

S-Nitrosylation Regulates Cell Survival and Death in the Central Nervous System

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Abstract Nitric oxide (NO), which is produced from nitric oxide synthase, is an important cell signaling molecule that is crucial for many physiological functions such as neuronal death, neuronal survival, synaptic plasticity, and vascular homeostasis. This diffusible gaseous compound functions as an effector or second messenger in many intercellular communications and/or cell signaling pathways. Protein S-nitrosylation is a posttranslational modification that involves the covalent attachment of an NO group to the thiol side chain of select cysteine residues on target proteins. This process is thought to be very important for the regulation of cell death, cell survival, and gene expression in the central nervous system (CNS). However, there have been few reports on the role of protein S-nitrosylation in CNS disorders. Here, we briefly review specific examples of S-nitrosylation, with particular emphasis on its functions in neuronal cell death and survival. An understanding of the role and mechanisms underlying the effects of protein S-nitrosylation on neurodegenerative/neuroprotective events may reveal a novel therapeutic strategy for rescuing neurons in neurodegenerative diseases.

Keywords Nitric oxide · S-Nitrosylation · CNS · Death · Survival

Introduction

Nitric oxide (NO) is a gas that is freely permeable to the cellular plasma membrane; thus, it does not need a biological receptor to affect intracellular communication and/or signal transduction mechanisms. NO has dual effects: low levels of NO that are produced under physiological conditions stimulate many normal intracellular signaling pathways, whereas high levels lead to cellular injury [1]. Several neuronal models have demonstrated the neuroprotective actions of NO. For example, in dorsal root ganglia neurons, NO protects neurons after peripheral nerve injury [2]. In a cerebellar granule cell model, serum deprivation-induced cell death was rescued by NO donors [3]. Another neuronal cell model of trophic factor deprivation in spinal cord motor neurons demonstrated that NO exerted its neuroprotective effects by regulating the cyclic guanosine monophosphate (cGMP)-dependent signaling pathway [4]. NO activated the phosphatidylinositol 3-kinase (PI3K)/Akt survival signaling pathway and promoted cell survival in a 6-hydroxydopamine (6-OHDA)-induced apoptotic neuronal model of rat pheochromocytoma PC12 cells [5]. In a previous study, we proposed that NO-activated protein kinase activity is involved in neuritogenesis [6]. Furthermore, an NO donor and cGMP analog promoted neurite outgrowth in PC12h cells, whereas an NO synthase inhibitor, NO scavenger, and cGMP-dependent protein kinase inhibitor all inhibited neuritogenic action. These results suggest that neuritogenic actions are mediated by NO-dependent cGMP signaling [7, 8]. A similar result was reported for PC12 cells, in which cell differentiation was mediated by NO-cGMP-dependent angiotensin II binding to the angiotensin II receptor [9]. In hippocampal neurons, estradiol-stimulated dendritic branching was induced by the NO-cGMP pathway [10].

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In contrast, excessive NO formation has emerged as an important mediator of neurotoxicity in a variety of disorders of the nervous system. When NO is present at higher than normal physiological levels, it can initiate a neurotoxic cascade such as glutamate signaling. A well-known mechanism for NO production involves activation of *N*-methyl-D-aspartate (NMDA)-type glutamate receptors. In the cascade, the glutamate neurotransmitter stimulates NMDA receptors, which stimulates calcium ion (Ca^{2+}) influx into the cell via receptor-associated ion channels, in turn activating neuronal nitric oxide synthase (NOS) to produce NO. Treatment by NOS inhibitors or removal of arginine from the medium prevented NMDA neurotoxicity in a cortical neuron culture system [11]. A quencher of NO, hemoglobin prevents NMDA-induced neuronal damage [12]. NO is more harmful under pathological conditions that involve reactive oxygen species production, such as peroxynitrite. Dysfunction and structural dysregulation of neuronal network activity are fundamental causes of neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease, and amyotrophic lateral sclerosis (ALS). A marker of nitrosative stress, nitrotyrosine formation has been reported in patients with AD and PD [13–15]. In this review, we summarize the role of NO in regulating cell survival and cell death in the central nervous system (CNS).

Role of NOS Isoforms in the CNS

NO is an organic gas that is ubiquitously synthesized by NOS. In mammalian cells, NOS is subclassified into three types: neuronal NOS (nNOS or NOSI), inducible NOS (iNOS or NOSII), and endothelial NOS (eNOS or NOSIII). The functions of NO differ depending upon the cell type and enzyme isotype. nNOS is involved in neurotransmission by creating retrograde signaling between synapses. At the synapses, nNOS is coupled to the NMDA receptor via postsynaptic density-95 protein complexes [16]. Upon glutamate stimulation of the NMDA receptor, Ca^{2+} enters the cytoplasm through the ion channel, where in conjunction with calmodulin, it triggers nNOS activation and NO production [17]. Low levels of NO that are produced under physiological conditions stimulate many normal intracellular signaling pathways. In contrast, overstimulation of the NMDA receptor and subsequent Ca^{2+} influx induce pathological signaling, resulting in neural damage and death through production of toxic levels of NO [18]. A primary activator of nitrosative stress in AD is the release of excess Ca^{2+} into the cytosol following the overstimulation of NMDA receptors. nNOS mediates the long-term regulation of synaptic transmission in the CNS, the central regulation of blood pressure, smooth muscle relaxation,

and vasodilation via peripheral nitrenergic nerves. It is also implicated in neuronal cell death in cerebrovascular stroke [19]. The latter effect may be related to NO biosynthesis by nNOS that is dependent upon the tetrahydrobiopterin cofactor, the concentration of which is gradually decreased in the caudate nucleus of PD patients [20].

iNOS, which is upregulated by acute inflammatory stimuli, is strongly implicated in the pathogenesis of neurodegenerative diseases. Pathological levels of NO via iNOS induction in activated glial cells lead to various neurodegenerative diseases. NO is further oxidized to nitrite peroxynitrite and free radicals to highly interact with thiols and the ion-sulfur center of various enzymes [21], resulting in apoptosis, neurotoxicity, neuronal degeneration, and numerous CNS disorders. Activation of iNOS requires gene transcription, and its induction can be influenced by endotoxin and cytokines such as lipopolysaccharides, interleukin (IL)-1 β , IL-2, interferon- γ (IFN- γ), and tumor necrosis factor (TNF). Although the endogenous factors that induce iNOS expression in the aging brain are unknown, TNF levels in the cerebrospinal fluid and peripheral circulation, as well as IL-1 β and IFN- γ levels in monocytes are increased by aging [22]. iNOS expression was induced in the hippocampal dentate gyrus of an ischemia brain model [23]. In addition, alterations in NO release were observed from neutrophils obtained from PD patients and the increased expression of iNOS was reported in 6-OHDA- and lipopolysaccharide-induced models of PD [13]. iNOS induction in astroglial cells rather than microglial cells is involved in its pathological status in AD [14] and PD [15]. Co-culture of iNOS-expressing astrocytes and neurons immediately caused damage to the neuronal respiratory chain and loss of ATP [24].

The major function of eNOS is regulation of vascular smooth muscle relaxation via a cGMP-mediated signal transduction pathway. eNOS is mostly expressed in endothelial cells, although it has also been found in cardiac monocytes, platelets, and certain neurons of the brain. Ca^{2+} -activated calmodulin is important for the regulation of eNOS activity. eNOS produces NO in a pulsatile manner, with eNOS activity increasing when intracellular Ca^{2+} levels rise. It has been reported that eNOS is induced under inflammatory conditions [25]. eNOS is involved in neuronal functions such as long-term potentiation in the hippocampus and NMDA-stimulated gamma-aminobutyric acid release in various regions of the brain [26].

Protein S-Nitrosylation

Because NO is a free gas, it can easily penetrate the biological membrane. The superoxide radical shows high affinity for NO. The reaction between NO and the superoxide

anion induces the production of peroxynitrite [27], which is a highly reactive molecule that can cause extensive damage to proteins, lipids, and DNA. NO interacts with thiols, which are organosulfur compounds that contain a carbon-bonded sulfhydryl (–C–SH or R–SH) group. Several actions of NO, including neurotransmission and vasodilation, are mediated by the activation of soluble guanylate cyclase and subsequent elevation of cGMP levels [28], whereas other actions are mediated by *S*-nitrosylation of the free cysteine group of proteins [28]. Since Jaffery and Snyder [29] discovered the “biotin-switch assay” (Fig. 1) for protein *S*-nitrosylation, more than 1000 proteins have been identified as *S*-nitrosylation target proteins (Table 1). *S*-nitrosylation can mediate neuroprotective or neurotoxic effects, depending upon the action of the targets [30].

Targets of *S*-Nitrosylation in Modulating Cell Function and Mediating Protection in the CNS

NMDA receptors mediate Ca^{2+} entry into the cell to regulate physiological processes such as synaptic plasticity and memory, but overstimulation by glutamate or other agonists can cause neuronal cell death. *S*-nitrosylation of the redox modulatory site of the NMDA receptor resulted in down-regulation of NMDA receptor activity [18]. Thus, under excitotoxic conditions, *S*-nitrosylation of NMDA receptors can provide neuroprotective effects. The autonomous activity of Ca^{2+} /calmodulin-dependent protein kinase II α (CaMKII α) mediates excitotoxic neuronal cell death during ischemia [31]. NO-induced *S*-nitrosylation of CaMKII α also directly generates autonomous activity [32], which may be involved in excitotoxic neuronal cell death. Type 1 ryanodine receptor (RyR1) is the Ca^{2+} -induced Ca^{2+} release channel in the endoplasmic reticulum (ER) [33]. During epileptic seizures, *S*-nitrosylation of RyR1 causes Ca^{2+} release from the ER via the RyR1, causing seizure-dependent neuronal cell death [34].

Mitogen-activated protein kinase kinase 4 (MKK4) is activated via phosphorylation and regulates cell proliferation, differentiation, survival, and migration in response to cellular stress [35]. In the ischemia/reperfusion brain,

MKK4 is *S*-nitrosylated by endogenous NO. Inhibition of *S*-nitrosylation and activation of MKK4 results in decreases in NMDA receptor-mediated neuronal degeneration and apoptosis [36]. The Src homology 2 domain-containing phosphatase (SHP-2) increases neural progenitor cells and neuronal survival through activation of extracellular signal-regulated kinase 1/2 (ERK1/2) [37]. Excitotoxic injuries induce *S*-nitrosylation of SHP-2, resulting in increased susceptibility to NMDA receptor-mediated excitotoxicity by inhibition of ERK1/2 activation [38]. Cyclooxygenase (COX)-2 is involved in NMDA receptor-mediated neurotoxicity via the generation of prostaglandins. This neurotoxicity is due to activation of COX-2 by *S*-nitrosylation after selective binding of nNOS to COX-2 [39]. Peroxiredoxin 2 (Prx 2) is one of the antioxidant enzymes that are abundantly present in mammalian neurons. *S*-nitrosylation of this enzyme interrupts the normal redox cycle via inhibition of peroxidase activity, resulting in the accumulation of cellular peroxides and neuronal cell death [40]. In addition to Prx2, Prx1 is also nitrosylated by NO/*S*-nitrosylation donors [41], promoting disulfide formation on Prx1 catalytic cysteines and disrupting the oligomeric structure of Prx1, ultimately leading to loss of peroxidase activity [41].

FLICE inhibitory protein (FLIP) and Bcl-2 are key apoptosis regulatory proteins. FLIP inhibits mitochondrial-mediated apoptosis through the inhibition of caspase-8 and Fas-associated death domain binding. *S*-nitrosylation of FLIP and Bcl-2 suppresses their ubiquitination and proteasomal degradation, subsequently enhancing antiapoptotic activity through their stabilization [42, 43]. Proliferating cell nuclear antigen (PCNA) interacts with caspase-9 and partially blocks its activation. *S*-nitrosylation of PCNA did not inhibit caspase-9 activation and apoptosis in rotenone-treated SH-SY5Y neuroblastoma cells [44]. X-linked inhibitor of apoptosis (XIAP) can inhibit pro-caspase activity via ubiquitination and degradation, thereby suppressing caspase-mediated apoptosis and promoting cell survival. Under cerebral ischemia–reperfusion condition, *S*-nitrosylation of XIAP occurs via the thioredoxin system [45]. *S*-nitrosylation of XIAP inhibits its E3 ubiquitin ligase activity, and induces apoptosis [46]. DJ-1 (PARK7) acts as a neuroprotective protein via modulation of multiple

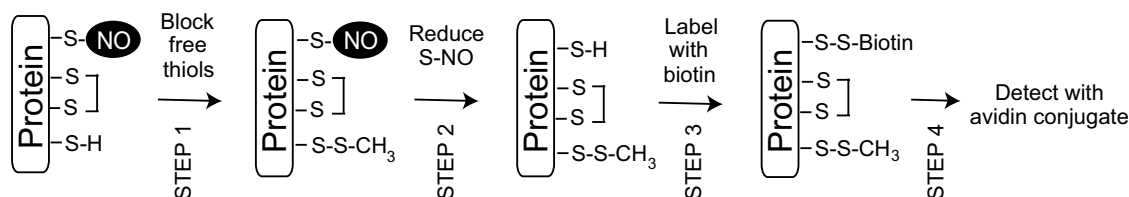


Fig. 1 Schematic of *S*-nitrosylated protein detection using the “biotin-switch assay”. *Step 1* Free thiols are blocked by a methylthiolating reagent. *Step 2* Reduction of *S*-nitrosylation bonds to thiols with

reductant. *Step 3* Newly reformed cysteines react with biotin-conjugated thiol-modifying reagent. *Step 4* Target biotinylated proteins are collected by avidin-coupled reagents

Table 1 Protein *S*-nitrosylation on cell protection and death in CNS

Protein name		Localization	Ref.
Excitotoxic cell death			
Neuroprotection	<i>N</i> -methyl-D-aspartate receptors (NMDARs)	Plasma membrane	[18]
Cell death	Type 1 ryanodine receptor (RyR1)	ER	[34]
	Mitogen-activated protein kinase kinase 4 (MKK4)	Cytosol	[36]
	Ca ²⁺ /calmodulin-dependent protein kinase II α isoform (CaMKII α)	Postsynaptic density	[32]
	Src homology-2 domain-containing phosphatase (SHP-2)	Cytosol	[38]
Redox imbalance			
Neuroprotection	Kleeh-like ECH-associated protein 1 (Keap1)	Cytosol	[67]
Cell death	Cyclooxygenase (COX)-2	Cytosol	[39]
	Peroxiredoxin 2 (Prx 2)	Cytosol	[40]
	Peroxiredoxin-1 (Prx1)	Cytosol	[41]
Caspase-mediated apoptosis			
Neuroprotection	Bcl-2	Mitochondria	[42]
	FLICE inhibitory protein (FLIP)	Cytosol	[43]
Cell death	Proliferating cell nuclear antigen (PCNA)	Nucleus	[44]
	X-linked inhibitor of apoptosis (XIAP)	Cytosol	[46]
PTEN/PI3K/Akt signaling			
Neuroprotection	Phosphatase and tensin homolog (PTEN)	Cytosol	[49]
	DJ-1 (PARK7)	Cytosol	[49]
Cell death	Akt	Cytosoll	[48]
Nuclear translocation of GAPDH			
Neuroprotection	GAPDH's competitor of SIAH protein enhances life (GOSPEL)	Cytosol	[53]
	B23/nucleophosmin	Nucleus	[55]
Cell death	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Cytosol	[50]
Accumulation of misfolded proteins			
Cell death	Protein disulfide isomerase (PDI)	ER	[57]
	PKR-like ER kinase (PERK)	ER	[58]
	Inositol-requiring enzyme 1 (IRE1)	ER	[58]
	Histone deacetylase 6 (HDAC6)	Cytosoll	[59]
	Parkin	Cytosoll	[60]
	c-Jun N-terminal kinase (JNK)	Cytosoll	[63]
	I κ B kinase (IKK) β	Cytosoll	[63]
	Insulin-degrading enzyme (IDE)	Cytosoll	[65]

cellular survival signaling pathways including the phosphatase and tensin homolog (PTEN)/PI3K/Akt cascade [47]. PTEN and Akt are nitrosylated and regulate Akt-mediated cell survival signaling [48]. Under low concentrations of NO, *S*-nitrosylation of PTEN leads to inhibition of its phosphatase activity and consequent Akt phosphorylation/activation, resulting in cell survival, proliferation, and growth. *S*-nitrosylated DJ-1 can be a source of NO group for PTEN [49]. Thus, transferring NO from DJ-1 to PTEN, results in enhanced neuroprotection. In contrast, high NO levels lead to *S*-nitrosylated Akt and inhibition of this neuroprotective pathway [48].

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is generally characterized as a housekeeping glycolytic enzyme. GAPDH undergoes *S*-nitrosylation, which

facilitates its binding to seven in absentia homolog 1 (SIAH1), an E3 ubiquitin ligase, resulting in nuclear translocation [50]. The nuclear GAPDH/SIAH1 complex augments p300/CBP-mediated acetylation of nuclear proteins, including p53, which regulates cell death [51]. GAPDH's competitor of SIAH protein enhances life (GOSPEL) is a novel GAPDH-binding protein expressed in the cytosol [52]. It competes with SIAH1 for binding to GAPDH upon *S*-nitrosylation, preventing nuclear translocation of GAPDH and resulting in neuroprotection [53]. B23/nucleophosmin is a nucleolar chaperone that plays a role in cellular stress responses [54]. It binds to both SIAH1 and GAPDH, disrupting the SIAH1-GAPDH complex in the nucleus in response to nitrosative stress. *S*-nitrosylation of B23 enhances B23-SIAH1 binding and mediates its

neuroprotective actions [55]. In addition, *S*-nitrosylated GAPDH may be an NO donor to B23 by transnitrosylation [55].

Protein disulfide isomerase (PDI) is an ER chaperone that is induced during ER stress, and is responsible for the formation of disulfide bonds in proteins [56]. *S*-nitrosylation of PDI inhibits its enzymatic and chaperone activities. The accumulation of misfolded and polyubiquitinated proteins results in persistent ER stress [57]. The three major ER stress-sensing proteins are protein kinase R-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6). *S*-nitrosylation of IRE1 α attenuates its ribonuclease activity, and *S*-nitrosylation of PERK activates its kinase activity and downstream phosphorylation/inactivation of eIF2 α . These *S*-nitrosylated proteins disrupt unfolded protein response signals and leads to ER stress-mediated cell death [58].

NO suppresses the deacetylase activity of histone deacetylase 6 (HDAC6) by *S*-nitrosylation in vitro and increases acetylated α -tubulin, a major substrate for HDAC6. HDAC6 plays a crucial role in promoting aggresome formation of misfolded/ubiquitinated proteins, which are pathological features common to neurodegenerative diseases. The accumulation of misfolded proteins and subsequent cell death may be partly dependent on HDAC6 inactivation via *S*-nitrosylation [59].

Parkin is an E3 ubiquitin ligase involved in the ubiquitination of proteins that are important in the survival of dopamine neurons in PD. Downregulated E3 ligase activity impairs ubiquitination and degradation of substrate proteins, potentially contributing to Lewy body formation and neuronal cell injury or death. *S*-nitrosylation inhibits the ubiquitin E3 ligase activity of parkin and its protective functions [60].

Autophagy is a major degradation process for misfolded proteins or damaged organelles. c-Jun N-terminal kinase (JNK) and I κ B kinase β (IKK β) are involved in the regulation of autophagic processes [61, 62]. *S*-nitrosylation of JNK1 or IKK β inhibits autophagosome formation due to inhibition of their kinase activities [63]. Insulin-degrading enzyme (IDE), a 110 kDa metalloendopeptidase, cleaves multiple short polypeptides including β -amyloid (A β) [64]. *S*-nitrosylation results in the complete inactivation and oligomerization of IDE, which may contribute to AD pathophysiology by decreasing A β degradation [65].

Antioxidative Effects of NO Through Keap1 *S*-Nitrosylation Signaling in the CNS

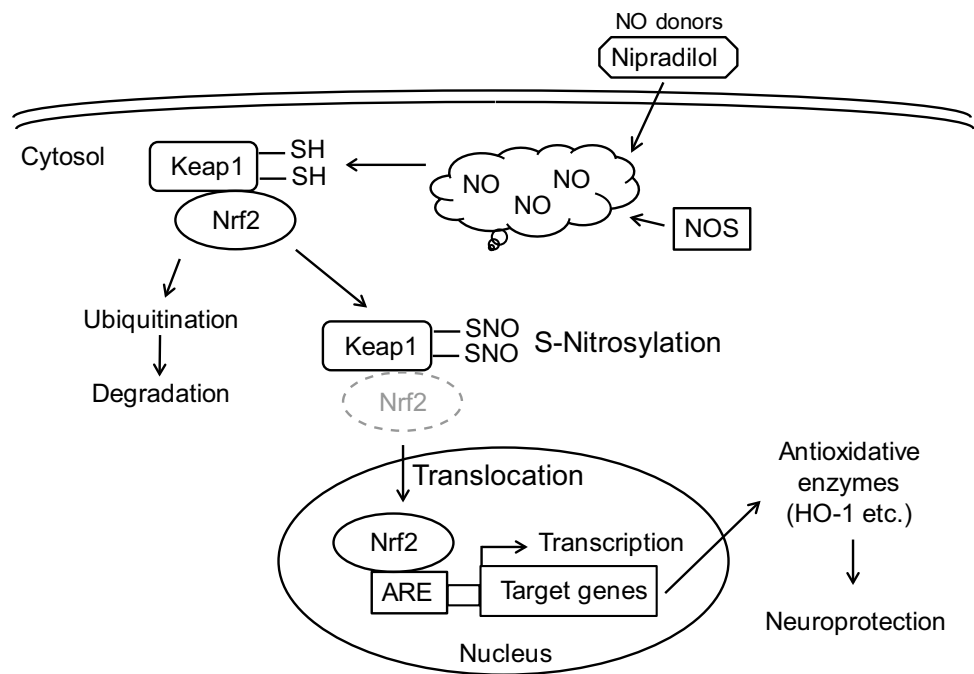
Because of their accessibility for experimental manipulations and relatively simple structure, the retina represent “approachable parts of the brain” that have long served as

an important model system for studying the mechanisms of cell survival or death in the CNS. Retinal ganglion cells (RGCs), the projection neurons of the retina, integrate information from photoreceptors via several types of interneurons, although some RGCs are also intrinsically photosensitive. While a growing number of *S*-nitrosylated proteins reportedly contribute to pathogenesis of brain diseases, there have been few studies on their role in retinal diseases. We investigated the role of protein *S*-nitrosylation by NO in RGC survival after optic nerve injury [66, 67]. Many reagents such as glyceryl trinitrate, sodium nitropruside, and *S*-nitrosothiols have been reported as NO donors [68]. Recently, NO donor drugs have been designed as NO-releasing compounds such as nipradilol (Nip), which has been registered as an antiglaucoma drug. The protective actions of Nip have been shown in various neuronal cells [69] including RGCs [70]. Mizuno et al. [71] demonstrated that Nip shows protective effects through NO-dependent mechanisms; however, the detailed mechanisms underlying these protective effects are not fully understood. Some lines of evidence have indicated that NO can attenuate RGC death [72] through a NO/cGMP-dependent pathway [73]. Moreover, Tomita et al. [74] reported that the beneficial effects of Nip on RGC death was not completely negated by inhibiting protein kinase G. Furthermore, Naito [75] reported that Nip attenuates hydrogen peroxide-induced lipid peroxidation. To determine if the neuroprotective actions of Nip are mediated by antioxidative processes via a NO/cGMP- or NO/*S*-nitrosylation-dependent mechanism, we examined the possible role of the Kelch-like ECH-associated protein 1 (Keap1). The Keap1 and NF-E2-related factor 2 (Nrf2) pathways regulate the expression of cytoprotective genes in response to oxidative stress or electrophilic stress [76]. Keap1 is a redox-sensor system protein that allows the activation of Nrf2 by modification including oxidation, alkylation, and *S*-nitrosylation [77]. Once activated, Nrf2 translocates to the nucleus from the cytosol, and binds to the antioxidant responsive element of target genes, driving the expression of antioxidative heme oxygenase-1 (HO-1) (Fig. 2). The Keap1 sensor protein contains 27 cysteine residues. Interestingly, NO activates the Keap1/Nrf2 pathway by *S*-nitrosylation of Keap1 in colon carcinoma cells [78]. Two reactive cysteines of Keap1, Cys 273 and Cys 288, have been identified as key sites of Nrf2 translocation activity [79].

NO/*S*-Nitrosylation-Dependent Antioxidative Protein Induction by Nip

NO can be directly involved in the modulation of HO-1 expression. NO-releasing agents can markedly increase HO-1 mRNA and protein expression in a variety of tissues

Fig. 2 Schematic diagram of the Keap1 *S*-nitrosylation-dependent antioxidative signaling pathway. NO donors and nipradilol induce *S*-nitrosylation of Keap1 and antioxidative enzyme (e.g., HO-1) expression through activation of the Nrf2/ARE signaling pathway



including neuronal cells [80]. The protective effects of Nip are dependent upon newly synthesized antioxidative proteins, such as HO-1. A previous study by our group showed that after 4 h of Nip treatment, HO-1 mRNA and protein expression increased in RGC-5, a retinal neural cell line [81]. HO-1 is an antioxidative protein that degrades intracellular heme to free iron, carbon monoxide, and biliverdin, which is then converted to bilirubin. Bilirubin acts as a strong endogenous reactive oxygen species scavenger and attenuates the final production of lipid peroxidation, 4-hydroxy-2-nonenal. These results indicate that the protective effect of Nip is dependent upon NO-induced HO-1 induction. In addition, a previous study showed that Nip promotes translocation of Nrf2 into the nucleus, which was inhibited by NO scavengers and an *S*-nitrosylation inhibitor (dithiothreitol). Translocated Nrf2 was bound to the E1 enhancer of HO-1 promoter as an antioxidative responsive element [66]. These results indicate that the Nip-mediated translocation of Nrf2 to the nucleus is dependent upon NO/*S*-nitrosylation.

Conclusions

In this review, we briefly summarized the role of NO as a neurotoxicant and as a neuroprotector in CNS disorders. We focused on protein *S*-nitrosylation, which plays an important role in both neuroprotective and neurotoxic effects and is involved in the pathogenesis of a number of neurodegenerative disorders such as AD and PD. Because there have been few reports on protein *S*-nitrosylation in

CNS disorders, elucidating the specific targets of *S*-nitrosylation and understanding their regulatory mechanisms could lead to the development of therapeutic strategies and targets for the treatment of neurodegenerative diseases.

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