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FORUM REVIEW ARTICLE

Critical Roles of Glutaredoxin in Brain Cells—Implications for Parkinson's Disease

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Abstract

Significance: Glutaredoxin (Grx)1, an evolutionarily conserved and ubiquitous enzyme, regulates redox signal transduction and protein redox homeostasis by catalyzing reversible S-glutathionylation. Grx1 plays different roles in different cell types. In Parkinson's disease (PD), Grx1 regulates apoptosis signaling in dopaminergic neurons, so that loss of Grx1 leads to increased cell death; in microglial cells, Grx1 regulates proinflammatory signaling, so that upregulation of Grx1 promotes cytokine production. Here we examine the regulatory roles of Grx1 in PD with a view toward therapeutic innovation.

Recent Advances: In postmortem midbrain PD samples, Grx1 was decreased relative to controls, specifically within dopaminergic neurons. In *Caenorhabditis elegans* models of PD, loss of the Grx1 homologue led to exacerbation of the neurodegenerative phenotype. This effect was partially relieved by overexpression of neuroprotective DJ-1, consistent with regulation of DJ-1 content by Grx1. Increased GLRX copy number in PD patients was associated with earlier PD onset; and Grx1 levels correlated with levels of proinflammatory tumor necrosis factor-α in mouse and human brain samples. *In vitro* studies showed Grx1 to be upregulated on proinflammatory activation of microglia. Direct overexpression of Grx1 increased microglial activation; silencing Grx1 diminished activation. Grx1 upregulation in microglia corresponded to increased neuronal cell death in coculture. Overall, these studies identify competing roles of Grx1 in PD etiology.

Critical Issues: The dilemma regarding Grx1 as a PD therapeutic target is whether to stimulate its upregulation for neuroprotection or inhibit its proinflammatory activity.

Future Directions: Further investigation is needed to understand the preponderant role of Grx1 regarding dopaminergic neuronal survival. *Antioxid. Redox Signal*. 30, 1352–1368.

Keywords: glutaredoxin, Parkinson's disease, neuroprotection, neuroinflammation

Introduction

PARKINSON'S DISEASE (PD) IS a growing economic burden internationally due to the increasing population of elderly people and the necessity for extensive long-term health care for individuals with PD. Being the second most common neurodegenerative disease among the elderly, PD affects 1% of people older than 65 years, and 5% of people older than 85 years. PD is distressful for the patients and their families, manifesting symptoms that include bradykinesia, resting tremor, rigidity, and postural instability. Loss of dopaminergic (DA) neurons in the *substantia nigra* (98) is the primary cause of movement disorders; however, PD patients also exhibit several other complications, including impaired cognition and dementia.

The major risk factor for PD is advanced age (65), which is accompanied by many changes that could contribute to the onset and/or progression of the disease, including diminution of antioxidant defense mechanisms, dysregulation of cytosolic calcium homeostasis, compromised mitochondrial membrane integrity, increased mutations in mitochondrial DNA, and impaired mitochondrial electron transport with decreased production of ATP. Overall, mitochondrial functional impairment, increased oxidative stress, and abnormal protein aggregation are common characteristics of PD (69). Increased oxidative stress pertinent to neurodegeneration in

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PD has been ascribed to increased dopamine turnover, diminished glutathione (GSH) content, and increased levels of free iron in the *substantia nigra* (82).

Notably, GSH deficiency is considered to be one of the initial changes contributing to onset of PD (29). Samples of brain tissue from PD patients have displayed decreased GSH content in the dopaminergic neurons of the *substantia nigra* (71, 86, 111) due to formation of glutathione disulfide (GSSG) and glutathionyl conjugates, without diminution of GSH synthesis. While specific genetic mutations and environmental exposures (*e.g.*, pesticides) have been implicated as potential causes of PD, most cases of PD are classified as sporadic (of unknown origin).

Rare familial forms of PD have been defined by mutations in a number of genes, including DJ-1, LRRK2, Parkin, PTEN-induced putative kinase 1 (PINK1), α-synuclein, and ubiquitin carboxyl-terminal esterase L1 (UCH-L1) (111). The proteins expressed by these genes participate in regulation of various signaling pathways that control apoptosis, autophagy, calcium homeostasis, mitochondrial fission/fusion, respiration, reactive oxygen species (ROS) production, and transport processes (23, 132, 133). Mutations in these proteins account for only rare occurrences of inherited PD; however, oxidative stress associated with PD may result in post-translational oxidative modifications of these same proteins and contribute more broadly to sporadic PD. For example, PINK1 is a protein kinase, like many other protein kinases (*e.g.*, mitogen-activated protein kinase, protein kinases A and C [PKA, PKC]), susceptible to deactivation by cysteine modification (106); likewise, the active site cysteine of the E3 ubiquitin ligase Parkin is subject to oxidative modification.

Overexpression of α-synuclein in Drosophila results in neuronal death, which can be mitigated by enhancing synthesis of GSH (81); on the contrary, the rate of α-synuclein aggregation is accelerated by GSSG (61). DJ-1, which is characterized as protective of dopaminergic neurons by acting as an antiapoptotic agent and redox sensor under reducing conditions, instead is subject to diminished function under oxidative conditions, apparently due to accelerated degradation (52, 104). The leucine-rich repeat kinase 2 (LRRK2) protein possesses both a kinase mitogen-activated protein kinase kinase kinase domain and Ras GTPase-like domain (21). Analogs of these LRRK2 domains (*e.g.*, apoptosis signal regulating kinase 1 [ASK1] and hRas) are known to be altered functionally under oxidative stress conditions (73), suggesting that LRRK2 also may be sensitive to redox modification.

Neuroinflammation is a common characteristic of neurodegenerative diseases, including PD; and loss of dopaminergic neurons in the *substantia nigra* and striatum correlates with overt inflammation. The primary immune cells of the central nervous system (CNS), namely the microglia residing mainly in gray matter, are specifically implicated in neurodegeneration (30), as they become activated and launch an immune response, releasing chemokines, cytokines, and trophic factors. Indeed, increased levels of cytokines (TNF-α, interleukin-1β [IL-1β], and interleukin-6 [IL-6]), compared to controls, are observed in *postmortem* samples of *substantia nigra* tissue from PD patients, and also in cerebrospinal fluid and peripheral blood mononuclear cells from PD patients (92). Since tumor necrosis factor-alpha (TNF-α) is considered a major mediator of the neuroinflammatory responses that can lead to dopaminergic neuronal degeneration, it is remarkable that mutation in the gene that promotes increased production of TNF-α is correlated with early-onset PD (115).

As this introduction has conveyed, two key aspects of PD have been emphasized, namely (i) the susceptibility of dopaminergic neurons to oxidative stress and cell death; and (ii) the role of inflammatory activation of resident immune cells of the brain in promoting degeneration of the dopaminergic neurons. These major influences on PD development are depicted in Figure 1.

In this context, redox signaling and sulfhydryl homeostasis, involving reversible post-translational modification of proteins in brain cells, are important factors in the regulation or dysregulation of their cellular functions (36). These redox regulatory processes are mediated by thiol/disulfide oxidoreductase (TDOR) enzymes, which catalyze thiol/disulfide interchange reactions involving reversible oxidation and reduction of cysteine residues. Glutaredoxin (Grx) and thioredoxin (Trx) are the two primary TDOR enzymes that maintain sulfhydryl homeostasis and mediate redox signaling (74, 136). The substrate selectivities of each of these enzymes are indicative of distinct physiological functions. Grx specifically reduces protein-glutathione mixed disulfides (protein-SSG) (49), whereas Trx primarily reduces intramolecular and intermolecular disulfide bonds (49). The two enzyme systems carry out distinct, but complementary functions, likely acting synergistically to maintain thiol status in various types of cells (Fig. 2).

The focus of this review is on Grx, which has been implicated in both of the key aspects of PD; as a potential neuroprotective mediator in dopaminergic neurons on the one hand and as a proinflammatory mediator in microglial cells on the other hand. Thus, this review considers the ''yin and yang'' of Grx homeostasis in brain cells and the role of this enzyme in the progression of PD and potential therapeutic interventions.

Overview

Grx1 is an evolutionarily conserved and ubiquitously expressed enzyme that regulates redox signal transduction and repairs protein oxidation by reversing the oxidative modification of protein cysteine residues known as S-glutathionylation (protein-SSG) (102). Grx1 is implicated in the regulation of many important cellular processes, apparently playing different key roles in different cell types.

The main function of Grx1 is to deglutathionylate proteins and restore the reduced state (protein-SH), thereby contributing to sulfhydryl homeostasis and serving to regulate the steady-state levels of protein-SSG in redox signaling networks (32). Loss of Grx1 activity and reversibility of S-glutathionylation result in increased levels of protein-SSG, implicated in many diseases, including neurodegenerative diseases (34, 57, 89, 108). Since the chief function of Grx1 is to catalyze deglutathionylation, manipulation of its content in cells has been used effectively to document regulatory pathways that involve S-glutathionylated intermediates. For example, IKK-SSG and p-65-SSG have been identified as regulatory intermediates in the nuclear factor kappa B (NFκB) signaling pathway in different contexts (91, 96, 107).

Regulation by Grx1 has been shown also for other proteins representing a broad range of cellular functions; examples include the transcription factor nuclear factor-1 (NF-1) (14), the signaling intermediate Ras (1), the contractile protein actin (126), the apoptosis mediators serine/threonine protein

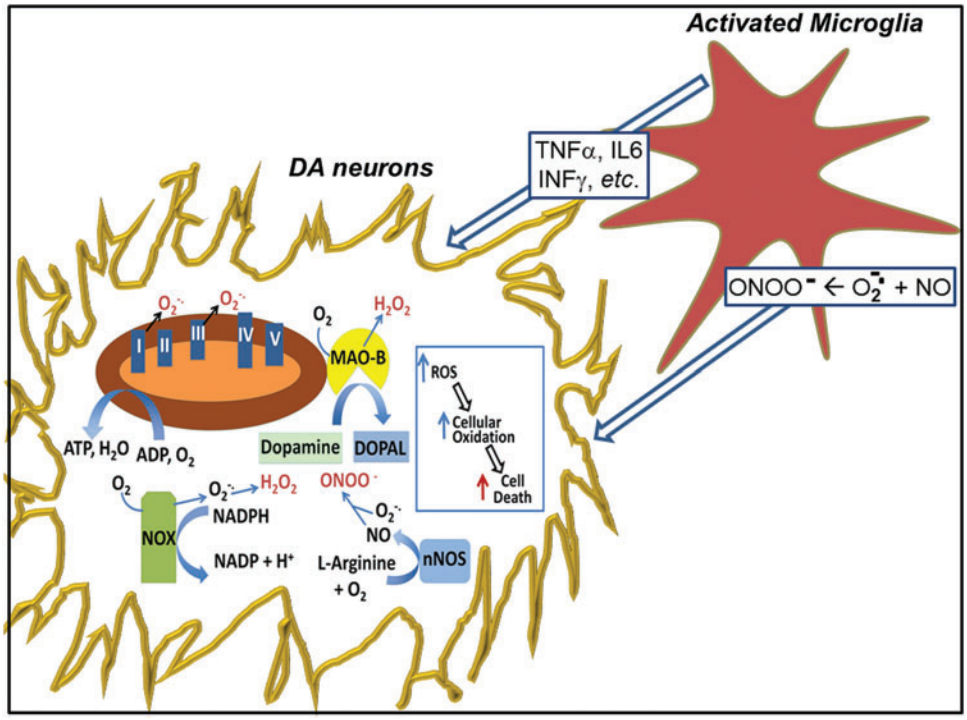

FIG. 1. Dopaminergic neurons are especially susceptible to oxidative stress internally and *via* assault from activated microglia that promote inflammation. The scheme depicted here represents an activated microglial cell and a neuron. Microglia are considered the ''resident macrophages'' of the CNS. The microglia are activated by cytokines, and they produce cytokines as well as ROS and RNS that promote oxidative stress extracellularly and thereby mediate injury of neuronal cells. Various insults affecting the CNS, such as environmental and genetic factors, or changes in homeostatic mechanisms due to aging, can lead to activation of the CNS-immune system, including microglia activation, astrocyte proliferation, and lymphocyte recruitment. These events may in turn induce the production of ROS or RNS and drive the expression of inflammatory factors. The inflammatory mediators can affect the vitality of neurons directly as well as stimulate the CNS-immune cells to amplify proinflammatory signals that induce neurotoxic effects. Uncontrolled, chronic inflammation can result in loss of neurons and progression of neurodegenerative diseases. DA, dopaminergic; CNS, central nervous system; IL-6, interleukin-6; NOX, NADPH oxidase; ROS, reactive oxygen species; RNS, reactive nitrogen species; TNF-α, tumor necrosis factor-alpha. Color images are available online.

kinase or protein kinase B (Akt) (79, 88) and cJun (60), and the ion transporters K(ATP) channel (134) and cystic fibrosis transmembrane conductase chloride channel (128). In each case, Grx1 was shown to restore the original functional state of the modified protein. These examples convey the breadth of regulation of cell functions by reversible S-glutathionylation and highlight the potential for dysregulation if the activity of Grx activity is altered.

As depicted in Figure 3, many previous studies [reviewed in (10)] have shown that *Grx1 regulates the S-glutathionylation status and activity of mediators of cell death and cell survival,* which are implicated in many disease situations. A few examples are displayed in Figure 3, where deglutathionylation catalyzed by Grx1 alters the function of key regulators such as NFκB. In the context of PD, Grx1 appears to regulate apoptosis signaling in DA neurons, so that loss of Grx1 in these cells leads to increased cell death (see the Grx as a Potential Neuroprotective Mediator section).

Focusing on the immune cells of the brain, inflammatory signaling pathways involve many mediators that are subject to sulfhydryl redox regulation (Fig. 4). In particular, Figure 4 [adapted from (36)] depicts inflammatory signaling pathways involving mediators that are subject to reversible S-glutathionylation, implicating a key role for Grx1. As shown, S-glutathionylation can affect several different mediators in pathways that lead to activation of NFκB, including the NFκB subunit protein (65kDa) (p65) and p50 subunits. In contrast to its role in neurons, Grx1 appears to regulate inflammatory signaling in microglial cells, so that upregulation of Grx1 promotes proinflammatory cytokine production (see the Grx as a Neuroinflammatory Mediator section). The challenge is to understand the net effect of the regulatory roles of Grx1 in PD to strategize its potential value as a therapeutic target.

While it has been implied in the discussion sections of many published articles (54, 57, 58, 101) that elevation or supplementation of Grx1 would have a protective role in PD development, no definitive study has been conducted on Grx1 overexpressing animals to test that hypothesis. More likely, the role of Grx1 in PD progression may not be so straightforward. Grx1 diminution/overexpression may have cell-type-specific effects (apoptosis *vs.* inflammation). Moreover, Grx1 levels may exert differential effects on the protein S-glutathionylation status of different proteins in different contexts. Below is a discussion of these considerations.

Grx as a Potential Neuroprotective Mediator

As indicated above, the etiology of PD is currently unclear; however, oxidative stress and redox dysfunction are generally believed to play key roles in PD pathogenesis and

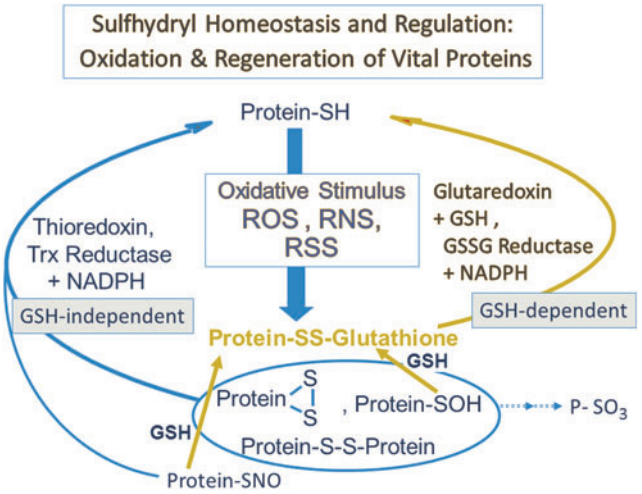

FIG. 2. Glutaredoxin and thioredoxin systems contribute synergistically to sulfhydryl homeostasis. Repair of sulfhydryl modifications is an important aspect of sulfhydryl homeostasis. This function is carried out by the TDOR enzyme systems. The Trx—TR system and the system that comprised Grx coupled to GSH and GR catalyze disulfide reduction reactions and reactivation of oxidatively modified sulfhydryl proteins. Grx is highly selective for glutathione-containing mixed disulfides, that is, protein-SSG. Thus, reversible protein-SSG formation by Grx may protect vital proteins from irreversible damage and serve as a regulatory mechanism. The Trx system favors reduction of intramolecular disulfides *via* a Trx-(S-S) intramolecular disulfide intermediate, and it is indiscriminate with mixed disulfide substrates. As described in the text, many of the proteins characteristically associated with neurodegenerative diseases are subject to oxidative modification and potential regulation *via* these enzyme systems. GR, GSSG reductase; Grx, glutaredoxin; GSH, glutathione; GSSG, glutathione disulfide; protein-SSG, S-glutathionylated protein; TDOR, thiol/disulfide oxidoreductase; TR, thioredoxin reductase; Trx, thioredoxin. Color images are available online.

progression. Aging and environmental factors predispose cells to adverse effects of redox changes. In addition, genetic mutations linked to PD have been observed to disrupt the redox balance (37, 53).

In contemporaneous studies in our laboratory (101) and that of Viji Ravindranath (104), we learned that knockdown of Grx1 in model DA neurons led to increased cell death, providing an indication of the potential neuroprotective role of Grx1. In more recent studies (52, 54), we examined the levels of Grx1 in postmortem midbrain samples from PD patients and found that Grx1 content is decreased relative to controls, specifically within the DA neurons. Total Grx1 levels as well as a number of Grx1-positive and TH-positive neurons were found to be decreased in *substantia nigra* of PD patients (54). As microglia account for between 1% and 10% of cells in the *substantia nigra*, depending on which marker is used to identify microglia (77), total Grx1 levels in the *substantia nigra* are not reflective of Grx1 levels in the microglia. The collective observations from our laboratory point to a cell-type-dependent role for Grx1 in neurodegeneration, in which Grx1 may play opposing yet balanced roles in neuronal and microglial homeostasis. Specific overexpression of Grx1 in the microglia *in vivo* by adenoassociated virus, as in Ref. (22), would aid in investigating effects of microglial-specific Grx1 induction on neuronal viability *in vivo*. Assessing neuronal and microglial Grx1 levels in PD patients or PD animal models may help delineate cell-type-specific roles of Grx1 in PD progression.

We examined the consequences of loss of the Grx1 homologue in well-established *C. elegans* models of familial PD involving overexpression of pathogenic human LRRK2 mutants (G2019S or R1441C). Loss of the Grx1 homologue led to significant exacerbation of the neurodegenerative phenotype in *C. elegans* overexpressing the LRRK2 mutants. Re-expression in the DA neurons of the active but not a catalytically inactive form of the Grx1 homologue rescued the exacerbated phenotype. Analogous results were observed

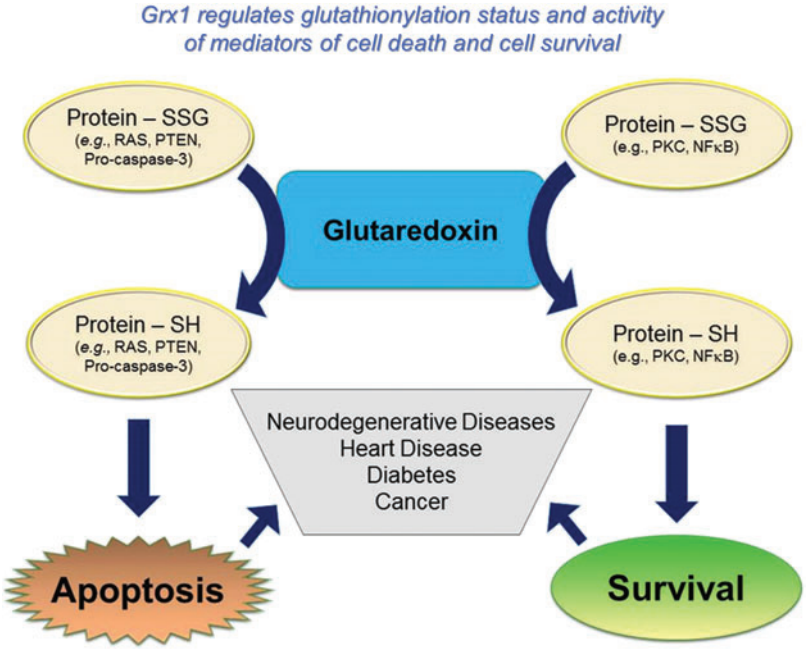

FIG. 3. Grx1 regulates S-glutathionylation status and activity of mediators of cell death and cell survival. Some examples of mediators that are known to be susceptible to reversible S-*glutathionylation and regulation by Grx1 are pictured here.* Adapted from Allen and Mieyal (10). NFκB, nuclear factor kappa B; PKC, protein kinase C. Color images are available online.

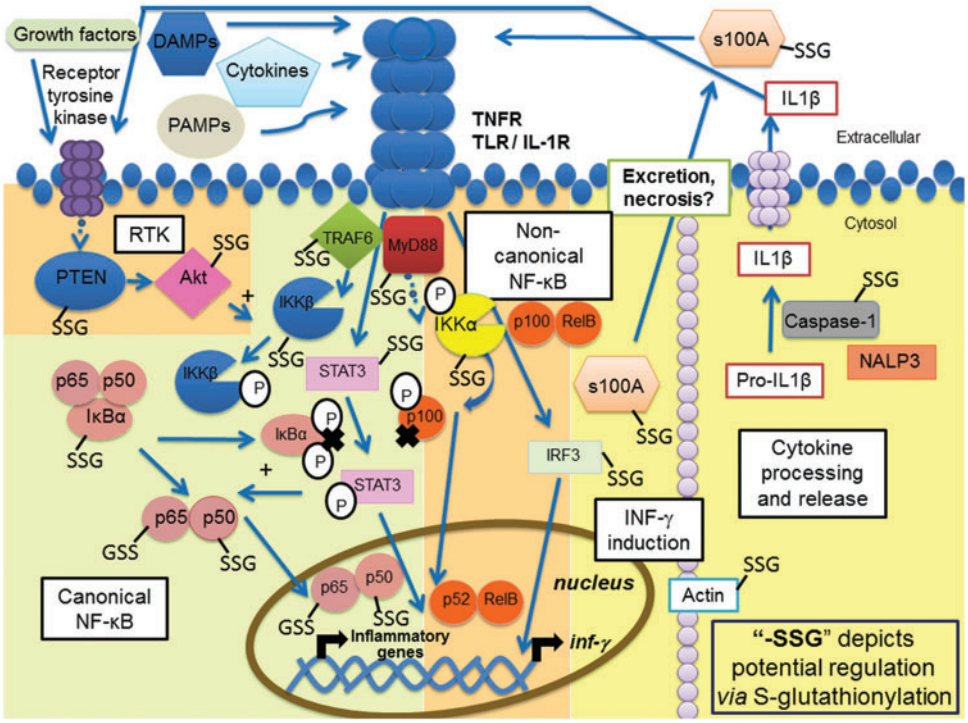

FIG. 4. Pathways of proinflammatory signaling have many mediators that are subject to regulation by reversible S-glutathionylation and Grx1. Akt, serine/threonine protein kinase or protein kinase B; IKKβ, I-kappaB kinase-beta; IL-1B, interleukin-1B; p65, NFκB subunit protein (65kDa); TLR, Toll-like receptor. Color images are available online.

in other *C. elegans* models, including overexpression of human α-synuclein and overexpression of tyrosine hydroxylase (a model of sporadic PD), revealing a novel neuroprotective role of Grx against DA neurodegeneration in models of familial and sporadic PD (54). However, it is important to note in this context that the catalytic properties of the *C. elegans* Grx1 homologue (GLRX-10) have not been characterized as yet. Therefore, further investigations of the catalytic mechanism of the *C. elegans* Grx1 homologue are needed to fully characterize this enzyme as a model for hGrx1. It is also worth noting that Grx activity was not assessed directly in Grx1$^{-/-}$ *C. elegans* strains (54), so the relative catalytic efficiency of GLRX-10 and its glutathionyl specificity relative to hGrx1 are not documented. Nevertheless, our studies in *C. elegans* provide reasonable basis to expect that Grx1 loss in DA neurons *in vivo* increases their susceptibility to degeneration.

Having obtained evidence for a neuroprotective role of Grx1, we set out to examine potential targets of Grx1 regulation in DA neurons, focusing on proteins that have been both implicated in familial PD and found to be sensitive to oxidative modification, providing a unifying mechanistic basis for links between idiopathic and familial PD. Our primary target was DJ-1 (52). We focused first on DJ-1, because previous data suggested a link between Grx1 and DJ-1.

DJ-1 is an atypical peroxiredoxin, molecular chaperone, and transcription factor; its mutation with a loss of function or deletion is linked to autosomal recessive PD (38). In another context, oxidation of DJ-1 was interpreted to target it for degradation (125). DJ-1 knockout mice show increased sensitivity to oxidative stress (59); but it is not known if DJ-1 is oxidized in sporadic PD and if such oxidation of DJ-1 would change its function or accelerate its degradation. In previous studies of model neuronal cells in culture, loss of Grx1 was reported to result in a loss of DJ-1 protein content; and mutation of cysteine 106 of DJ-1 ablated the dependence of DJ-1 content on Grx1 activity (103). *These results suggested that loss of Grx1 may promote S-glutathionylation of DJ-1, leading to its accelerated degradation.*

We found diminished DJ-1 content in midbrain samples from *Grx*$^{-/-}$ mice. Furthermore, with model DA neuronal cells (SH-SY5Y), we observed decreased DJ-1 protein content in response to agents (H_2O_2 and diamide) that promote protein-SSG formation; with isolated DJ-1, we identified two distinct sites of S-glutathionylation (52). Overexpression of DJ-1 in the DA neurons partly compensated for the effect of knockout of the Grx1 homologue in a *C. elegans in vivo* model of PD. Overall, these results suggest a novel Grx1-mediated regulatory mechanism for DJ-1 content *in vivo* potentially linked to PD. However, the partial rescuing effect of DJ-1 overexpression suggests that other key proteins regulated by reversible S-glutathionylation also contribute to the neuroprotective effect mediated by Grx1. Certainly, additional studies are necessary to fully delineate the neuroprotective mechanism of Grx1.

Grx as a Neuroinflammatory Mediator

We hypothesized that Grx1 and reversible S-glutathionylation would play a role in neuroinflammation based on analogy to previous work by us and others in other contexts (Fig. 5). In model studies of diabetic retinopathy using rat retinal glial cells, we found a twofold induction of Grx1 in response to high glucose, similar to the observed increase in Grx activity in samples of retinal tissue from streptozotocin diabetic rats compared to controls (108). Corresponding to Grx1 upregulation in retinal glial cells, there was a concomitant proinflammatory response; namely, an increase in NFκB activation and expression of the proinflammatory intercellular adhesion molecule-1 (ICAM-1). The proinflammatory response was mimicked when Grx1 was selectively upregulated *via* adenoviral infection of the cells in normal glucose.

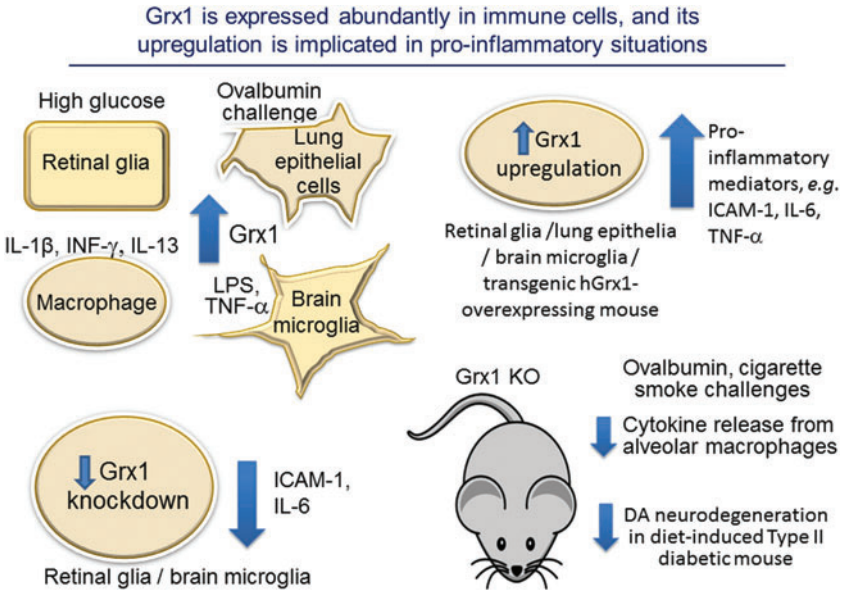

FIG. 5. Grx1 is expressed abundantly in immune cells, and its upregulation is implicated in proinflammatory situations. Grx1 is expressed abundantly in immune cells (26) and has been found to be upregulated in various situations where cytokine production is enhanced, such as hyperglycemia in retinal glial cells, a model of diabetic retinopathy (104); in lung epithelial cells in response to various inflammatory stimuli (4, 5, 97); and in CNS microglial cells in response to LPS and TNFα (35). ICAM-1, intercellular adhesion molecule-1; LPS, lipopolysaccharide. Color images are available online.

Conversely, knockdown of Grx1 in retinal glial cells in high glucose prevented ICAM-1 elevation. Collectively, these results suggested that Grx1 regulates the reversible S-glutathionylation and activity of the NFκB signaling pathway. Supporting this interpretation, I-kappaB kinase-beta (IKKβ) was documented to be glutathionylated specifically on Cys-179, identifying it as a potential control point regulated by Grx1 in NFκB-mediated ICAM-1 expression. Moreover, conditioned medium from Grx1-overexpressing retinal glial cells contained more IL-6 relative to control cells, and it induced upregulation of Grx1 and ICAM-1 in freshly cultured rat endothelial cells and retinal glial cells, indicating paracrine and autocrine transmission of the proinflammatory response to upregulation of Grx1 (107). As depicted in Figure 5, abundant Grx1 is found in immune cells (4, 87), and Grx1 has been observed to be upregulated in various situations where cytokine production was enhanced, such as retinal glial cells in high glucose, microglial cells in response to lipopolysaccharide (LPS) and TNFα (35), and lung epithelial cells in response to various inflammatory stimuli (4, 5, 97). As depicted (Fig. 5), knockout or knockdown of Grx1 in various contexts has led to diminished proinflammatory effects of the corresponding stimulants (3, 34, 93, 104); and independent adenoviral upregulation of Grx1 was documented to promote increased formation of proinflammatory mediators in the absence of stimulants (34, 104).

Neuroinflammation and redox dysfunction are recognized factors in PD pathogenesis; and diabetes is implicated as a potentially predisposing condition. Remarkably, upregulation of Grx1 is implicated in regulation of inflammatory responses in various disease contexts, including diabetes. We investigated the potential impact of Grx1 upregulation in the CNS on dopaminergic viability. For example, Grx1 content in mouse and human brain samples correlated with levels of the proinflammatory cytokine TNF-α; and we found that increased GLRX copy number in PD patients was associated with earlier PD onset. These findings prompted mechanistic *in vitro* studies. Accordingly, treatment with LPS, or TNF-α, led to upregulation of Grx1 content and activity in microglia. Furthermore, upregulation of Grx1 *via* adenoviral infection, titrated to the extent of induction by LPS, increased microglial activation analogously, whereas Grx1 knockdown diminished activation.

Using selective inhibitors/probes of the NFκB activation pathway, we discovered *glrx1* induction to be mediated by the Nurr1/NFκB axis, providing the first evidence for involvement of the Nurr1/CoREST complex in repression of *glrx1* induction (35). Moreover, we documented that upregulation of Grx1 in microglia promoted increased death of neuronal cells in coculture. Using a model of diet-induced insulin resistance in mice, we observed a correlation between upregulation of Grx1 in brain and loss of dopaminergic function; namely, decreased tyrosine hydroxylase (TH) content and diminution of TH-positive striatal axonal terminals. Such effects were not observed with like-treated Grx1 knockout mice. Our results indicate that Grx1 upregulation promotes neuroinflammation and consequent neuronal cell death *in vitro*, and synergizes with proinflammatory insults to promote DA loss *in vivo*.

As discussed above, upregulation of Grx1 propagates proinflammatory cytokine responses in various immune cell contexts, and Grx1 knockdown has been shown to decrease inflammatory activation of microglia (35), retinal glial cells (108), and alveolar macrophages (4). In these various contexts, Grx1 has been shown to be a positive regulator of NFκB, contributing to the propagation of inflammatory signaling (4, 34, 91, 92, 93, 104). Specifically, silencing of Grx1 expression prevents progression of inflammation-driven diseases, such as allergic airway disease, and dopaminergic neuronal loss subsequent to type II diabetes in animal models (4, 76), documenting the proinflammatory role of Grx1 *in vivo*.

Certainly, there are exceptions to the generalization regarding the role of Grx1 as a proinflammatory mediator. In fact, the opposite has been reported. For example, a recent report indicated that BALB/C$^{Grx1-/-}$ mice, lacking Grx1, were shown to mount an enhanced inflammatory response when challenged with house dust mites (43), suggesting that the role of Grx1 in inflammatory responses may be context and/or mouse strain dependent. In addition, overexpression

of Grx1 has been reported to have an anti-inflammatory effect in macrophages under particular conditions. Thus, overexpression of Grx1 prevented accumulation of protein-SSG in the monocytes of mice treated with low-density lipoprotein plus high glucose, and inhibited monocyte chemoattractant protein-1-induced monocyte chemotaxis in mice (121).

Impact of Grx1 overexpression

As described above, diminution/knockdown of Grx1 has been associated with increased apoptosis, characterizing Grx1 as a positive regulator of apoptosis (11, 20, 112); whereas global overexpression of Grx1 has produced mixed results in the context of disease tolerance. Grx1 overexpression in the C57BL/$6^{hGrx1TG}$ mouse was found to decrease cardiomyocyte cell death following ischemia/reperfusion injury (3). On the contrary, Grx1 overexpression in the lung was found to impair bacterial clearance and decrease survival on infection with *Pseudomonas aeruginosa* (11). C57BL/$6^{hGrx1TG}$ mice show transgene expression in the brain and heart, but not in the lung, liver, or kidney (3). We observed (35) that Grx1-overexpressing mice display a decrease in brain TH levels similar to that induced by treatment with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxin used as a chemical model of PD (16), suggesting that Grx1 elevation in the CNS may recapitulate a PD phenotype when combined with an appropriate secondary insult (in this case, prolonged feeding of a high-fat high-sugar [HFHS] diet).

C57BL/$6^{hGrx1TG}$ mice also presented with elevated levels of proinflammatory cytokines TNF-α and IL-6 (35), suggesting that the observed decrease in TH levels may be due, in part, to elevated neuroinflammation. C57BL/$6^{hGrx1TG}$ mice displayed enhanced NFκB activation, as shown by EMSA (3), as well as by decreased p65 S-glutathionylation and increased expression of p65 target genes (80). Including our observation of increased brain cytokine content (35), several lines of evidence point to C57BL/$6^{hGrx1TG}$ mice having enhanced baseline NFκB activation. Considering models of PD, MPTP has been observed to promote p65 activation in mouse brain (35), and NFκB inhibition has been found to ameliorate MPTP toxicity in animals (35) as well as to prevent 1-methyl-4-phenylpyridinium (MPP^+)-induced neuroblastoma cell death in culture (131). Elevated p65 activation has been documented in animal models of PD as well as in glia and neurons of PD patients (33, 47, 90). Therefore, we probed a database for samples of *substantia nigra* from PD patients for evidence of increased expression of NFκB target genes (Table 1). As shown in the Table, several key components of NFκB pathways are found to be elevated. Moreover, Grx1 was found to be induced in the brain after MPTP treatment of male Swiss albino mice (57), and this induction was found to be concomitant with TH neuronal loss. Although the authors of that study interpreted neuronal loss to be due to insufficient Grx1 in neurons, the results could also reflect the consequence of Grx1 elevation in the glia. Accordingly, the PD-like phenotypes observed in the MPTP-treated mice and the C57BL/$6^{hGrx1TG}$ mice might be due to elevated p65 signaling in the CNS. It would be of use to characterize dopaminergic impairments, microglial activation, and behavioral abnormalities in the C57BL/$6^{hGrx1TG}$ mouse to explore its potential as a mouse model of PD.

Potential alterations in DJ-1 content on Grx1 overexpression

DJ-1 is involved in both PD and T2D pathogenesis. Alterations in DJ-1 levels appear to disrupt both beta islet cells (51) and neuronal homeostasis (13). As described above, we have reported evidence for Grx1 controlling DJ-1 levels (52), suggesting that Grx1 overexpression in some contexts might lead to dysregulation of DJ-1 homeostasis. C57BL/6J^{hGrx1TG} mice that overexpress Grx1 did not display appreciable change in DJ-1 levels (52). However, DJ-1 levels were not assessed in transgenic mice under stress, such as the HFHS diet (type II diabetes [T2D]). It is possible that stress coupled with Grx1 overexpression would promote alterations in DJ-1 levels. In regard to HFHS/T2D, DJ-1 levels have been found to be diminished in T2D pancreatic islets; and DJ-1-deficient mice show decreased secretion from beta cells in response to aging or metabolic stress (50). DJ-1 deficiency decreases adipogenesis and reduces inflammation in response to the HFHS diet (50). Therefore, it is conceivable that elevated Grx1 and HFHS diet converge to disrupt DJ-1 homeostasis, thereby leading to the observed biochemical markers of early PD (35).

Potential anti-inflammatory effects of Grx1 overexpression

While elevated cytokine levels in the CNS have been associated generally with neurotoxic inflammation, certain cytokines, such as IL-6 and IL-1β, have been reported to exert neuroprotective effects in some contexts (7, 15, 105). We found elevated levels of IL-6 in brain samples from the C57BL/$6^{hGrx1TG}$ mice (35). IL-6 has been found elevated in

Table 1. Evidence for Elevated NFκB Signaling in *Substantia Nigra* of Parkinson's Disease Patients

p *value*	*Fold change*	*Gene symbol*	*Gene title*
0.00023	1.82	NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B cells 1
0.00025	1.51	IKBKB	Inhibitor of kappa light polypeptide gene enhancer in B cells, kinase beta
0.0025	1.02	IL1B	Interleukin 1, beta
0.0428	1.36	Interleukin 6	Interleukin 6

mRNA expression data for samples of *Substantia nigra* from PD patients ($n = 11$) and matched controls ($n = 15$) (accession number GSE20295) were obtained from the Gene Expression Omnibus database. Three control subjects (GSM606624, GSM606625, and GSM606626) were excluded from analysis as outliers. Data from remaining subjects were analyzed for differential expression using GEO2R.

brains of PD patients (78), suggesting that, despite potential neuroprotective properties, elevated IL-6 fails to protect TH neurons in PD patients. We found that Grx-overexpressing microglia secreted elevated levels of IL-6, as well as a number of proinflammatory (TNF-α, RANTES, IL-12, IL-13) and anti-inflammatory (IL-10) cytokines (35). However, proinflammatory neurotoxic effects appear to be dominant, as increased neuronal cell death occurred in co-culture with the microglia in which Grx1 was elevated, suggesting that elevated inflammatory cytokine signaling is sufficient to promote neuronal cell death despite potential anti-inflammatory effects. It is nevertheless conceivable that the C57BL/6^{hGrx1TG} mouse may be resistant to further neurodegeneration induced by neurotoxic stimuli, particularly considering that resistance to MPTP toxicity in female Swiss albino mice was attributed to their naturally elevated Grx1 levels relative to the male mice (57). It would be instructive to characterize effects of neurotoxins on further DA neuronal loss in the C57BL/6^{hGrx1TG} mice.

Thus, Grx1 within DA neurons may play a neuroprotective role; however, Grx1 within microglia may play a neuroinflammatory role. The dilemma regarding Grx1 as a therapeutic target for PD is whether to stimulate its upregulation as a protective measure or inhibit its proinflammatory activity. Much work is needed to sort out the preponderant role of Grx1 and to devise effective therapeutic intervention.

Influence of Grx1 levels on protein S-glutathionylation

Generally, elevation of Grx1 has been found to correspond to a decrease in global protein S-glutathionylation (5, 35), as well as a decrease in S-glutathionylation of individual target proteins; for example, p65 (35) and IKKβ (107). However, Grx1 has also been shown to *facilitate* S-glutathionylation in the presence of glutathione thiyl radicals (GS•) (116) under specific conditions. For example, Grx1 was found to promote p65 S-glutathionylation under hypoxic condition and GSH supplementation in a pancreatic cell line (91). Similarly, Grx1 overexpression increased S-glutathionylation of select protein targets and was shown to protect SK-N-SH dopaminergic cells from 6-hydroxydopamine (6-OHDA)-induced damage (100). Since S-glutathionylation of particular proteins may be a cell-protective mechanism, it follows that, under certain circumstances, Grx1 elevation, leading to decreased S-glutathionylation of such proteins, would sensitize cells to pro-PD insults. Our previous results (35) support this interpretation, as we observed an increased loss of DA neuronal projections in C57BL/6^{hGrx1TG} mice following prolonged HFHS feeding. Thus, Grx1 content may differentially drive protein S-glutathionylation, the vehicle of signal transduction by Grx1, depending on whether conditions favor deglutathionylation or S-glutathionylation (*i.e.*, the presence of GS•).

PD is a heterogeneous disease. As such, attempts to stratify PD based on symptom presentation (119) and/or biochemical signatures (28, 124) have been reported. We and others have documented correlations with PD incidence for both Grx1 elevation (35) and Grx1 diminution (54); however, alterations in content may drive distinct pathogenic processes of PD. Decrease in Grx1 content has been shown to impair mitochondrial complex I activity (58) and to exacerbate loss of DA neurons in *C. elegans* genetic models of PD (54). However, we have found Grx1 overexpression to result in decreased DA neuronal striatal projections (35), further exacerbated by prolonged HFHS diet. As described in Ref. (35), loss of DA neuronal projections is often attributed to increased phagocytosis by the microglia. It is therefore conceivable that Grx1 diminution could drive PD onset through mitochondrial impairment, while Grx1 overexpression could promote loss of neuronal projections *via* increased microglial activation. *Thus, Grx1 expression levels might serve as a unique classifier of PD subtypes.*

Glutathionylatable targets in apoptosis and inflammation

In this section, we provide an overview of apoptosis or inflammatory mediators documented to be regulated *via* S-glutathionylation. We have previously provided detailed overviews of mitochondrial proteins (102) as well as components of NFκB pathways (35, 109). Below, we provide information on other S-glutathionylatable targets in apoptosis and inflammation. In addition, we describe S-glutathionylatable proteins that have been specifically implicated in neuronal apoptosis.

Fas

Fas (CD95, TNFRSF6) is a death receptor, activation of which by TNF-α leads to apoptosis (reviewed in (11)). Fas has been implicated in neurodegeneration. LRRK2 has been shown to signal through activating Fas *via* binding to the death domain; this was shown to be dependent on caspase-8 (39). Fas and TNF-α have been implicated in EAE pathogenesis (19). Fas S-glutathionylation was first addressed in the context of lung infection by *P. aeruginosa* (11). Increased levels of Fas S-glutathionylation were proposed to drive apoptosis of epithelial cells and subsequent bacterial clearance. These findings suggest that Grx1 is implicated in epithelial remodeling.

Caspase-3

Caspase-3 was found to be glutathionylated (83) on Cys135 (active site) and on Cys45, both of which have been found to be inhibitory to its apoptotic activity. S-glutathionylation of these Cys residues on caspase-3 was achieved by addition of physiological levels of GSSG (46). Mutations of caspase-3 at Cys163, Cys184, and Cys220 led to increased cleavage, presumably due to decreased S-glutathionylation (83), although these sites were not identified as glutathionylated (46). Procaspase-3 has also been found adducted by biotinylated-GSSG, and treatment with GSSG was shown to inhibit cleavage into the active form. Procaspase-3 was also identified in rat liver, suggesting formation of this adduct *in vivo* in the absence of exogenous oxidative stimuli (46).

Grx1 knockdown *via* siRNA attenuated TNF-α-induced apoptosis in endothelial cells (83). However, the authors did not perform the converse experiment; that is, overexpression of Grx1 and determination whether the antiapoptotic effect would be reversed, or if there would be no effect. These data suggest that Grx1 knockdown would, in fact, dampen caspase-3 activity. Grx1 was shown to interact directly with caspase-3 both in epithelial cells and in an overexpression system (83). This interaction was shown to be negatively

affected by treatment with TNF-α. Grx1 overexpression prevented H_2O_2-induced caspase-3 cleavage in retinal pigment epithelium cells (67). Collectively, accumulated evidence indicates that Grx1 plays an antiapoptotic role in regulation of caspase-3 activity.

Akt

S-glutathionylation of Akt resulted from H_2O_2 treatment (67), and the extent of Akt S-glutathionylation was decreased in cells overexpressing Grx1. In another study, data suggest that Akt is S-glutathionylated on Cys296 and Cys310 (6). Remarkably, S-glutathionylation of Akt was shown to inhibit phospho-Akt formation. Grx1 knockdown in RPE cells did not appear to decrease Akt protein levels (67); however, similar experiment in Neuro2A cells decreased Akt protein levels (6). Thus, the effect of S-glutathionylation on the rate of degradation of Akt may be cell-type dependent and requires further investigation.

MyD88

MyD88 is an intracellular adaptor protein for the toll-like receptor (TLR) and IL-1R receptors. On ligation to the receptor, MyD88 recruits IRAK, which subsequently recruits the TRAF adaptor proteins *via* death domain dimerization, ultimately activating NFκB and AP-1 *via* TAK1/TAB. MyD88 has been shown to be essential for TNF-α and IL-6 production following stimulation of TLR/IL-1R (8). Remarkably, knockout of MyD88 has been shown to prevent TLR-mediated LRRK2 phosphorylation in astrocytes, implicating MyD88 in familial PD (27).

S-nitrosylation of MyD88 within the TLR interaction region at Cys-216 has been reported (48). This modification, dependent on endothelial nitrogen oxide synthetase expression, was found to diminish NFκB activation by LPS. The S-nitrosylation of MyD88 was abolished in a cell-free system by treatment with GSH (1 m*M*), suggesting that S-nitrosylation could serve as a precursor for S-glutathionylation of MyD88. Hence, we were prompted to seek direct evidence for S-glutathionylation of MyD88, which would implicate regulation by Grx1. In fact, we documented S-glutathionylation of MyD88 in BV2 murine microglial cells at rest [Fig. 6A; (75)], consistent with the interpretation that MyD88 would be activated *via* deglutathionylation by Grx1 and thereby contribute to the proinflammatory response as an upstream mediator that propagates the effect of Grx1 upregulation to initiate NFκB signaling, akin to other NFκB pathway members undergoing reversible S-glutathionylation.

Thus, as described above, Grx1 upregulation by LPS or *via* adenoviral overexpression led to a concomitant decrease of S-glutathionylation of p65 in the microglia (35). Since deglutathionylation of p65 has been found to correspond to increased transcriptional activity of p65 in several other contexts (5, 63, 66), we interpret our recent findings to indicate that upregulation of Grx1 activates microglia through activating NFκB, likely through deglutathionylation of MyD88 and p65 and potentially other S-glutathionylated components of the NFκB signaling network. Therefore, we hypothesize that MyD88 is regulated by S-glutathionylation, thereby acting as the upstream mediator that allows Grx1 induction to initiate NFκB signaling.

MyD88 inhibition decreases IL-6 release by LPS, but does not prevent Grx1 induction—for assessing impact of MyD88 on Grx1 induction and IL-6 release stimulated by LPS, BV2 murine microglia cells were treated with cell-permeable MyD88 inhibitory peptide (NBP2-29328; Novus Biologicals, Littleton, CO) or inactive analog for 24 h. Cells were then stimulated with 1 μg/mL LPS for 24 hr, and then, cells were lysed to assess Grx1 activity. We found that Grx1 induction by LPS does not appear to change in the presence of MyD88

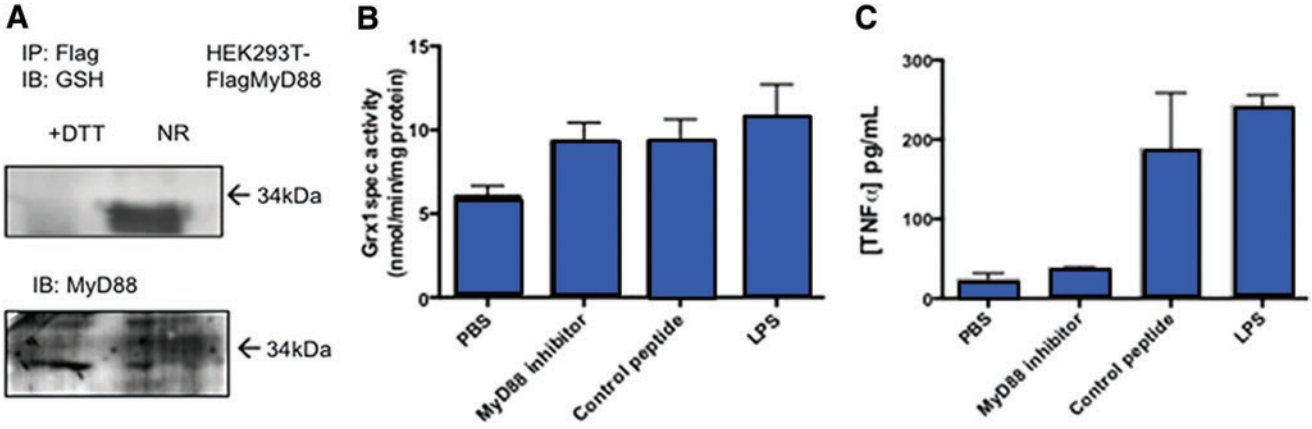

FIG. 6. MyD88 may be a novel target for Grx1-mediated microglial activation. For detection of MyD88 S-glutathionylation, HEK293T cells were cultured in low-glucose DMEM supplemented with 10% FBS. N-terminal FLAG tagged hMyD88 sequence cloned into the pc3.1 vector (48) (FLAG-MyD88) was a kind gift from Dr. Takeshi Into (Asahi University School of Dentistry, Japan). For transient FLAG-MyD88 expression, HEK293T cells were transfected with the FLAG-MyD88 plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Immunoprecipitation studies were conducted as described previously (35). Anti-FLAG M2 agarose was purchased from Sigma-Aldrich (St. Louis, MO). We detected glutathionylated Flag-MyD88 in the absence of stimulus **(A)**, suggesting that Grx1 may regulate NFκB signaling at the level of MyD88 activation. **(A)** Pulldown of Flag-tagged human MyD88 transiently expressed in HEK293T cells, probed for adducted glutathione, $n = 3$ +DTT–10 m*M* DTT added, NR, not reduced. **(B)** Grx1 activity assay in BV2 pretreated with MyD88 inhibitory peptide (Novus Bio) or control peptide for 24 h and treated with 1 μg/mL LPS for 24 h. **(C)** TNF-α levels in medium of cells in **(B)**, mean ± SD, $n = 2$. DTT, dithiothreitol. Color images are available online.

inhibitor (Fig. 6B), suggesting that MyD88 may contribute to propagating the effect of Grx1 upregulation, but it is not required for the mechanism of Grx1 induction in BV2 cells. Media samples were taken for IL-6 ELISA (BioLegend, San Diego, CA) according to the manufacturer's instructions. A decrease in LPS-stimulated IL-6 release was observed (Fig. 6C), suggesting that MyD88 is necessary for IL-6 release from TLR4 stimulation in BV2 cells, consistent with its role in activation of NFκB signaling.

Glutathionylatable Targets in Neuronal Apoptosis

Calcium channels

Dysregulation of calcium homeostasis has been identified as a likely contributor to neurodegenerative disease. In this regard, higher levels of cytosolic calcium have been reported to promote cellular oxidative stress (72), a condition favoring protein glutathionylation. In the context of models of PD, MPTP treatment of mice was found to induce elevated intracellular calcium and to increase neuronal cell death (62). Similarly, calcium levels are found to rise in the event of excitotoxic injury, leading to cell death. This increase in apoptosis can be blunted by inhibition of the calcium-transporting IP3 or ryanodine receptors (72). S-glutathionylation of the ryanodine receptor type 1 was found to occur in response to various treatments *in vitro*, corresponding to opening of the channel and release of calcium from the endoplasmic reticulum (12). With an *in vivo* cerebral ischemia rat model, S-glutathionylation of the ryanodine receptor type 2 was found to occur under ischemic conditions and result in hyperactivation of the channel, possibly promoting neuronal death (17). In addition, sarcoplasmic reticulum calcium ATPase (SERCA)2 is the chief regulator of Ca^{2+} reuptake, and its activity is known to be altered by reversible S-glutathionylation in other contexts (2), suggesting that its modulation may also contribute in the context of neurodegeneration.

TRPC5 (transient receptor potential channel, subfamily C, member 5) is another Ca^{2+} channel whose S-glutathionylation on Cys176 and Cys178 was shown to activate the channel, leading to elevated intracellular Ca^{2+}, which may contribute to increased neuronal death (45) Moreover, higher levels of TRPC5-SSG were found in striatal neurons of patients with Huntington's disease. Therefore, these findings suggest that TRPC5 S-glutathionylation is proapoptotic, and thus, Grx1 diminution may exert its proapoptotic effects in the neuron, at least in part, through TRPC5 S-glutathionylation. However, we are unaware of studies that have examined the S-glutathionylation status of the various calcium pumps in PD models.

Actin/tau

Actin has been shown to be regulated *via* S-glutathionylation (126), and in the context of neuronal degeneration, cytoskeletal S-glutathionylation has been implicated in axonal dying-back degeneration (18). Actin polymerization/depolymerization dynamically maintains cellular infrastructure and participates in cell growth, movement, and division. In particular, actin polymerization and function have been found to be redox regulated through Grx1-dependent reversible S-glutathionylation. Thus, under unstimulated conditions for NIH3T3 fibroblasts and A431 epithelial cells, actin is largely S-glutathionylated on the Cys 374 residue, inhibiting actin polymerization. Stimulation of these cells by growth factors (FGF and EGF, respectively) led to Grx-dependent deglutathionylation, increased polymerization, and migration/rearrangement of the intracellular actin (126, 127). Regarding its role in neurons, S-glutathionylation of actin appears to promote axonal and dendrite stability and favor neuronal survival in normal brain (113). As expected, dysregulation of actin S-glutathionylation has been implicated in neuronal diseases. For example, fibroblasts from patients with Friedreich's ataxia showed increased actin S-glutathionylation, and the actin filaments were disordered as a result of S-glutathionylation (85), concomitant with enlargement of the cells and cytoplasm. Postmortem brain samples from Alzheimer's disease (AD) patients have also displayed increased actin oxidation (9). Accordingly, it is reasonable to conclude that dysregulation of actin S-glutathionylation may contribute to neurodegenerative disease progression in several contexts.

Tau protein is known to play key roles in neurons, promoting neurite extension and axonal growth through dynamic microtubule assembly. Aggregation of Tau in neurofibrillary tangles is a prominent feature of AD, and neurofibrillary tangles have also been noted in PD. A particular cysteine residue (Cys-322) of Tau is required for its binding to microtubules. Accordingly, oxidation of Cys-322 *in vitro* has been reported to alter Tau's ability to dimerize (26). S-glutathionylation of Tau has been reported to accelerate its polymerization, displaying kinetics comparable to that of Tau dimer linked *via* intermolecular disulfides (26). In addition, assembled filaments generated from monomers of S-glutathionylated Tau appeared to more closely resemble AD fibrillary tangles than did the aggregates of Tau-S-S-Tau dimers. Nevertheless, it is not known whether Tau is actually oxidized in neurofibrillary tangles nor is it known whether oxidation of Tau, for example, S-glutathionylation, may alter its normal functions.

Systems analysis of pathways linked to Grx1 and neurodegeneration

To investigate potential shared pathways between neurodegenerative effects of Grx1 diminution and upregulation, we performed cluster analysis on a list of S-glutathionylatable targets known to be involved in apoptosis and inflammation. Notable common pathways are described below, identified from either the Gene Ontology collection or KEGG (Kyoto Encyclopedia of Genes and Genomes.

Gene ontology: response to interleukin 6

In our previous review (36), we briefly considered the role of IL-6 in PD. In the current context, IL-6 signaling is a likely contributor to the potentially opposing effects on PD progression of localized changes in Grx1 (the yin and yang of Grx1), as IL-6 has been reported to exert both proinflammatory and neurotrophic effects. IL-6 release from neurons has been proposed to be stimulated by neuronal activity (55), and neuron-derived IL-6 has been proposed to be required for facial nerve regeneration following axotomy (117). Circulating IL-6 levels have been found higher in PD patients compared to healthy controls, although this

association is weak. Astrocytes are considered to be the primary source of IL-6 in the CNS (122). Hypoxia/reoxygenation appears to increase IL-6 secretion in cultured brain slices (68). The opposing effects of IL-6 on DA neuronal survival resemble the opposing effects of Grx1 on this physiological process, which may provide a reasonable explanation of the seemingly disparate effects of Grx1 on DA neuronal homeostasis. It would be of interest to determine whether alterations in Grx1 content promote parallel or opposing alterations in IL-6 secretion from the various cell types of the brain, neurons *versus* microglia *versus* astrocytes.

Gene ontology: nucleotide binding LRR containing receptor pathways

NAIP contains an NOD domain and an LRR domain (25). NAIP inhibits caspase-9 activity, which may represent another layer of regulation by S-glutathionylation. XIAP (X-linked apoptosis inhibitory protein), a protein related to NAIP, has been shown to be regulated by S-nitrosylation, which was shown to inhibit its antiapoptotic action (120). XIAP is S-nitrosylated on the baculoviral IAP repeat residues, which bind to caspases and inhibit their function. This S-nitrosylation may, in fact, serve as a precursor to S-glutathionylation, analogous to SERCA (2) as discussed in our previous review (36). Multiple targets of proapoptotic action of Grx1 diminution have been identified (67). Therefore, NAIP represents another avenue *via* which decrease in Grx1 content may promote DA neuronal apoptosis.

Gene ontology: response to nicotine

Nicotine has been associated with decreased DA neuronal loss in PD animal models [reviewed in (93, 94)]; similarly, smoking is negatively associated with PD development in humans. Nicotine acts on nicotinic receptors, producing changes in Ca^{2+} signaling. Nicotine administration in rotenone-treated animals protects DA neurons [reviewed in (110)]. Treatment with targeted inhibitors suggested that nicotine may exert its neuroprotective effect through PI3K-Akt/PKB pathways (118). Nicotine administration in cultured neurons may also decrease unfolded protein response (114). In parallel, nicotinic receptor stimulation decreases L-induced dyskinesia (93, 135). This relationship held true for both existing dyskinesia and preventing dyskinesia (93). In clinical trials, there are conflicting results regarding the effect of nicotine on PD symptoms (93). This disparity may be explained, in part, by the apparent proinflammatory effects of nicotine (44). Akin to IL-6 (described above, in the Gene ontology: response to interleukin 6 section), effects of nicotine on DA homeostasis in PD may parallel the yin and yang of Grx1.

KEGG: TNF signaling pathway

TNF is a known activator of ASK1, which has been implicated in DA neuronal death. We have shown Grx1 to regulate ASK1 S-glutathionylation in SH-SY5Y cells (64), representing a potential avenue through which Grx1 regulates DA neuronal apoptosis. Antibodies against TNF prevented Trx1 oxidation (potentially *via* inhibiting TNF-driven mitochondrial ROS generation) and subsequent ASK1 activation (95). TNF-α is also considered an adipokine (described in more detail in the next section). Moreover, we have also shown that TNF-α can induce Grx1 in microglia, leading to microglial activation (35). Therefore, differential modulation of the TNF-α signaling pathway may represent an explanation for the duality of apoptotic and inflammatory effects of Grx1 in the context of PD.

KEGG: adipocytokine signaling pathway.

Adipocytokines are communication molecules secreted by the white adipose tissue, and they can also be expressed in the CNS [reviewed in (84)]. Adipocytokines have been implicated in regulating metabolic activity. However, evidence suggests that adipocytokines also play a role in neuronal survival and glial inflammation. Prominent adipocytokines are leptin, resistin, adiponectin, and TNF-α. Leptin can be produced in the cerebellum, cortex, and hypothalamus. Resistin is cysteine rich; in macrophages, resistin modulates NFκB signaling, and thus, cytokine release. Adiponectin also modulates NFκB signaling and promotes neuronal survival through the AMPK pathway. The Adipo-R1 receptor is expressed primarily in neurons, while Adipo-R2 is expressed weakly in astrocytes and neurons. Adiponectin increases astrocyte activation, and it has been shown to decrease release of inflammatory cytokines from the blood/brain barrier. Accordingly, deficiency in adiponectin is associated with increased inflammation (84).

Several findings in studies of models of PD suggest a protective role of the adipokines. For example, leptin administration to neurons challenged with 6-OHDA attenuated apoptosis (129), likely involving modulation of the MEK pathway. Furthermore, leptin administration into the substantia nigra of mice protected against coadministered 6-OHDA *in vivo* and increased levels of neurotrophic factors (129), consistent with the occurrence of leptin receptors (LepR) in the mouse midbrain (130). The neuroprotective effect of leptin has also been studied in model neurons treated with MPP^+, revealing a role for mitochondrial uncoupling proteins in maintaining ATP levels and the mitochondrial membrane potential in neuronal cells treated with MPP^+ [reviewed in (24, 40)].

Despite the accumulating evidence from studies of PD models, the role of adipokines in actual PD onset or progression is not clear. Thus, in a recent study, levels of leptin, resistin, and adiponectin were measured in blood samples from PD patients and found to be not significantly different from those obtained from non-PD controls (99). These results do not rule out changes in the adipokines in the CNS of PD patients, and so, further study should be aimed at determining the adipokine levels in cerebral spinal fluid or postmortem brain samples. Indeed, a leptin neuroprotective effect has been implicated in a study of PD patients undergoing treatment with deep-brain stimulation (DBS). Thus, DBS of the subthalamic nucleus resulted in weight gain and increased circulating levels of ghrelin and leptin (70). This result provides insight regarding a potential mechanism of neuroprotection involving leptin elicited by DBS.

Grx as a therapeutic target in inflammatory diseases

As described above, Grx1 knockdown has been shown to decrease inflammatory activation of microglia (35), retinal glial cells (108), and alveolar macrophages (4), documenting a proinflammatory role for Grx1; however, there are

exceptions where Grx1 may play an opposite role (41, 121), suggesting that the role of Grx1 in inflammatory response may be context and/or mouse strain dependent. We found Grx1 knockdown to inhibit LPS induction of *il6* (35, 75), consistent with Grx1 as a target for anti-inflammatory treatments. Grx1 diminution has been shown to contribute to apoptosis in some cell types, such as neurons (100, 101), retinal pigment epithelial cells (67), cardiomyocytes (31), and lung epithelial cells (11). *Therefore, cell-specific delivery of a Grx1 inhibitor is desirable to control potential off-target effects*. $Grx1^{-/-}$ mice (41) are viable and appear to have a normal life span, indicating that selective, local diminution of its activity may be tolerated.

The absence of Grx1 (*e.g.*, $Grx1^{-/-}$ mice) has been associated with variable anti-inflammatory effects (5, 42), suggesting that Grx1 may govern a specific inflammatory signature. We found that Grx1 knockdown prevented *il6* induction, but not that of *tnfa* or *il1b* (75). These results are consistent also with our earlier report of Grx1 knockdown in BV2 cells decreasing LPS-stimulated secretion of IL-6 but not TNF-α (35). Interestingly, $C57BL/6^{Grx1-/-}$ mice displayed decreased whole-brain levels of both *tnfa* and *il6* compared to WT controls (35, 75), suggesting that Grx1 knockdown in the microglia *versus* whole brain differentially regulates *tnfa* expression. Further investigation is needed to elucidate the main glutathionylated targets regulated by Grx1 in the microglia, which may explain apparent specificity for Grx1 regulation of *il6* induction.

Inflammation is a complex biological process involving a number of physiological systems. As such, therapies that effectively target the majority of interacting systems may represent an effective treatment strategy. A recent study of ours (72; manuscript under invited revision) is consistent with this interpretation. Thus, we discovered an inhibitor of Grx1 *via* rapid screening, identifying ''CWR-J02,'' a chloroacetamido agent that inhibits Grx1 by covalently adducting the active site cysteine, as documented by mass spectrometry. J02 treatment of intact model microglia (BV2 cells) inhibited intracellular Grx1 with an IC50 value essentially the same as that for deactivation of the isolated enzyme in the presence of 1 m*M* GSH. Treatment of the microglia with J02 concomitantly blocked production of proinflammatory cytokines. A proteomic analysis of J02-adducted proteins identified many J02-reactive proteins, including Grx1 and several mediators of inflammatory activation.

Taken together, our data identify J02 as an intracellularly effective Grx1 inhibitor that likely elicits its anti-inflammatory action in a synergistic manner by also disabling other proinflammatory mediators. Notably, the covalent mode of action and the presence of the alkyne group on the J02 molecule make it relatively facile to screen-related derivatives for increased Grx1 selectivity and intracellular potency in future studies. Accordingly, despite its rather broad selectivity, J02 may be a useful lead compound for further development as an anti-inflammatory agent.

Our proteomic data identified J02 as modifying and likely inhibiting a number of proteins belonging to the NFκB family. These proteins have been shown to be inactivated by S-glutathionylation (36). As J02 adduction onto reactive cysteines may mimic S-glutathionylation, J02 likely inactivates these target proteins. This effect could synergize with increased S-glutathionylation of these proteins due to inactivation of Grx1, thus highlighting J02 as a unique thiol-directed anti-inflammatory agent.

Some of the most used anti-inflammatories, such as nonsteroidal anti-inflammatory drugs (123) and glucocorticosteroids (56), owe their efficacy partly to targeting multiple pathways. Targeting multiple pathways also provides the potential benefit of decreased likelihood for adverse effects due to over-deactivation of a single pathway. Pathway analysis found J02 to potentially affect a wide variety of cellular processes pertinent to the inflammatory response and redox signaling, such as regulation of metabolic processes, translation, gene expression, cell redox homeostasis, and actin cytoskeleton remodeling. Combined partial effects on these processes may allow J02 or its analogs to be effective anti-inflammatory agents while limiting adverse effects.

In contrast to targeting the proinflammatory role of Grx1 in microglia *via* inhibition or knockdown, an alternative therapeutic approach would be to augment the activity of Grx1 and its neuroprotective role in DA neurons. Conceptually, increasing the activity of Grx1 could be accomplished by screening for neuron-selective inducers of the enzyme or by genetic engineering utilizing viral vectors or CRSPR/CAS9 technology.

Conclusions

Current research on Grx and its potential roles in PD presents a complex situation where Grx1 likely plays a primary neuroprotective role in dopaminergic neurons, and maintenance or enhancement of Grx1 activity in these brain cells would diminish the progression of PD. In contrast, Grx1 appears to play a primary proinflammatory role in microglia that are responsible for neuroinflammation, so diminution of Grx1 in these brain cells would diminish the progression of PD. Thus, a targeting Grx1 in the context of therapy of PD is a challenging prospect, requiring consideration of cell-selective manipulation of Grx1 content or activity.

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Abbreviations Used

6-OHDA = 6-hydroxydopamine
AD = Alzheimer's disease
Akt = serine/threonine protein kinase (*aka* protein kinase B)
ASK1 = apoptosis signal regulating kinase 1
CFTR = cystic fibrosis transmembrane conductance regulator
CNS = central nervous system
DA = dopaminergic
DBS = deep-brain stimulation
Grx = glutaredoxin
$GS^{\bullet}$ = glutathione thiyl radicals
GSH = glutathione
GSSG = glutathione disulfide
HFHS = high fat high sugar
ICAM-1 = intercellular adhesion molecule-1
IKKβ = I-kappaB kinase-beta
IL-1B = interleukin-1B
IL-6 = interleukin-6
KEGG = Kyoto Encyclopedia of Genes and Genomes
LPS = lipopolysaccharide
LRRK2 = leucine-rich repeat kinase 2
MPP+ = 1-methyl-4-phenylpyridinium
MPTP = 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NF-1 = nuclear factor-1
NFκB = nuclear factor kappa B
NOX = NADPH oxidase
p53 = protein 53 tumor suppressor
p65 = NFκB subunit protein (65kDa)
PD = Parkinson's disease
PINK1 = PTEN-induced putative kinase 1
PKC = protein kinase C
ROS = reactive oxygen species
SERCA = sarcoplasmic reticulum calcium ATPase
T2D = type II diabetes
TDOR = thiol/disulfide oxidoreductase
TH = tyrosine hydroxylase
TLR = Toll-like receptor
TNF-α = tumor necrosis factor-alpha
TRPC5 = transient receptor potential channel, subfamily C, member 5
Trx = thioredoxin
XIAP = X-linked apoptosis inhibitory protein