial dysfunction, and cellular aging. Recently, we and our colleagues have shown that excessive generation of free radicals and related molecules, in particular nitric oxide species (NO), can trigger pathological production of misfolded proteins, abnormal mitochondrial dynamics (comprised of mitochondrial fission and fusion events), and apoptotic pathways in neuronal cells. Emerging evidence suggests that excessive NO production can contribute to these pathological processes, specifically by S-nitrosylation of specific target proteins. Here, we highlight examples of S-nitrosylated proteins that regulate misfolded protein accumulation and mitochondrial dynamics. For instance, in models of Parkinson's disease, these S-nitrosylation targets include parkin, a ubiquitin E3 ligase and neuroprotective molecule, and protein-disulfide isomerase, a chaperone enzyme for nascent protein folding. S-Nitrosylation of protein-disulfide isomerase may also be associated with mutant Cu/Zn superoxide dismutase toxicity in amyotrophic lateral sclerosis. Additionally, in models of Alzheimer's disease, excessive NO generation leads to the formation of S-nitrosylated dynamin-related protein 1 (forming SNO-Drp1), which contributes to abnormal mitochondrial fragmentation and resultant synaptic damage. *Antioxid. Redox Signal.* 14, 1479–1492.

Introduction

MANY NEURODEGENERATIVE DISEASES manifest excessive generation of reactive oxygen species (ROS), such as superoxide anion ($O_2^{\bullet -}$), and reactive nitrogen species (RNS), including nitric oxide ($NO^{\bullet}$), which contribute to neuronal cell injury and death (10, 79, 84). *N*-methyl-D-aspartate (NMDA)–type glutamate receptors have been linked to ROS and RNS production in the nervous system. Overactivation of NMDA receptors causes excessive influx of Ca^{2+} ions, which generates ROS and activates neuronal NO synthase (nNOS) (48). Reaction of the NO group with critical cysteine thiols of target proteins results in the formation of S-nitrosoproteins and can thus regulate protein function (81). Lipton and Stamler initially discovered and characterized this biochemical process on the NMDA receptor itself (NO inhibits excessive NMDA receptor activity *via* S-nitrosylation) (81), and the activity of potentially hundreds or even thousands of other proteins may well be modulated in this fashion. Analogous to phosphorylation, they coined the term "S-nitrosylation," indicating a biological effect of the chemical reaction of S-nitrosation. S-Nitrosylation can mediate either protective or neurotoxic effects depending on the action of the target protein. Additionally, NADPH oxidase as well as mitochondrial respiration produce free radicals, principally superoxide anion ($O_2^{\bullet -}$), upon NMDA receptor activation (19). Superoxide anion thus generated from both mitochondrial and non-mitochondrial (*e.g.*, NADPH oxidase) sources reacts rapidly with free radical NO to form the very toxic product peroxynitrite ($ONOO^-$) (81) (Figs. 1 and 2).

In recent work, we have shown that S-nitrosylation and further oxidation of critical cysteine residues can lead to protein misfolding. Misfolded proteins form aggregates in many neurodegenerative diseases, and soluble oligomers of these aberrantly folded proteins are thought to adversely affect cell function by interfering with normal cellular processes or initiating cell death signaling pathways (91). As examples, α-synuclein and synphilin-1 in Parkinson's disease (PD), and amyloid-β (Aβ) and tau in Alzheimer's disease (AD) form toxic oligomers of non-native secondary structures. The formation of larger aggregates may be an attempt of the cell to wall off these toxic proteins. Protein aggregation is also a

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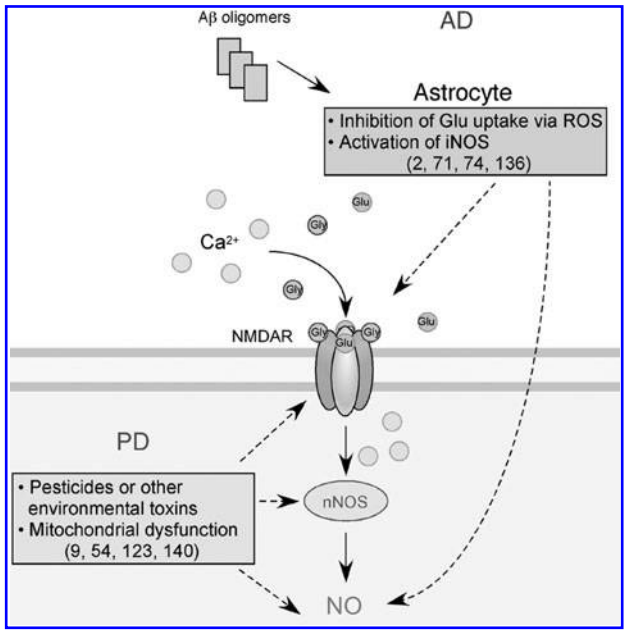

FIG. 1. Possible mechanisms whereby Ca^{2+} signaling contributes to NO generation in neurodegenerative conditions. Hyperactivation of *N*-methyl-D-aspartate receptors (NMDARs) by glutamate (Glu) and glycine (Gly) induces excessive Ca^{2+} influx and activation of neuronal nitric oxide synthase (nNOS). nNOS produces NO from L-arginine. In Alzheimer's disease (AD), soluble oligomers of amyloid-β (Aβ) peptide, thought to be a key mediator in AD pathogenesis, can facilitate neuronal NO production in both NMDAR-dependent and NMDAR-independent manners (2, 71, 74, 136). In Parkinson's disease (PD), mitochondrial dysfunction caused by pesticides, herbicides, or other environmental toxins can trigger NO production, possibly *via* mitochondrial pathways and the NMDAR/Ca^{2+} cascade (9, 54, 123, 140). Note that in addition to reactive nitrogen species, reactive oxygen species (ROS) are also produced in response to Aβ and pesticides.

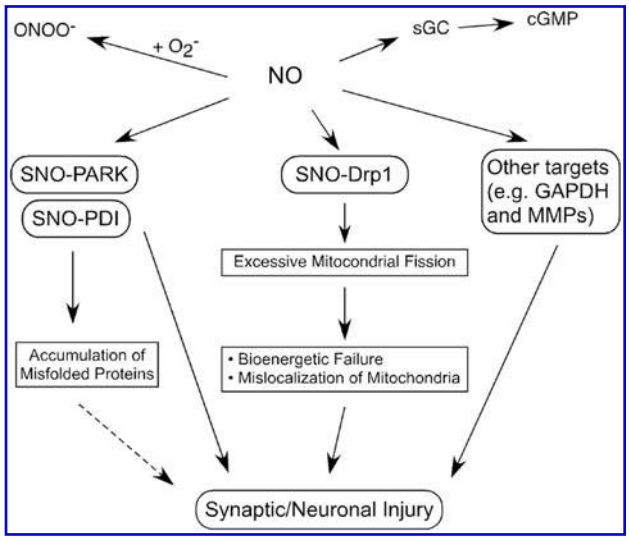

FIG. 2. Overproduction of NO triggers formation of S-nitrosylated (SNO) proteins. NO produced by nNOS reacts with sulfhydryl groups to form SNO proteins. Physiological levels of NO mediate neuroprotective effects, at least in part, by S-nitrosylating the NMDAR and caspases, thus inhibiting their activity. In contrast, we postulate that overproduction of NO can be neurotoxic *via* S-nitrosylation of Parkin (forming SNO-PARK), protein-disulfide isomerase (PDI) (forming SNO-PDI), GAPDH, MMP-2/9, PrxII, and COX-2. S-Nitrosylated parkin and PDI contribute to neuronal cell injury by triggering accumulation of misfolded proteins. S-Nitrosylation of dynamin-related protein 1 (Drp1) (forming SNO-Drp1) causes excessive mitochondrial fragmentation in neurodegenerative conditions. NO also activates soluble guanylate cyclase (sGC) to produce cyclic guanosine-3′,5′-monophosphate (cGMP), and cGMP can activate cGMP-dependent protein kinase. Peroxynitrite ($ONOO^-$), derived from reaction of NO and superoxide anion ($O_2^{\bullet -}$), can oxidize vicinal sulfhydryl groups to disulfide bonds and can also nitrate tyrosine residues to form 3-nitrotyrosine.

signature of Huntington's disease (a polyQ disorder), amyotrophic lateral sclerosis (ALS), and prion disease (32).

Sporadic forms of neurodegenerative diseases, rather than single gene mutation, constitute the vast majority of cases, and pathologic protein misfolding in these diseases may be the result of posttranslational changes to the protein engendered by nitrosative and/or oxidative stress, which can thus mimic the more rare genetic variants of the disease (145). Here we focus on specific examples that show the critical roles of S-nitrosylation of ubiquitin E3 ligases, for example, parkin, and endoplasmic reticulum (ER) chaperones, such as protein-disulfide isomerase (PDI), in accumulation of misfolded proteins in neurodegenerative diseases (31, 123, 140) (Fig. 1). We also review recent findings on S-nitrosylation of dynamin-related protein 1 (Drp1), which can contribute to the pathological fragmentation of mitochondria (Fig. 1).

Generation of ROS/RNS by Ca^{2+} Influx Through NMDA Receptor Channels in Response to Glutamatergic Signaling

The amino acid glutamate functions as the major excitatory neurotransmitter and is present at millimolar concentrations in the adult central nervous system. Ca^{2+} stimulates release of glutamate from the presynaptic nerve terminal into the synaptic cleft, where it diffuses to postsynaptic receptors on an adjacent neuron. Normal excitatory neurotransmission is essential for synaptic development and plasticity as well as learning and memory. In contrast, excessive glutamate excitation plays a role in a variety of neurological disorders ranging from acute hypoxic-ischemic brain injury to chronic neurodegenerative diseases. Survival pathways appear to be mediated *via* NMDA receptor synaptic activity, whereas neuronal damage may be mediated by excessive extrasynaptic activity (see below for further discussion of synaptic *versus* extrasynaptic NMDA receptor activity) (95, 100). Severe overstimulation of excitatory receptors can cause necrotic cell death, whereas less fulminant or chronic overstimulation can cause apoptotic or other forms of cell death (3, 12, 21).

Glutamate receptors in the nervous system are divided into two groups, ionotropic (representing ligand-gated ion channels) and metabotropic (coupled to G-proteins). Ionotropic glutamate receptors are represented by three separate classes, NMDA, α-amino–3-hydroxy-5 methyl-4-isoxazole propionic acid, and kainate; each receptor type is named for the synthetic ligands that selectively activate them. Functional NMDA receptors are likely to be heterotetramers *in vivo*,

containing NR1, NR2 (with NR2A-NR2D subtypes), and, in some cases, NR3 subunits (26). In neurons, both synaptic and extrasynaptic sites express functional NMDA receptors. Synaptic NMDA receptor activity generally signals to molecular pathways promoting neuronal survival, for example, by enhancing the expression of antioxidative enzymes (100). Conversely, excessive activation of extrasynaptic NMDA receptors mediates molecular pathways that trigger neurotoxicity associated with accumulation of misfolded proteins (95). Evidence suggests that the NR2A subtype is predominantly located at synaptic sites, whereas NR2B is enriched at extrasynaptic sites but this differential localization is not exclusive; the subcellular localization of NR2 subtypes at best can only explain in part the differential effects of synaptic and extrasynaptic NMDA receptor activity on neuronal survival (79). *Via* postsynaptic density (PSD)-95 protein complexes, synaptic NMDA receptors are known to couple to multiple intracellular signaling molecules, including nNOS, located just beneath the postsynaptic membrane (68, 115). Recently, extrasynaptic NMDA receptors were also reported to be associated with PSD-95 (103), suggesting that nNOS/NO toxicity can play a role in cell death pathways triggered by extrasynaptic NMDA receptors.

NMDA receptors, unlike most other glutamate receptors, are highly permeable to Ca^{2+}. Depolarization relieves blockade of NMDA receptor-coupled ion channels by Mg^{2+} (89). Ca^{2+} influx promotes many normal intracellular signaling pathways, but excessive influx promotes pathological signaling, contributing to cell injury and death *via* production of free radicals and related molecules such as ROS and NO, as well as other enzymatic processes (12, 21, 40, 81, 84) (Figs. 1 and 2). Excitotoxicity is defined as neuronal damage caused by excessive activation of glutamate receptors (96) and is at least partly mediated by excessive Ca^{2+} influx through NMDA receptor-associated ion channels (26, 79, 84). Increased levels of neuronal Ca^{2+}, in conjunction with the Ca^{2+}-binding protein, calmodulin, trigger the activation of nNOS and subsequent generation of NO from the amino acid L-arginine (1, 18, 115) (Figs. 1 and 2). Three subtypes of NOS have been identified; the two constitutive forms of NOS—nNOS and endothelial NOS—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. For example, activated glial cells may produce neurotoxic amounts of NO *via* iNOS expression in various neurodegenerative diseases. All three isoforms are widely distributed in the brain. Each NOS isoform contains an oxidase domain at its amino terminal and a reductase domain at its carboxy terminal, separated by a Ca^{2+}/CaM binding site (1, 16, 18, 46, 52). Constitutive and inducible NOS are also further distinguished by CaM binding; nNOS and endothelial NOS bind CaM in a reversible Ca^{2+}-dependent manner and are thus activated by Ca^{2+}. In contrast, iNOS binds CaM so tightly at resting intracellular Ca^{2+} concentrations that its activity does not appear to be affected by transient variations in Ca^{2+} concentration. To terminate iNOS-mediated NO production, microglia may redistribute iNOS to the aggresome for inactivation (70).

A connection between RNS and mitochondrial dysfunction in neurodegenerative diseases, especially PD, has recently been postulated (10, 11). Pesticides and other environmental toxins specifically inhibit mitochondrial complex I, generating excessive ROS/RNS, thus contributing to aberrant protein accumulation (31, 123, 140). In animal models, administration of complex I inhibitors, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, 6-hydroxydopamine, rotenone, and paraquat, recapitulates many features of sporadic PD, including degeneration of dopaminergic neurons, overproduction and aggregation of α-synuclein, accumulation of Lewy body-like intraneuronal inclusions, and impairment of behavioral functions (10, 11). Studies such as these strongly suggest a relationship between RNS and protein misfolding. Each may be the pathogenic trigger for the other in neurodegenerative diseases, but the mechanism has not yet been determined.

Alternatively, accumulation of ROS can trigger caspase activation resulting in synaptic damage and apoptosis (1). This process can be exacerbated by the action of endogenous neurotoxic electrophiles such as the prostaglandin derivative 15d-PGJ2 and catecholamine metabolites (including dopamine) (1). Such electrophiles compromise the reductive capacity of the cell by binding reduced cysteine residues, such as in glutathione, through a reaction called S-alkylation.

Nitrosative Stress, Protein Misfolding, Synaptic Injury, and Neuronal Cell Death

NO participates in cellular signaling pathways that regulate broad aspects of brain function, including synaptic plasticity, normal development, and neuronal cell death (40). These effects were thought to be largely achieved by activation of guanylate cyclase to form cyclic guanosine-3′,5′-monophosphate, but emerging evidence suggests that a more prominent reaction of NO is S-nitros(yl)ation of regulatory protein thiol groups (48, 62, 81). S-Nitrosylation is the covalent addition of an NO group to a cysteine thiol/sulfhydryl (RSH or, more properly, thiolate anion, RS^-) to form an S-nitrosothiol derivative (R-SNO). Such regulatory modifications are broadly found in mammalian, plant, and microbial proteins. We and our colleagues have found that a consensus motif of nucleophilic residues (generally an acid and a base) surround a critical cysteine, increasing the susceptibility of the sulfhydryl to S-nitrosylation (56). This process is counterbalanced by denitrosylation by means of thioredoxin/thioredoxin reductase, class III alcohol dehydrogenase, PDI, intracellular glutathione, and other mechanisms. We first identified the physiological relevance of the redox-based mechanisms by which NO and related RNS exert seemingly paradoxical effects in the central nervous system (81). NO is neuroprotective through S-nitrosylation of NMDA receptors and caspases, yet is neurodestructive through formation of peroxynitrite or S-nitrosylation of matrix metalloproteinase-9, GAPDH, and other targets, as discussed below (53, 55, 81) (Fig. 2).

Accumulating evidence suggests that S-nitrosylation is analogous to phosphorylation in regulating the biological activity of many proteins (31, 53, 55, 56, 81, 118, 123, 140). However, the chemistry of NO is much more complex. NO is often a good "leaving group," resulting in further oxidation of the thiol to a disulfide bond between neighboring (vicinal) cysteine residues. Alternatively, as NO leaves because of another reaction partner, the thiol group can react with ROS to yield sulfenic (-SOH), sulfinic ($-SO_2H$), or sulfonic ($-SO_3H$) acid derivatives on the cysteine residue of the protein (53, 123, 140). S-Nitrosylation may also possibly produce a nitroxyl

disulfide, in which the NO group is shared by proximate cysteine thiols (60).

At low (physiological) levels, NO can mediate neuroprotective functions. For example, our group first identified the physiological relevance of S-nitrosylation by showing that NO reacts with the NMDA receptor to downregulate its excessive activity (79–81). Specifically, we found that five different cysteine residues on extracellular domains of the NMDA receptor could react with NO. One of these, located at cysteine residue #399 (Cys399) on the NR2A subunit of the NMDA receptor, mediates $\geq$90% of the effect of NO under our experimental conditions (30). 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 (82). As opposed to ambient air with an oxygen tension of 150 torr, a pO_2 of 10–20 torr is found in normal brain, and even lower levels occur under hypoxic/ischemic conditions. We recently found that as the oxygen tension is lowered the NMDA receptor becomes more sensitive to inhibition by S-nitrosylation (121). In sum, NO inhibits hyperactivation of the NMDA receptor through S-nitrosylation, providing neuroprotective affects under excitotoxic conditions.

In contrast, we and other colleagues have also studied the consequence of excessive (pathophysiological) generation of oxidative/nitrosative reaction. For example, recent evidence suggests that the presence of excessive NO-related species may play a significant role in the process of protein misfolding. Increased nitrosative and oxidative stress are associated with chaperone and proteasomal dysfunction, resulting in accumulation of misfolded aggregates (62, 145). However, until recently little was known regarding the molecular and pathogenic mechanisms underlying contributions of NO to the formation of aggregates such as amyloid plaques in AD or Lewy bodies in PD. We and others recently presented physiological and chemical evidence that S-nitrosylation modulates the ubiquitin E3 ligase activity of parkin (31, 83, 140). Additionally, we found that S-nitrosylation regulates the chaperone and isomerase activities of PDI (123), contributing to protein misfolding and neurotoxicity in models of neurodegenerative disorders.

Another reaction of NO, in this case with superoxide anion, to form peroxynitrite can contribute to nitration of tyrosine residues, which may also potentially contribute to dysfunctional protein folding and neuronal cell injury. For instance, nitration of α-synuclein and tau affects oligomer formation *in vitro*. Further, nitrated α-synuclein and tau selectively accumulate in inclusion bodies in PD and neurofibrillary tangles in AD brains (50, 112, 113, 124). Collectively, these findings support the proposition that S-nitrosylation cysteine residues and possibly nitration or tyrosine residues can influence protein aggregation and neurotoxicity.

NO has also been reported to regulate mitochondrial function. Under physiological conditions, the NO-cyclic guanosine-3′,5′-monophosphate pathway induces mitochondrial biogenesis through peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) (94). In contrast, increased nitrosative stress can result in defects in mitochondrial function. For example, NO/S-nitrosylation affects mitochondrial respiration by reversibly inhibiting complexes I and IV (14, 22, 33, 34, 39), and the formation of SNO complex I may be implicated in PD (27, 28, 61). Mitochondria thus compromised will release ROS, and this in turn could contribute to brain aging and/or pathological conditions associated with neurodegenerative diseases. Additionally, increased nitrosative stress can elicit dysfunction of mitochondrial dynamics (fission and fusion events) (8, 15, 144). Although the exact mechanism whereby NO contributes to excessive fragmentation of mitochondria remains enigmatic, our recent findings have shed light on the molecular events underlying this relationship, particularly in AD. Specifically, we have recently discovered (patho)physiological and chemical evidence that S-nitrosylation modulates the GTPase activity of the mitochondrial fission protein, Drp1 (dynamin related protein 1), thus contributing to mitochondrial fragmentation. We found that excessive mitochondrial fragmentation results in bioenergetic impairment, synaptic damage, and eventually frank neuronal loss in models of AD (29).

Protein Misfolding and Aggregation in Neurodegenerative Diseases

Healthy neurons generally show no accumulation of protein aggregates, indicating that the appearance of such structures is a response to pathological stresses. Considerable evidence suggests that misfolded or otherwise abnormal proteins are produced even in healthy cells. The difference can largely be accounted for by cellular mechanisms for quality control, such as molecular chaperones, the ubiquitin-proteasome system (UPS), and autophagy/lysosomal degradation. A reduction in molecular chaperone or proteasome activities under pathological conditions can result in deposition and accumulation of aberrant proteins either within or outside of cells in the brain. Several mutations in molecular chaperones or UPS-associated enzymes are known to contribute to neurodegeneration (36, 91). For example, a reduction in proteasome activity was found in the substantia nigra of PD patients (90), and overexpression of the molecular chaperone HSP70 prevented neurodegeneration *in vivo* in models of PD (6). Aggregated proteins were first considered to be pathogenic. However, recent evidence suggests that macroscopic aggregates are an attempt by the cell to sequester aberrant proteins, whereas soluble (micro-) oligomers of such proteins are the most toxic forms (18).

S-Nitrosylation of Parkin and the UPS

Studies of rare mutations have revealed key components of the mechanism for protein aggregation and pathology in PD, including sporadic forms of the disease. Such studies revealed that mutated α-synuclein is a major constituent of Lewy bodies in PD patient brains, and that mutant forms of the ubiquitin E3 ligase parkin or the ubiquitin carboxy-terminal hydrolase UCH-L1 (a deubiquinating enzyme) may result in UPS dysfunction and also result in hereditary forms of PD. Formation of polyubiquitin chains on a peptide constitutes the signal for proteasomal degradation. The cascade of activation (E1), conjugation (E2), and ubiquitin-ligase (E3)-type enzymes catalyzes the conjugation of the ubiquitin chain to the proteins marked for degradation. Individual E3 ubiquitin ligases play a key role in the recognition of specific peptide substrates (114).

Parkin is a member of a large family of E3 ubiquitin ligases. Parkin contains a total of 35 cysteine residues, many of which coordinate structurally important zinc atoms, which are often involved in catalysis (88). Parkin recruits substrate proteins as well as an E2 enzyme (*e.g.*, UbcH7, UbcH8, or UbcH13). Mutations in the gene encoding parkin have been associated with autosomal recessive juvenile PD. In this case, mutations underlying this disorder usually do not produce Lewy bodies. However, other mutations in parkin resulting in adult onset PD have been associated with Lewy body formation. Mutations in both alleles of the parkin gene will cause dysfunction in its activity, although not all mutations result in loss of parkin E3 ligase activity (36). Additionally, wild-type parkin can mediate the formation of nonclassical and nondegradative lysine 63-linked polyubiquitin chains (76, 77). Parkin can also monoubiquitinate Eps15, HSP70, and itself, possibly at multiple sites. These activities may explain why some parkin mutations result in the formation of Lewy bodies, whereas others do not. Synphilin-1 (α-synuclein interacting protein) is a well-characterized substrate for parkin ubiquitination, and is found in Lewy body-like inclusions in cultured cells when coexpressed with α-synuclein. Accumulation of these proteins portends a poor prognosis for the survival of dopaminergic neurons in familial PD and possibly also in sporadic PD.

PD is the second most prevalent neurodegenerative disease and is characterized by the progressive loss of dopamine neurons in the substantia nigra pars compacta. Aberrant protein accumulation is observed in patients with genetically encoded mutant proteins, and recent evidence from our and other laboratories suggests that nitrosative/oxidative stress acts as a potential causal factor for protein misfolding in the much more common sporadic form of PD. Nitrosative/oxidative stress can mimic hereditary PD by promoting protein misfolding in the absence of a genetic mutation (31, 83, 140). In fact, S-nitrosylation and further oxidation of parkin result in a dysfunctional enzyme and disruption of UPS function (31, 140). We found that nitrosative stress produces S-nitrosylation of parkin (forming SNO-parkin) in rodent models of PD and in brains of human patients with PD and the related α-synucleinopathy, diffuse Lewy body disease. Initially, S-nitrosylation of parkin stimulates its ubiquitin E3 ligase activity, which may contribute to Lewy body formation. Subsequently, with time we found that the E3 ligase activity of SNO-parkin decreases, resulting in UPS dysfunction (83, 140) (Fig. 3). Importantly, S-nitrosylation of parkin on critical cysteine residues also compromises its neuroprotective activity (31). It is likely that S-nitrosylation influences the enzymatic functions of similar ubiquitin E3 ligases, suggesting that this process may be involved in a number of degenerative disorders.

In addition to parkin, recent studies on rare genetic forms of PD have found that mutations in the genes encoding PINK1 (*PARK6*), α-synuclein (*PARK1/4*), DJ-1 (*PARK7*), leucine-rich repeat kinase-2 (LRRK2) (*PARK8*), or ATP13A2 (*PARK9*) are also associated with PD pathology (13, 69, 72, 97, 105, 110, 125, 147). NO can bind to cysteine residue(s) of DJ-1 to form an S-nitrosothiol and may affect its dimerization (63). However, the exact mechanism of SNO-DJ1 contributing to PD pathogenesis remains to be elucidated. It remains possible that S-nitrosylation may regulate the activity of additional PD-related gene products, such as PINK1, LRRK2, and ATP13A2

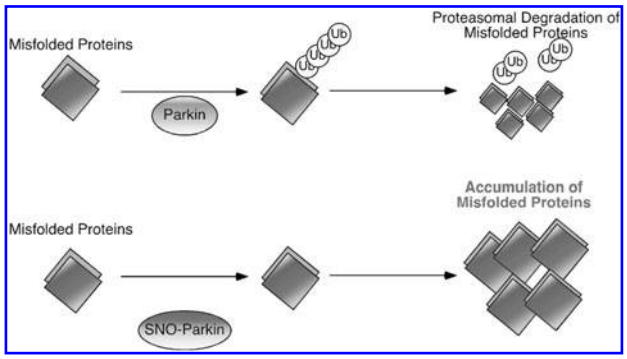

FIG. 3. Possible mechanism whereby S-nitrosylated parkin contributes to the accumulation of aberrant proteins and neuronal damage. The ubiquitin E3 ligase parkin is believed to be involved in ubiquitin-proteasome-dependent degradation of misfolded proteins (*top*). S-Nitrosylation of parkin alters its E3 ligase activity, thus inducing ubiquitin-proteasome system dysfunction (*bottom*). Thus, S-nitrosylation of parkin (forming SNO-Parkin) can contribute to neuronal cell injury in part by triggering accumulation of misfolded proteins.

(but not α-synuclein because it lacks cysteine residues), but further studies will be needed to investigate this possibility.

S-Nitrosylation of PDI Mediates Protein Misfolding and Neurotoxicity in Cell Models of PD and AD

In the ER, PDI facilitates proper protein folding by introducing disulfide bonds into proteins (oxidation), breaking disulfide bonds (reduction), and catalyzing thiol/disulfide exchange (isomerization), thus facilitating disulfide bond formation, rearrangement reactions, and structural stability (87). 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. 4). Several mammalian PDI homologs, such as ERp57 and PDIp, also localize to the ER and may manifest similar functions (35). Increased expression of PDIp in neuronal cells under conditions mimicking PD suggests the possible contribution of PDIp to neuronal survival (35). In many neurodegenerative disorders and cerebral ischemia, the accumulation of immature and denatured proteins results in ER dysfunction (35), but upregulation of PDI represents an adaptive response promoting protein refolding and may offer neuronal cell protection (35). We recently reported that S-nitrosylation of PDI (to form SNO-PDI) disrupts normal protein folding and this neuroprotective role (123).

The ER normally manifests a relatively positive redox potential in contrast to the highly reducing environment of the cytosol and mitochondria. This redox environment in the ER can influence the stability of protein S-nitrosylation and oxidation reactions (45). Excessive NO is known to create ER stress by disruption of Ca^{2+} homeostasis. One possible mechanism is the increased activity of the ER Ca^{2+} channel-ryanodine receptor through S-nitrosylation (137). We have

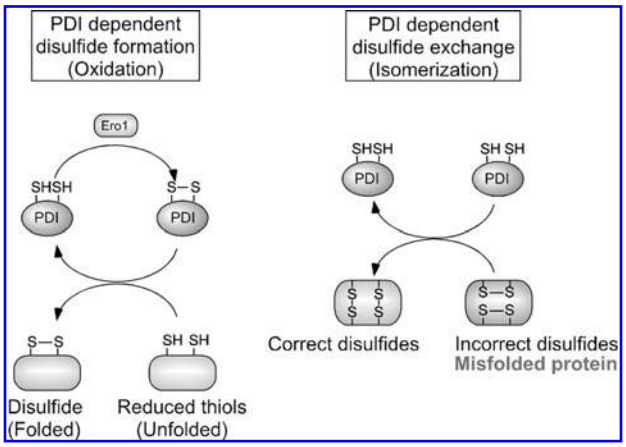

FIG. 4. Molecular mechanisms of PDI-dependent oxidative protein folding in the endoplasmic reticulum (ER). *Left:* Oxidized PDI (*top*), which contains a disulfide bond at its active site, catalyzes the formation of a disulfide bond in a substrate protein (*bottom*), resulting in the reduction of PDI. 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. *Right:* 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 (*top*) converts these incorrect disulfide bonds to their correct native 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 (*bottom*). For simplicity, the redox state of only one PDI active site is shown.

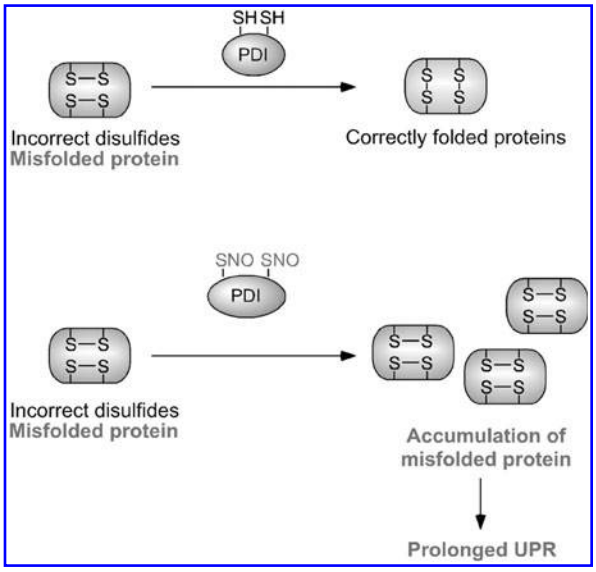

FIG. 5. Possible mechanism whereby S-nitrosylated PDI contributes to the accumulation of aberrant proteins and neuronal damage. PDI is known to associate with nascent or misfolded proteins and to refold them correctly (*top*). Under conditions of nitrosative stress, the decreased chaperone and isomerase activity of PDI due to S-nitrosylation enhances accumulation of misfolded proteins, thus activating a prolonged unfolded protein response (UPR) and neuronal cell death (*bottom*). In this manner, S-nitrosylation of PDI (forming SNO-PDI) can contribute to neuronal cell injury, in part by triggering accumulation of misfolded proteins.

recently reported that excessive NO, as well as rotenone exposure, which is known to lead to PD, can lead to S-nitrosylation of the active-site thiols of PDI, inhibiting its isomerase and chaperone activities (123). S-Nitrosylation of PDI prevented its attenuation of neuronal cell death triggered by ER stress, misfolded proteins, or proteasome inhibition. Also, PDI was S-nitrosylated in the brains of virtually all cases we examined of sporadic AD and PD. These results suggest that SNO-PDI can mediate protein misfolding and consequent neuronal cell death or injury (Fig. 5).

The activity of the UPS and molecular chaperones normally decline with age (102). Since we have not found detectable levels of SNO-parkin and SNO-PDI in normal aged brain but only in disease states (31, 123, 140), it is likely that S-nitrosylation of these and similar proteins is a key mechanism contributing to neurodegenerative conditions.

PDI activity in ALS and prion disease

Recently, PDI has also been implicated in the pathophysiology of familial ALS (5). 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 intrasubunit disulfide bond. Biochemical evidence suggests that the disulfide-reduced monomer of mutant SOD1 (mtSOD1) forms inclusion bodies (4, 43, 47, 108, 122), and aggregates of misfolded mtSOD1 are commonly associated with the disease, as seen at postmortem examination. In addition, although wild-type SOD1 is found predominantly in the cytoplasm, mtSOD1 forms monomers or insoluble high molecular weight multimers within the ER (67). Intriguingly, PDI has been found to colocalize and bind to intracellular aggregates of mtSOD1. Also, upregulation of the unfolded protein response in the ER is observed in mtSOD1 mice. Recent studies have shown that inhibition of PDI activity with bacitracin can increase aggregation of mtSOD1 in neuronal cells, and that regulation of endogenous PDI activity by reticulons protects against neurodegeneration (5, 139). In contrast, overexpression of PDI decreases mtSOD aggregation and mtSOD1-induced neuronal cell death. Taken together, these findings suggest that ER stress may contribute to the pathophysiology of familial ALS, and that increased PDI activity may reduce mtSOD1 aggregation and promote neuronal survival (5, 127, 139). Moreover, S-nitrosothiol levels were also found to be abnormal in the spinal cords of mtSOD1 transgenic mice (116), including elevated SNO-PDI (127) (Fig. 6). Sporadic ALS patients were also reported to manifest increased SNO-PDI levels, suggesting that S-nitrosylation of PDI may contribute to the pathogenesis of sporadic ALS (127). It will be important to determine whether SNO-PDI is involved in protein aggregation and motoneuron injury in ALS in the absence of SOD1 mutations.

Additionally, transmissible spongiform encephalopathies, 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

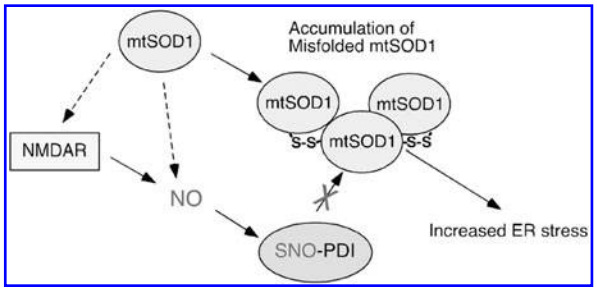

FIG. 6. Possible mechanism whereby S-nitrosylated PDI contributes to the accumulation of aberrant mutant Cu,Zn superoxide dismutase (mtSOD1) and neuronal damage in familial amyotrophic lateral sclerosis. Amyotrophic lateral sclerosis-linked mtSOD1 is prone to form high-molecular-weight complexes (or aggregates) that contain non-native disulfide bonds. Accumulation of mtSOD1 is known to trigger ER stress. Although most mtSOD1 is present in the cytoplasm, recent lines of evidence suggest that mtSOD1 may be secreted *via* the ER-Golgi pathway. Further, the ER resident protein PDI binds to both wild-type SOD1 and mtSOD1, thereby reducing mtSOD1 aggregation and toxicity (5, 127). Since S-nitrosylation inhibits the enzymatic activity of PDI, overproduction of NO may contribute to mtSOD1 toxicity *via* the formation of SNO-PDI. Excessive NMDAR activity may contribute to mtSOD1 toxicity *via* production of NO to facilitate SNO-PDI formation (130).

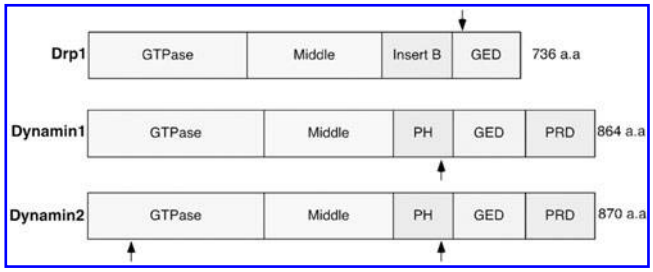

FIG. 7. Schematic structure of the domains of Drp1 and Dynamin1/2. The length and topology of amino acids for each region are shown. Each protein has a GTPase domain, a middle domain, and a GTPase effector domain (GED). Dynamin1/2 also contains a pleckstrin homology (PH) domain and a highly basic C-terminal proline-rich domain (PRD). *Arrows* indicate S-nitrosylated cysteine residues (Cys644 of Drp1, Cys 607 of Dynamin1, and Cys 60 and 607 of Dynamin2). S-Nitrosylation of these proteins upregulates their GTPase activity (29, 64, 128).

prolonged unfolded protein response due to PrP misfolding in the ER may contribute to neuronal dysfunction (57, 58, 142). This ER stress response is mainly associated with upregulation of GRP58, an ER chaperone with PDI-like activity, suggesting that this chaperone may play an important role in the cellular response to prion infection (58). In fact, *in vitro* studies on GRP58, 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.

Mitochondrial Fission/Fusion Machinery in Nerve Cells

Neurons are particularly vulnerable to mitochondrial defects because they require high levels of energy for their survival and specialized function. Mitochondrial biogenesis, an event that produces new mitochondria, is required in regions that demand high concentrations of ATP, especially the synapse. The distribution of mitochondria at the nerve terminal can control synaptic transmission and structure (25, 73, 75).

In healthy neurons, the fission/fusion machinery proteins maintain mitochondrial integrity and insure their presence at critical locations. These proteins includes Drp1 and Fis1, acting as fission proteins, and mitofusin (Mfn) and Opa1, operating as fusion proteins (143). In both familial and sporadic neurodegenerative conditions, abnormal mitochondria regularly appear in the brain as a result of dysfunction in the fission/fusion machinery. Genetic mutations in Mfn2 can cause Charcot-Marie-Tooth disease, a hereditary peripheral neuropathy that affects both motor and sensory neurons (66, 149). Mutations in Opa1 cause autosomal dominant optic atrophy, characterized by the loss of retinal ganglion cells and the optic nerve, representing their axons (41). Recently, Waterham *et al.* described a heterozygous, dominant-negative mutation of Drp1 in a patient whose symptoms were broadly similar to those of Charcot-Marie-Tooth neuropathy and autosomal dominant optic atrophy (135). Drp1 includes four distinct structural domains: an N-terminal GTPase domain, a dynamin-like middle domain, an insert B domain, and a C-terminal GTPase effector domain (Fig. 7). The mutation (Ala 395 to Asp) was found in the middle domain of Drp1. This case study further suggested that a defect in mitochondrial fission may have more severe consequences than those of fusion defects, since the Drp1 mutation caused a much earlier onset (prenatal) and fatal outcome. Additionally, it is apparent that the balance between fission and fusion is critical for normal function of mitochondria and determination of phenotype in disease. These fission/fusion proteins are widely expressed in human tissues, clearly supporting the notion that neurons are particularly sensitive to mitochondrial dysfunction.

Dysregulation of mitochondrial dynamics in AD

An estimated 26 million people globally have AD, which is thought to be the most common form of dementia. In AD brains, neuronal loss in the hippocampus and cerebral cortex mainly accounts for the cognitive decline. Degenerating AD brains contain aberrant accumulations of misfolded, aggregated proteins—Aβ and tau—which can adversely affect neuronal connectivity and plasticity, and trigger cell death signaling pathways. These aggregates are recognized as either intracellular neurofibrillary tangles, which contain hyperphosphorylated tau, or extracellular plaques, which contain Aβ. β-Secretase and γ-secretase proteolytically cleave amyloid precursor protein in its transmembrane region to generate Aβ. It is currently thought that soluble oligomers of misfolded protein are pathogenic and that the large aggregates may actually be an attempt by the cell to wall off the aberrant proteins (although such aggregates could potentially be toxic by location or if not contained by the proper chaperones). A recent study showed that an N-terminal fragment of amyloid

precursor protein may also contribute to neurodegeneration in AD models (93).

Emerging evidence suggests that mitochondrial dysfunction plays a prominent role in the pathogenesis of AD (133). Analyses of autopsy and biopsy samples revealed that mitochondria isolated from AD brains exhibit diminished respiratory capacity (101), and that AD neurons contain a number of mitochondria with fractured cristae (59). Additionally, electron-microscopic studies have described an increase in mitochondrial fragmentation in human AD brains (7, 131). In cell-based experiments, Aβ production resulted in the appearance of fragmented and abnormally distributed mitochondria (8, 132), suggesting that Aβ (possibly in the form of soluble oligomers) may trigger excessive mitochondrial fission in AD patients. Pathological forms of tau may also contribute to mitochondrial fragmentation in AD brains since expression of caspase-cleaved tau induced mitochondrial fission in a calcineurin-dependent manner (107).

Similarly, dysfunction in mitochondrial integrity is associated with PD (126). For instance, the Parkinsonian neurotoxins, rotenone and l-methyl-4-phenyl-pyridinium ion, which inhibit complex I of the mitochondrial electron transport chain, can induce excessive mitochondrial fragmentation and cell death (8). Additionally, multiple groups recently observed that a deficiency in familial PD-related proteins, such as parkin and PINK1, led to the appearance of mitochondrial pathology (44, 51, 86, 106). Exogenous expression of mitochondrial fusion proteins, Mfn2 and OPA1, or dominant negative Drp1 rescued the altered mitochondrial morphology, suggesting that parkin or PINK1 deficiency promoted mitochondrial fragmentation (86). Additionally, Drp1 seems to activate autophagy/mitophagy pathways for morphologic remodeling of mitochondria in PINK1-deficient neuroblastoma cells (38). Further, PINK1 escorts parkin to damaged mitochondria, where parkin ubiquitinates the voltage-dependent anion channel 1 and promotes autophagic clearance of damaged mitochondria (mitophagy) (49). In contrast, the PINK1/Parkin pathway also appears to promote mitochondrial fission and has also been reported to inhibit mitochondrial fusion in *Drosophila* (42, 106, 138). For example, the *Drosophila* PINK1/parkin pathway induces Mfn ubiquitination, which results in lower Mfn levels and thus the appearance of small-sized mitochondria (148). Thus, although further studies will be needed to understand the significance of these findings for the pathogenesis of PD, it is clear that PD-associated genes influence mitochondrial dynamics.

S-Nitrosylation of Drp1 Mediates Mitochondrial Fission and Neurotoxicity in Cell Models of AD

In addition to rare hereditary mutations in the genes encoding mitochondrial fission and fusion proteins, recent studies have demonstrated that posttranslational modification of these proteins can contribute to altered mitochondria dynamics. For example, phosphorylation, ubiquitination, sumoylation, and proteolytic cleavage of Drp1 regulate mitochondrial fission by affecting Drp1 activity, at least in cell culture systems (17, 23, 37, 65, 92, 120, 134, 141). Excessive activation of mitochondrial fission or fusion proteins by posttranslational modification was posited to contribute to neurodegeneration by compromising mitochondrial function. Along these lines, we recently reported that excessive NO can lead to S-nitrosylation of Drp1 at Cys644 (29) (Fig. 8). Cys644 resides within the GTPase effector domain of Drp1, which influences both GTPase activity and oligomer formation of Drp1 (85, 104, 109, 146). S-Nitrosylation of Drp1 (forming SNO-Drp1) induces formation of Drp1 dimers, which function as building blocks for tetramers and higher order structures of Drp1, and activates Drp1 GTPase activity; however, substitution of Cys644 to Ala (C644A) abrogated these effects of NO.

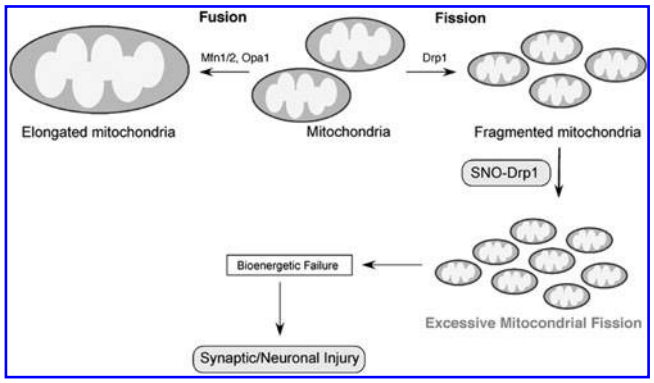

FIG. 8. Possible mechanism whereby S-nitrosylated Drp1 contributes to abnormal mitochondrial function and neuronal damage. In healthy neurons, mitochondria continuously undergo fission and fusion to maintain their integrity and insure their presence at critical locations. Mitochondrial fission is driven by fission proteins such as Drp1 and fis1. Components involved in mitochondrial membrane fusion include Opa1 and mitofusin (Mfn)1/2. The NO group reacts with a critical cysteine thiol on Drp1 by S-nitrosylation to form SNO-Drp1. SNO-Drp1 contributes to synaptic and neuronal injury by leading to excessive mitochondrial fission/fragmentation and bioenergetic impairment.

We further demonstrated that exposure to oligomeric Aβ peptide results in formation of SNO-Drp1 in cell culture models. Moreover, we and our colleagues have observed that Drp1 is S-nitrosylated in the brains of virtually all cases of sporadic AD (29, 131). To determine the consequences of S-nitrosylated Drp1 in neurons, we exposed cultured cerebrocortical neurons to the physiological NO donor, S-nitrosocysteine, or to Aβ oligomers and found that both induced SNO-Drp1 formation and led to the accumulation of excessively fragmented mitochondria. Moreover, mutation of a specific cysteine residue in Drp1 (C644A) prevented these effects of S-nitrosocysteine or Aβ on mitochondrial fragmentation, consistent with the notion that SNO-Drp1 triggered excessive mitochondria fission or fragmentation. Finally, in response to Aβ, SNO-Drp1-induced mitochondrial fragmentation caused synaptic damage, an early characteristic feature of AD, and eventually apoptotic neuronal cell death. Importantly, blockade of Drp1 nitrosylation [using the Drp1(C644A) mutant] prevented Aβ-mediated synaptic loss and neuronal cell death, suggesting that SNO-Drp1 may represent a potential therapeutic target to protect neurons and their synapses in AD. Thus, the posttranslational modification of S-nitrosylation can mimic the effect of rare genetic mutations in contributing to the AD phenotype.

Unlike AD and PD, HD is a purely genetic disease caused by mutations that result in CAG expansion in the first exon of

the Huntingtin (Htt) gene, with more than ~35 CAGs being pathogenic and resulting in polyglutamine expression. Mitochondrial dysfunction is also associated with pathogenesis of HD (111). Respiratory electron transport chain activity and ATP levels are decreased in mitochondria from HD patients and Htt transgenic mice (98, 117, 119). Additionally, the complex II inhibitor, 3-nitropropionic acid (3-NP), causes a movement disorder similar in many respects to HD (20). Moreover, mutant Htt directly impairs mitochondrial membrane potential, calcium homeostasis, and mitochondrial axonal trafficking (24, 99). Recently, Wang *et al.* demonstrated that expression of mutant Htt sensitizes cells to oxidative stress-induced mitochondrial fission and reduces ATP levels by inhibiting mitochondrial fusion (129). Overexpression of the Drp1 dominant-negative K38A or the fusion protein Mfn2 reduces mutant Htt-induced mitochondrial fragmentation as well ATP loss and cell death. Importantly, RNAi against Drp1 also reduces the motility defect in a worm model of HD. In addition, 3-NP exposure induces increased mitochondrial fragmentation in an NMDA receptor-dependent manner in cerebrocortical neurons (78). Remarkably, we have also observed S-nitrosylation of Drp1 in HD brains similar to that seen in AD brains, raising the possibility that this redox event may play a pathogenic role in HD in addition to AD. Collectively, these studies suggest that alterations in mitochondrial dynamics may be involved in the pathogenesis of HD.

Conclusions

Sporadic forms of neurodegenerative diseases can be caused by excessive NMDA receptor activation and/or mitochondrial dysfunction that results in excessive nitrosative and oxidative stress. These pathological processes can result in malfunction of the UPS and/or molecular chaperones and contribute to abnormal protein accumulation and neuronal damage. Here we have described a mechanistic link between free radical production, protein misfolding, abnormal mitochondrial dynamics, and neuronal cell injury in neurodegenerative disorders such as PD and AD. The elucidation of NO-mediated S-nitrosylation of parkin, PDI, and Drp1 in neurodegenerative disease may promote development of new drugs to prevent aberrant protein misfolding or excessive mitochondrial fission by targeted disruption of this process.

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Abbreviations Used

Aβ = amyloid-β
AD = Alzheimer's disease
ALS = amyotrophic lateral sclerosis
cGMP = cyclic guanosine-3′,5′-monophosphate
Drp1 = dynamin-related protein 1
ER = endoplasmic reticulum
GED = GTPase effector domain
Htt = Huntingtin
iNOS = inducible nitric oxide synthase
Mfn = mitofusin
mtSOD1 = mutant Cu,Zn superoxide dismutase
NMDA = *N*-methyl-D-aspartate
nNOS = neuronal NOS
NO = nitric oxide
PD = Parkinson's disease
PDI = protein disulfide isomerase
PrP = prion protein
RNS = reactive nitrogen species
ROS = reactive oxygen species
SNO = S-nitrosylated
UPR = unfolded protein response
UPS = ubiquitin-proteasome system

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