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Review

Molecular mechanisms of nitrosative stress-mediated protein misfolding in neurodegenerative diseases

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Abstract. Nitrosative and oxidative stress, associated with the generation of excessive reactive oxygen or nitrogen species, are thought to contribute to neurodegenerative disorders. Many such diseases are characterized by conformational changes in proteins that result in their misfolding and aggregation. Accumulating evidence implies that at least two pathways affect protein folding: the ubiquitin-proteasome system (UPS) and molecular chaperones. Normal protein degradation by the UPS can prevent accumulation of aberrantly folded proteins. Molecular chaperones – such as protein-disulfide isomerase, glucose-regulated protein 78, and heat shock proteins – can provide neuroprotection from aberrant proteins by facilitating proper folding and thus preventing their aggregation. Our recent studies have linked nitrosative stress to protein misfolding and neuronal cell death. Here, we present evidence for the hypothesis that nitric oxide contributes to degenerative conditions by *S*-nitrosylating specific chaperones or UPS proteins that would otherwise prevent accumulation of misfolded proteins.

Keywords. *S*-Nitrosylation, protein-disulfide isomerase, molecular chaperone, ubiquitination, parkin, Parkinson's disease, NMDA receptors.

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 (HAD), multiple sclerosis, and ischemic brain injury, to name but a few [1–5]. 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 [6, 7]. A well-established model for NO production entails a central role of the *N*-methyl-D-aspartate (NMDA)-type glutamate receptors in 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 [8, 9]. Importantly, normal mitochondrial respiration also generates free radicals, principally ROS, and one such molecule, superoxide anion

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(O_2^-) reacts rapidly with free radical $NO^\bullet$ to form the very toxic product peroxynitrite ($ONOO^-$) [10, 11].

An additional feature of most neurodegenerative diseases is accumulation of misfolded and/or aggregated proteins [12–15]. These protein aggregates can be cytosolic, nuclear, or extracellular. Importantly, protein aggregation can result from either (i) a mutation in the disease-related gene encoding the protein, or (ii) post-translational changes to the protein engendered by nitrosative/oxidative stress [16]. 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 (i) ubiquitin E3 ligases such as parkin, or (ii) endoplasmic reticulum (ER) chaperones such as protein-disulfide isomerase (PDI) is critical for the accumulation of misfolded proteins in neurodegenerative diseases such as PD, AD and other conditions [17–20].

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 [12, 13]. 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 diseases with inclusions include Huntington's (polyQ), ALS, and prion disease [14]. The above-mentioned aggregates 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 [13, 21, 22].

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 multi-step 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 [9, 23].

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 [25, 26]. This form of toxicity is mediated at least in part by excessive activation of NMDA-type receptors [6, 7, 27], resulting in excessive Ca^{2+} influx through a receptor's 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 [6, 11, 28–31]. Intracellular Ca^{2+} triggers the generation of NO by activating nNOS in a Ca^{2+}/calmodulin (CaM)-dependent manner [8, 9]. It is currently thought that overstimulation of extrasynaptic NMDA receptors mediates this neuronal damage, while, in contrast, synaptic activity may activate survival pathways [32, 33]. 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 [30, 31, 34].

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 [9, 35]. 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. 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.

Recently, a novel cellular mechanism for Ca^{2+}-dependent release of NO was discovered in dorsal root ganglion neurons and pancreatic acinar cells. This Ca^{2+}-dependent NO release occurs not as a result of *de novo* synthesis by NO but instead via liberation of NO from an *S*-nitrosothiol (SNO) pool, whereby NO is reversibly bound to specific cysteine residues (see below for additional chemical information regarding this reaction). Interestingly, NOS-independent release of NO was mediated by calpain (a Ca^{2+}-dependent thiol protease) but not by CaM or protein kinase C (PKC) [36]. This finding leads to the question of whether glutamatergic signaling in neuronal cells can also trigger NO release from SNO pools.

Recent studies further pointed out the potential connection between ROS/RNS and mitochondrial dysfunction in neurodegenerative diseases, especially in PD [3, 37]. Pesticide and other environmental toxins that inhibit mitochondrial complex I result in oxidative and nitrosative stress, and consequent aberrant protein accumulation [17, 18, 20, 38, 39]. Administration to animal models of complex I inhibitors, such as MPTP, 6-hydroxydopamine, rotenone, and paraquat, which result in overproduction of ROS/RNS, reproduces many of the features of sporadic PD, such as dopaminergic neuron degeneration, up-regulation and aggregation of α-synuclein, Lewy body-like intraneuronal inclusions, and behavioral impairment [3, 37]. 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 [40].

Increased nitrosative and oxidative stress are associated with chaperone and proteasomal dysfunction, resulting in accumulation of misfolded aggregates [16, 41]. 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 [28, 42–44]. 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 [8, 11, 41, 45–47]. *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) surrounds a critical cysteine, which increases the cysteine sulfhydryl's susceptibility to *S*-nitrosylation [48, 49]. 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) [11, 50–57]. 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 [11, 17, 18, 49, 56–64]. 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 [18, 20, 56, 65]. Alternatively, *S*-nitrosylation may possibly produce a nitroxyl disulfide, in which the NO group is shared by close cysteine thiols [66].

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 [67, 68]. 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 [69–72]. 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 (i) ubiquitin E3 ligase activity of parkin [17–19], and (ii) chaperone and isomerase activities of PDI [20], contributing to protein misfolding and neurotoxicity in models of neurodegenerative disorders. Additionally, Cohen et al. [73] recently demonstrated that insulin/insulin-like growth factor-I (IGF-I) signaling, which influences longevity and lifespan in many species in part via down-regulation of ROS/RNS generation, can affect aggregation of toxic proteins such as Aβ. This finding potentially provides an additional link between ROS/RNS production during the normal aging process and protein aggregation in neurodegenerative conditions.

Parkin and the UPS

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 [74].

One piece of direct evidence for UPS involvement in PD arises from the discovery that mutations in the parkin gene cause autosomal recessive juvenile Parkinsonism (ARJP). 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. RING finger domains are comprised of a varying number of histidine and cysteine residues that coordinate a structurally important zinc atom often involved in catalysis [75]. Parkin has two RING finger domains separated by an "in between RING" (IBR) domain. 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 (either an increase or a decrease) and are responsible for many cases of ARJP as well as rare adult forms of PD. 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 [76], 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 [77]. Other substrates for parkin include parkin-associated endothelin receptor-like receptor (Pael-R) [78], cell division control related protein (CDCrel-1) [79], cyclin E [80], p38 tRNA synthase [81], synaptotagmin XI [82], α/β tubulin heterodimers[83], and 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 [84], and the second, binding partners of parkin, such as CHIP [85] and BAG5 [86]. CHIP enhances the ability of parkin to inhibit cell death through up-regulation of parkin-mediated ubiquitination, while BAG5-mediated inhibition of parkin E3 ligase activity facilitates neuronal cell death. In addition, Fallon et al. [87] recently reported another mechanism for parkin-mediated neuronal survival via a proteasome-independent pathway [87]. 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 (PI3K)/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 [88]. Interestingly, a recent study suggested that a novel ubiquitin-ubiquitin ligase activity of Uch-L1 might also be important in the pathogenesis of PD [89]. 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 [17–19]. For example, *S*-nitrosylation and further oxidation of parkin or Uch-L1 result in dysfunction of these enzymes and thus of the UPS [17, 18, 90–93]. 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 [18, 19, 94] (Fig. 1). 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 [17]. These mechanisms involve *S*-nitrosylation of critical cysteine residues in the first RING domain of parkin [18]. Nitrosative stress has also been suggested to effect parkin misfolding and concomitantly compromise its protective function [95]. 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) [96]. 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 [97]. La Voie et al. [96] showed that DA quinone can attack one or more cysteine residues in the RING domain(s) of parkin, forming a covalent adduct that abrogates its E3 ubiquitin ligase activity. DA quinone also reduced the solubility of parkin, possibly inducing parkin misfolding after disruption of the RING domain(s). 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.

In addition to its E3 ligase activity, parkin can tightly bind to and stabilize microtubules in cultured cells [83, 98]. Recent studies have suggested that microtubules can deliver misfolded proteins to the perinuclear area to form a single large inclusion, termed the "aggresome," when proteasome activity is down-regulated [99, 100]. The interaction between parkin and microtubules may possibly facilitate the transport of misfolded proteins, which are ubiquitinated by parkin, to the aggresome. It will be interesting to determine whether *S*-nitrosylation or DA modification of parkin can affect its association with microtubules and thereby regulate accumulation of misfolded proteins.

Unfolded protein response (UPR) and PDI

The ER normally participates in protein processing and folding but undergoes a stress response when immature or misfolded proteins accumulate [101–104]. ER stress stimulates two critical intracellular responses (Fig. 2). 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 transmembrane sensor proteins are involved in the UPR: pancreatic ER kinase (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 [105–108]. The second ER stress response, termed ER-associated degradation (ERAD), specifically recognizes terminally misfolded proteins for retro-translocation across the ER membrane to the cytosol, 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

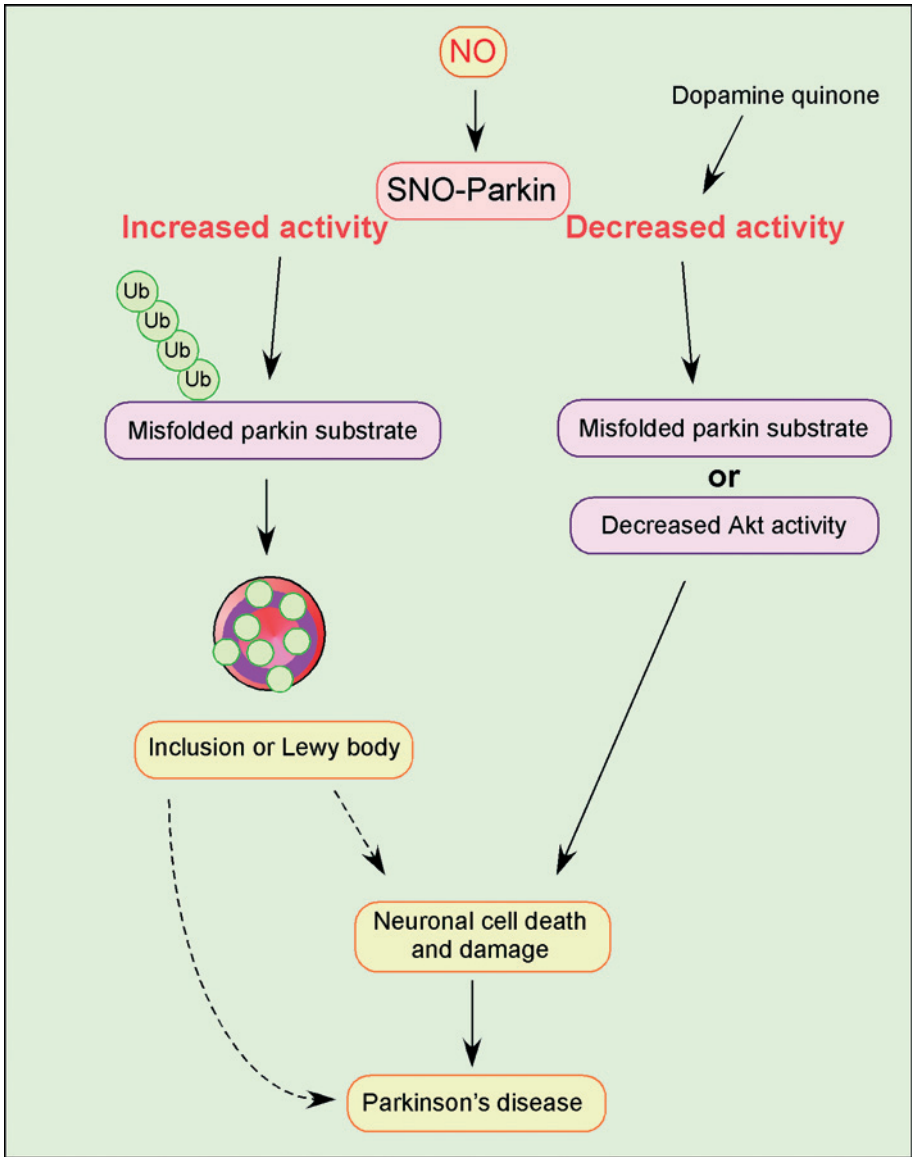

Figure 1. Possible mechanism of *S*-nitrosylated parkin (SNO-Parkin) contributing to the accumulation of aberrant proteins and damage or death of dopaminergic neurons. Nitrosative stress leads to *S*-nitrosylation of parkin, and, initially, to a dramatic increase followed by a decrease in its E3 ubiquitin ligase activity [17–19]. 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. [137]. The subsequent decrease in parkin activity may allow misfolded proteins to accumulate. Down-regulation of parkin may also result in decreased Akt neuroprotective activity because of enhanced epidermal growth factor receptor (EGFR) internalization [87]. Dopamine quinone can also modify cysteine thiols of parkin and reduce its activity [96]. Ub: ubiquitin.

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 catalyzes thiol/disulfide exchange, thus facilitating disulfide bond formation, rearrangement reactions, and structural stability [109]. PDI has four domains that are homologous to thioredoxin (TRX) (termed a, b, b', and a'). 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 [110–113]. 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" [114]. Hydrophobic residues line the inside surface of the "U," facilitating interactions between PDI and misfolded proteins.

In many neurodegenerative disorders and cerebral ischemia, the accumulation of immature and denatured proteins results in ER dysfunction [115–118], but up-regulation of PDI represents an adaptive response promoting protein refolding and may offer neuronal cell protection [116, 119–121]. In a recent study, we reported that the *S*-nitrosylation of PDI (to form SNO-PDI) disrupts its neuroprotective role [20].

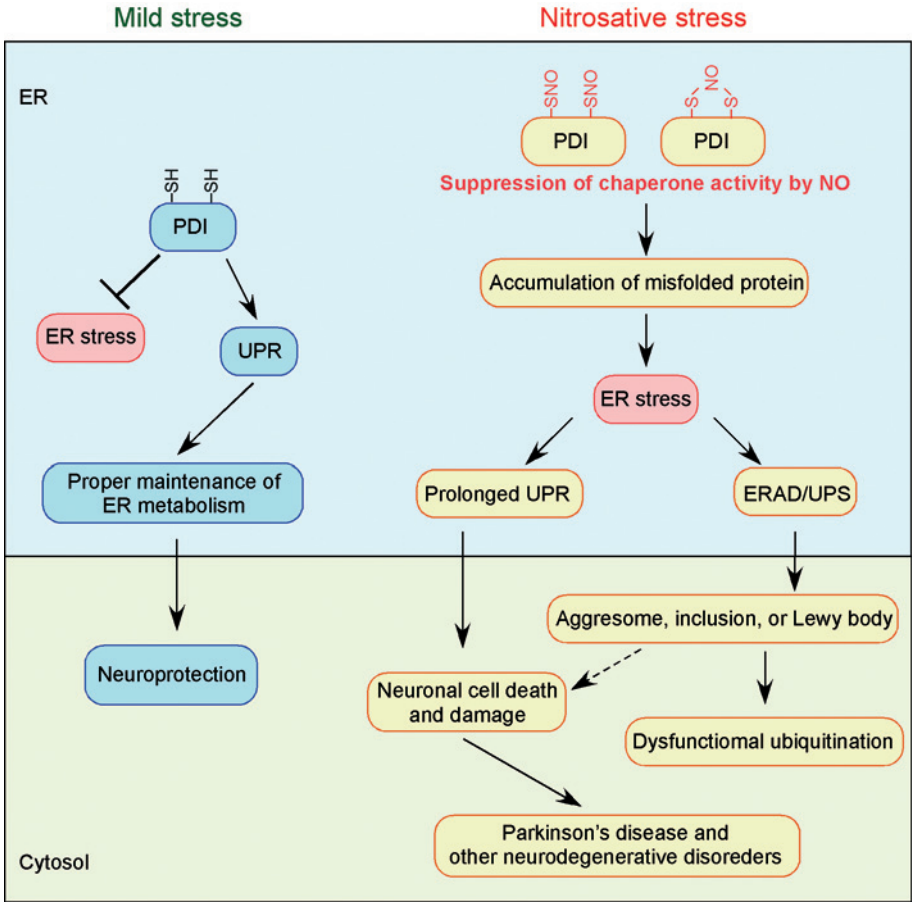

Figure 2. Possible mechanism of *S*-nitrosylated protein-disulfide isomerase (SNO-PDI) contributing to the accumulation of aberrant proteins and neuronal cell damage or death. Endoplasmic reticulum (ER) stress is triggered when misfolded proteins accumulate within the ER lumen, inducing the unfolded protein response (UPR). The UPR is usually a transient homeostatic mechanism for cell survival, while prolonged UPR elicits neuronal cell death. PDI modulates the activity of UPR sensors by mediating proper protein folding in the ER. 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 [101–104]. 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 [20, 122].

S-Nitrosylation of PDI mediates protein misfolding and neurotoxicity in cell models of PD or AD

In contrast to the highly reducing environment of the cytosol and mitochondria, the ER manifests a relatively positive redox potential (*e.g.*, the ratio of reduced:oxidized glutathione is approximately 3:1). This redox environment can contribute to the stability of protein *S*-nitrosylation or oxidation [122]. Recently, we reported that excessive NO can lead to *S*-nitrosylation of the active site thiol groups of PDI, and this reaction inhibits both its isomerase and chaperone activities [20]. 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. Additionally, it is possible that vicinal (nearby) cysteine thiols reacting with NO can form nitroxyl disulfide [66], and such reaction may potentially occur in the catalytic side of PDI to inhibit enzymatic activity. 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 an 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 [20, 77]. *S*-Nitrosylation of PDI also prevented its attenuation of neuronal cell death

triggered by ER stress, misfolded proteins, or proteasome inhibition (Fig. 2). Further evidence suggested that SNO-PDI may in effect transport NO to the extracellular space, where it could conceivably exert additional adverse effects [60].

In addition to PDI, *S*-nitrosylation is likely to affect critical thiol groups on other chaperones, such as HSP90 in the cytoplasm [123] and possibly GRP in the ER. Normally, HSP90 stabilizes misfolded proteins and modulates the activity of cell signaling proteins including NOS and calreticulin [13]. 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 [124, 125]. Martínez-Ruiz et al. [123] 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 [126]. These findings suggest that the activity of the UPS and molecular chaperones may decline in an age-dependent manner [127]. Given that we have not found detectable quantities of SNO-parkin and SNO-PDI in normal aged brain [17, 18, 20], 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 [118]. 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 [128–132], and aggregates of misfolded mtSOD1 are commonly associated with the disease, as seen at postmortem 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 [133]. Atkin et al. [118] recently showed that inhibition of PDI activity with bacitracin can increase aggregation of mtSOD1 in neuronal cells. Moreover, PDI co-localized and bound to intracellular aggregates of mtSOD1. Up-regulation 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 [134]. 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 [121, 135, 136]. This ER stress response is mainly associated with up-regulation of Grp58, an ER chaperone with PDI-like activity, suggesting that this chaperone may represent a cellular response to prion infection [121]. In fact, *in vitro* studies on Grp58, either overexpressing (via transfection) or down-regulating (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.

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. Similarly,

molecular analyses of NO-mediated dysfunction of other UPS proteins and molecular chaperones should provide further therapeutic insights.

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