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Thioredoxin as a Neurotrophic Cofactor and an Important Regulator of Neuroprotection

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Abstract

Oxidative stress has been implicated in the pathogenesis of a wide variety of neuronal diseases, including ischemic neuronal injury, Alzheimer's disease, and Parkinson's disease. Thioredoxin reduces exposed protein disulfides and couples with peroxiredoxin to scavenge reactive oxygen species. Nerve growth factor (NGF) has profound effects on neurons, including promotion of survival and differentiation via multiple signaling pathways. As for the NGF-induced neurite outgrowth, the CREB-cAMP responsive element (CRE) pathway is important to the activation of immediate-early genes such as *c-fos.* Thioredoxin is upregulated by NGF through ERK and the CREB-CRE pathway in PC12 cells. Thioredoxin is necessary for NGF signaling through CRE leading to *c-fos* expression and also plays a critical role in the NGF-mediated neurite outgrowth in PC12 cells. Therefore, thioredoxin appears to be a neurotrophic cofactor that augments the effect of NGF on neuronal differentiation and regeneration. NGF acts also as a neuronal survival factor. Previous reports showed that thioredoxin exerts a cytoprotective effect in the nervous system. The cytoprotective effect is mediated by enhancing the action of NGF, via the regulation of antiapoptotic signaling, or through its antioxidative stress activity.

Index Entries: Oxidative stress; neurite outgrowth; thioredoxin; nerve growth factor (NGF); PC12 cells; differentiation; redox; cAMP responsive element (CRE); apoptosis

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NGF Signal

Neurotrophins—including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophins 3 and 4/5 (NT3 and NT4/5)—have profound effects on neurons, including promotion of survival and differentiation *(1,2).* Neurotrophin signals are mediated by the Trk receptor tyrosine kinase and the p75 neurotrophin receptor *(3).* PC12 cells are pheochromocytoma cells and have been widely used to analyze the mechanism of NGF signaling, as they differentiate into sympathetic neuron-like cells upon NGF treatment and acquire neuronal characteristics *(4,5).* NGF has been shown to activate multiple signaling pathways in PC12 cells *(3,6,7).* The phosphatidylinositol 3 (PI3)-kinase pathway was implicated in NGF-dependent and serum-dependent survival of PC12 cells *(8),* whereas the extracellular signal-regulated kinase/mitogen-activated protein kinase (Erk/MAP) cascade mediates NGF-supported survival in PC12 cells. As for the NGF-induced neurite outgrowth, the signal is initiated by the binding of NGF to its high-affinity receptor, TrkA, on the plasma membrane *(6,9)* and transduced by activation of the ras and MAPK cascade *(10,11).* NGF treatment in PC12 cells leads to the activation of immediate-early genes (IEGs), such as *c-fos,* which are believed to be critical to the action of NGF *(12,13).* NGF activates the *c-fos* gene through several elements, including the serum response element *(14,15)* and CRE *(16,17).* NGF activates *c-fos* transcription via phosphorylation of CREB *(16–18).* SRE is regulated by the binding of SRF and a subset of Ets binding factors, known as ternary complex factor, such as Elk-1 and SAP-1 *(14,15,19,20).* CRE is regulated by the CREB/CREM/ATF families of transcription factors *(21).* The importance of CREB in neurite outgrowth in PC12 has been shown *(22,23).* Because neurotrophins prevented neuronal death in many experimental systems, they are considered as potential therapeutic agents for neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and peripheral polyneuropathies, as well as neurological and psychiatric pathologies such as ischemia, epilepsy, depression, and eating disorders *(24).*

The Thioredoxin System

Oxidative stress has been implicated in the pathogenesis of a wide variety of neuronal diseases, including ischemic neuronal injury, Alzheimer's disease, and Parkinson's disease. The maintenance of a cellular reducing environment is important to counteract oxidative stress. The cellular reducing environment is provided by two mutually interconnected systems: the thioredoxin system and the glutathione system.

We have identified and cloned a human thioredoxin from a human T-cell leukemia virus type I (HTLV-I)-positive cell line *(25,26)* and analyzed its role in the mammalian system. Thioredoxin is a small protein with two redox-active cysteine residues in an active center (Cys-Gly-Pro-Cys-) and operates together with NADPH and thioredoxin reductase as an efficient reducing system for exposed protein disulfides. Thioredoxin is present in many different prokaryotes and eukaryotes and appears to be present in all living cells *(27).* Several cytokine-like factors, such as 3B6-IL-1, eosinophil cytotoxicity-enhancing factor (ECEF), T-cell hybridoma MP6-derived B-cell growth factor, and early pregnancy factor, are identical or related to thioredoxin. Mammalian thioredoxin 2 has high homology with thioredoxin and has an active site C-G-P-C with thiol-reducing activity and is specifically localized in mitochondria *(28).* Conditional in vitro knockdown of thioredoxin 2 resulted in apoptosis, showing that thioredoxin 2 is essential for cell survival and plays an important regulatory role in mitochondrial apoptosis *(29).* Families of thioredoxin-dependent peroxidases (peroxiredoxin) scavenge intracellular hydrogen peroxide *(30,31).* Thus, the thioredoxin system is composed of several related molecules forming a network of interaction, maintaining the cellular reducing environment and protecting cells from oxidative stress.

Homozygous mutants carrying a targeted disruption of the thioredoxin gene died shortly after implantation, suggesting that thioredoxin expression is essential for early differentiation and morphogenesis in the mouse embryo *(32).* Thioredoxin transgenic mice display various phenotypes such as elongated life span and protection against ischemic injury, acute lung failure, diabetes mellitus, and toxicity caused by environmental stressors. As oxidative stress has been implicated in these conditions, thioredoxin seems to play an important role in protection against oxidative stress-associated diseases.

Recent topics involving the thioredoxin system include reports of thioredoxin-binding proteins. Using a yeast two-hybrid system, we isolated thioredoxin-binding protein/Vitamin D_3 upregulated protein 1 (TBP-2/VDUP-1), which was originally reported as an upregulated gene in HL-60 cells stimulated by 1α, 25-dihydroxyvitamin D3 *(33).* The interaction of thioredoxin with TBP-2/VDUP1 was observed in vitro and in vivo. Expression of TBP-2 disappeared in some transformed cell lines and overexpression of TBP-2 resulted in growth suppression *(33a),* suggesting that TBP-2/VDUP1 plays an important regulatory role in growth control. Ichijo et al. demonstrated that thioredoxin interacts with apoptosis-signaling kinase-1 (ASK-1) *(34),* a MAP-kinase kinase kinase, using a yeast two-hybrid system. ASK-1 is an upstream kinase of MKK3/6 or MKK4/7. ASK-1 activation leads to the activation of p38 MAPK, JNK. ASK-1 is known to be an important regulator of apoptosis. When thioredoxin is oxidized, ASK1 is dissociated from the oxidized thioredoxin and activated to induce an apoptosis signal *(35)* (Fig. 1). Recent reports showed that ASK-1 is required for the apoptosis induced by reactive oxygen species (ROS) or endoplasmic reticulum (ER) stress. Primary neurons derived from ASK-1 knockout mice were resistant to ER stress-induced cell death. ASK-1-mediated apoptosis plays an important regulatory role in polyglutamine disease *(36).*

The reduction of cysteine residues of various nuclear factors is known to be important for DNA-protein or RNA-protein interaction. Redox factor-1 (Ref-1) *(37),* the glutathione (GSH) system, and the thioredoxin (TRX) system maintain an intranuclear reducing environment to favor this function. Free sulfhydryl groups of iron-responsive element (IRE)-binding protein (IRE-BP) are required for the specific interaction between IRE-BP and IRE *(38).* DNA binding of the Fos-Jun heterodimer was modulated by reduction-oxidation (redox) of a single conserved cysteine residue in the DNA-binding domains of the two proteins *(39).* Ref-1 was identified as a factor facilitating AP-1 DNA binding *(37,40).* Ref-1 is identical to the formally described apurinic/apyrimidinic (AP) endonuclease *(41,42),* although the redox and DNA-repair activities of Ref-1 are encoded by nonoverlapping domains *(43).* DNA binding of NFκB to the κB site was also proven to be regulated by redox status *(44).* Several other transcription factors—including c-Myb, Ets, nuclear receptors like glucocorticoid receptors or estrogen receptors, a member of the Runt family, and p53—have been shown to be modulated by the cellular redox state *(45–49).* The interaction between HIF and coactivators may also be a target of redox regulation. The C-terminal activation domain of hypoxia-inducible factor 1α and its related factor has a specific cysteine. The expression of TRX and Ref-1 enhanced the interaction of these factors with a coactivator, CREB-binding protein (CBP)/p300 *(50).*

Regulatory Mechanism of Thioredoxin Gene Expression

Thioredoxin is expressed in all living cells. The thioredoxin gene has several SP-1 binding sites in the gene regulatory region, characteristic of housekeeping genes. However, we have often observed a cell-type-specific expression of thioredoxin, such as that in dendritic cells and activated macrophages *(51).* In addition, thioredoxin expression is induced by a variety of stressors, including viral infection, mitogens, phorbol myristate acetate (PMA), X-ray and ultraviolet irradiation *(52),* hydrogen per-

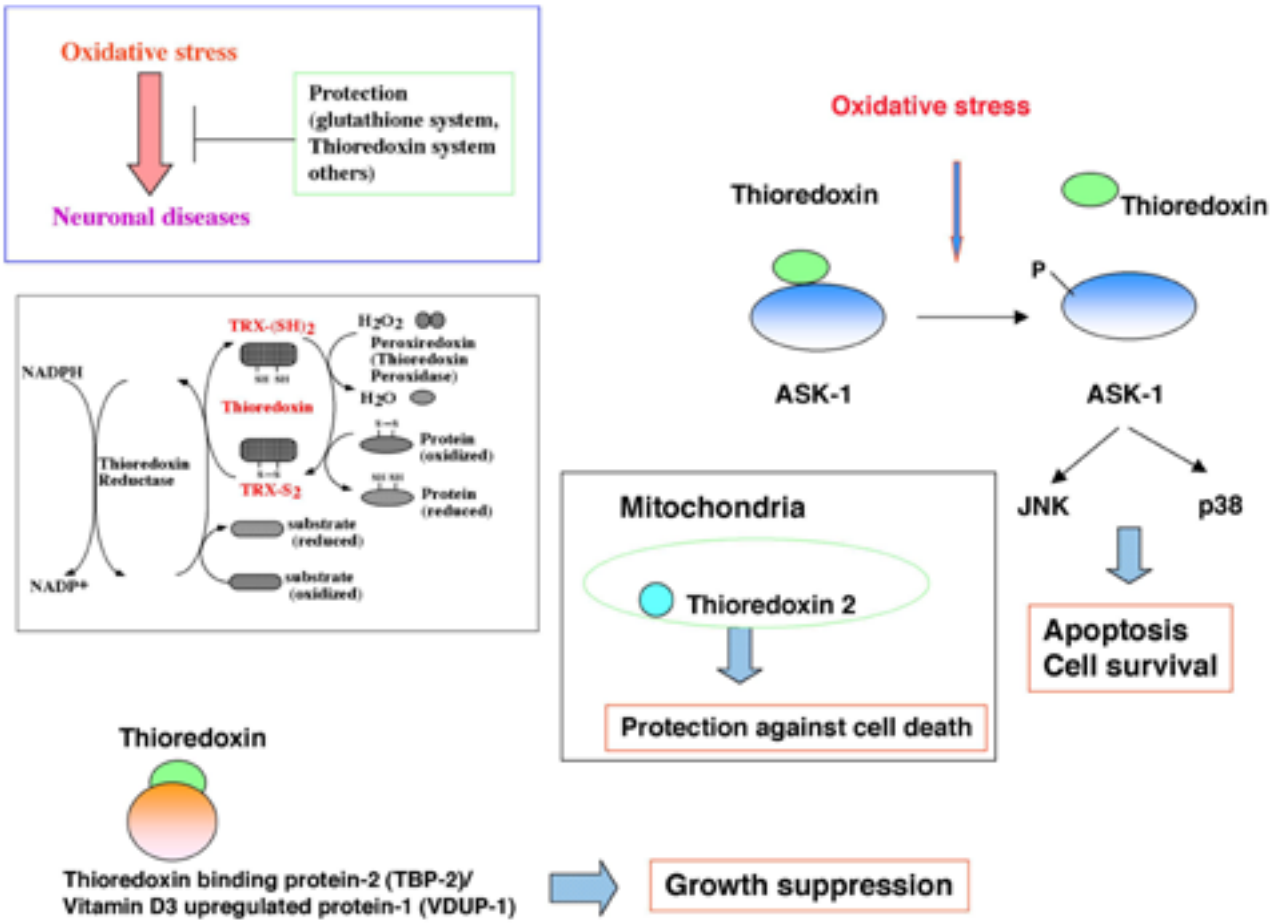

Fig. 1. Oxidative stress has been implicated in the pathogenesis of neuronal diseases. The thioredoxin system is composed of several related molecules forming a network of interaction with its active site cysteine residues, maintaining the cellular reducing environment and protecting cells from oxidative stress. Thioredoxin operates together with NADPH and thioredoxin reductase as an efficient reducing system for exposed protein disulfides and cooperates with families of thioredoxin-dependent peroxidases (peroxiredoxin) to scavenge intracellular hydrogen peroxide. Mammalian thioredoxin 2 is specifically localized in mitochondria. Thioredoxin also exerts its role through interaction with its binding proteins. Thioredoxin binding protein/vitamin D_3 upregulated protein 1 (TBP-2/VDUP1) plays an important regulatory role in growth control. Thioredoxin also interacts with apoptosis signaling kinase-1, a MAP-kinase kinase kinase that is known to be an important regulator of apoptosis. When thioredoxin is oxidized in response to oxidative stress, ASK1 is dissociated from the oxidized thioredoxin and activated to induce an apoptotic signal.

oxide, and ischemic reperfusion *(53)*. In the erythroleukemic cell line K562, thioredoxin is induced transcriptionally by hemin *(54)*. The major stimulatory effect on the thioredoxin gene by hemin is regulated through the antioxidant responsive element (ARE) *(55)*. The ARE was regulated by a switch of binding complexes including NF-E2p45/small Maf protein, Nrf2/small Maf protein, and the Jun/Fos families of proteins. Among them, the transcription factor Nrf2 was revealed to play a major regulatory role in the heme-mediated gene

activation of thioredoxin *(55).* Heme seems to be an important endogenous signal mediator and regulates various cellular functions. Heme is a prosthetic group of various important biomolecules such as hemoglobin, catalase, and cytochrome p450. Hemin is an oxidized form of heme. Hemin treatment is known to cause the differentiation of erythroid lineage cells and induce hemeoxygenase-1 gene expression. The release of heme from hemoglobin *(56)* and the release of iron from the heme moiety of cytochrome p450 *(57)* are implicated in the pathogenesis of reperfusion injury.

Groups of redox-regulating enzymes such as γ-glutamylcysteine synthetase, NAD(P)H: quinone oxidoreductase, and glutathione S-transferase Ya are known to contain the ARE *(58)*/electrophile-responsive element (EpRE) *(59)* for responding to electrophile-targeting xenobiotics. The Cap'n'Collar transcription factors including NF-E2p45, Nrf1 and Nrf2 *(60–62)* form heterodimers with small Maf proteins such as MafK, MafF, and MafG *(63),* binding to the ARE/EpRE/Maf-recognition element (MARE) *(64).* The importance of Nrf2 in the gene activation induced by oxidative stress has been shown in several studies. The hemeoxygenase-1 gene and γ-glutamylcysteine synthetase subunit genes are regulated by Nrf2 *(65,66).* The AREs of the thioredoxin gene and hemeoxygenase-1 gene display a strong resemblance. Administration of TRX protein can protect cells from ischemic reperfusion injury *(67,68),* and TRX expression is induced by ischemic reperfusion (reviewed in *69*). Hemeoxygenase-1 plays a role in endothelial cell damage caused by reperfusion injury. It is reported that thioredoxin expression enhances hemeoxygenase-1 expression *(70).* These results suggest that thioredoxin and hemeoxygenase-1 play coordinated roles in protection against a subset of oxidative stress and against pathological conditions such as reperfusion injury (Fig. 2). Activation through the ARE of the thioredoxin gene seems to constitute a positive feedback loop. Cap'n'Collar transcription factors such as Nrf2 and members of the Jun/Fos families of transcription factors have conserved cysteine residues that can be regulated by the redox status. Therefore, induction of thioredoxin may favor for the activation through the ARE *(70).* A recent report showed that NGF treatment elevates the levels of both MafK transcripts and protein. Interference with MafK expression or activity by interfering RNA and dominant negative strategies suppressed NGF-promoted outgrowth and maintenance of neurites by PC12 cells. Therefore, the authors suggested a role for MafK as a novel regulator of neuronal differentiation *(71).* Although the significance of the study remains unclear and awaits further analysis, an interconnection between NGF signaling and activation through ARE buttery genes could be postulated.

Thioredoxin in the Nervous System

In the nervous system, thioredoxin expression is reported to be augmented under various experimental conditions, most of which are associated with oxidative stress. Thioredoxin expression is induced in astroglia in the gerbil brain after transient global ischemia *(72).* Substantial upregulation of the mRNA and protein expression of thioredoxin is observed in rat motor neurons following hypoglossal nerve axotomy *(73).* Using a rat transient cerebral artery occlusion model, thioredoxin immunoreactivity and mRNA was found to be enhanced in the perifocal ischemic region *(74).* Thioredoxin expression is induced in the retinal pigment epithelium following retinal ischemia-reperfusion injury *(75,76),* in both neural retina and retinal pigment epithelium after exposure to light *(77).* Thioredoxin expression was induced by cyclic AMP analogs in retinal pigment epithelial cells *(78).*

In the regulatory region of the thioredoxin gene, there is a putative cyclic AMP responsive element (CRE). NGF induces thioredoxin expression at the protein and mRNA levels in PC12 cells. The NGF responsive region was positioned from –263 to –217 in the thioredoxin gene as determined by a luciferase assay *(79).* This region contained a CGTCA sequence that bears resemblance to the consensus CRE *(80).*

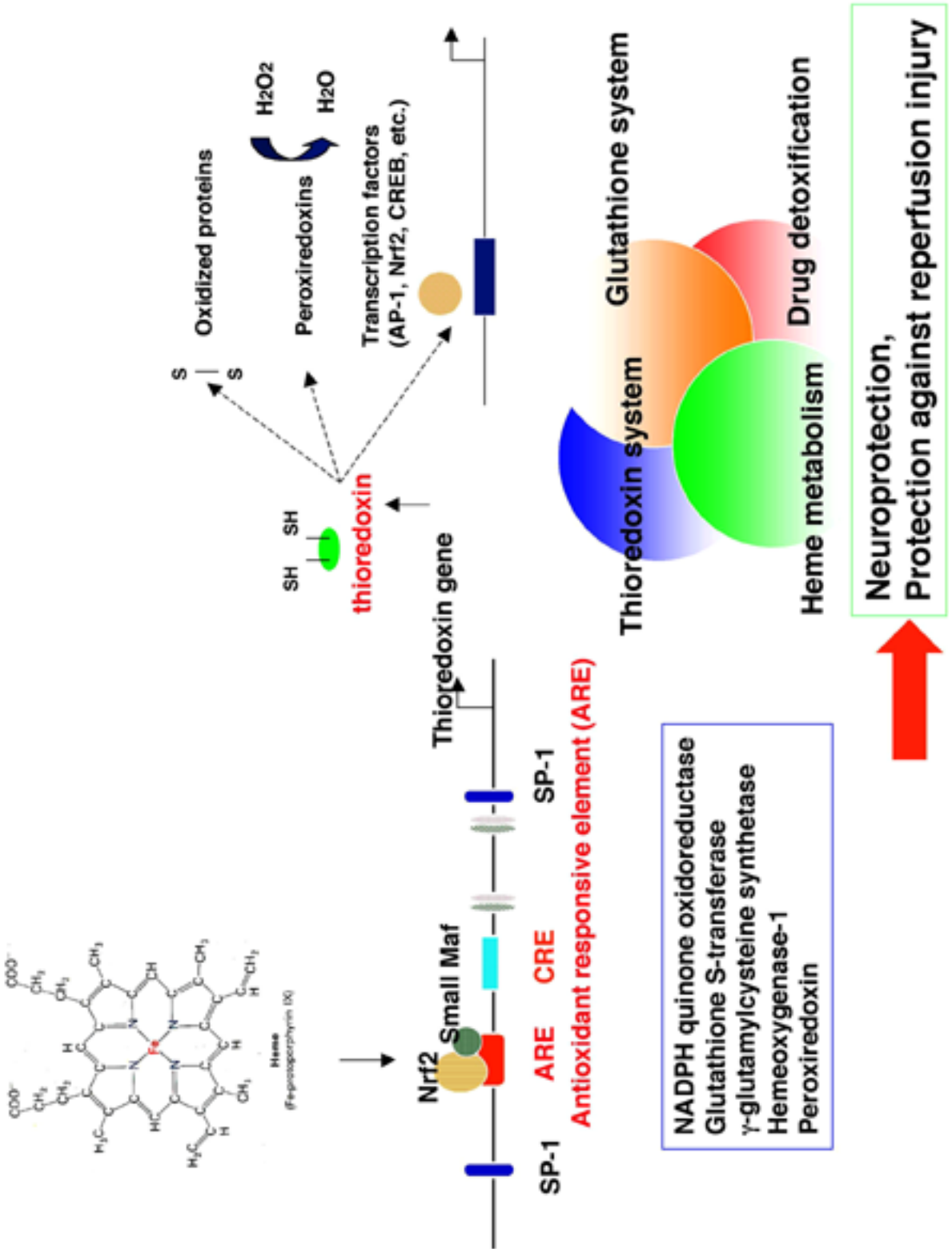
Neuroprotection,
Protection against reperfusion injury
Glutathione system
Thioredoxin system
Drug detoxification
Heme metabolism
NADPH quinone oxidoreductase
Glutathione S-transferase
γ-glutamylcysteine synthetase
Hemeoxygenase-1
Peroxiredoxin
Antioxidant responsive element (ARE)
SP-1
ARE
CRE
SP-1
Nrf2
Small Maf
Thioredoxin gene
thioredoxin
SH
SH
S
S
Oxidized proteins
Peroxiredoxins
H2O2
H2O
Transcription factors
(AP-1, Nrf2, CREB, etc.)

Fig. 2. Heme is implicated in the pathogenesis of reperfusion injury. In the erythroleukemic cell line K562, hemin induced thioredoxin gene expression through the antioxidant responsive element (ARE), regulated by the transcription factor Nrf2. The genes for hemeoxygenase-1 and redox-regulating enzymes such as γ-glutamylcysteine synthetase also contain the ARE and are regulated by Nrf2. Heme metabolism, the thioredoxin system, the glutathione system, and drug detoxification system seem to have an interconnecting relationship with co-coordinated roles against a subset of oxidative stress. Activation through the ARE of the thioredoxin gene may constitute a positive feedback loop. The reduction of cysteine residues of various nuclear factors by redox factor-1 (Ref-1), the glutathione (GSH) system, and the thioredoxin (TRX) system favors interaction between transcription factors and DNA or between transcription factors and coactivators.

Insertion of a mutation in the sequence abrogated NGF-induced thioredoxin gene expression. An electrophoretic mobility shift assay showed that CREB binds to the sequence. In addition, an ERK inhibitor PD98059 suppressed NGF-induced thioredoxin expression. NGF-induced CRE activation is mediated by extracellular signaling regulated protein kinase (ERK) *(81).* These results demonstrate that the thioredoxin gene is induced by NGF through the ERK and CRE-CREB cascade *(79).* NGF also increased activity of γ-glutamylcysteine synthetase (GCS), the rate-limiting enzyme for glutathione synthesis *(82),* indicating a link between the thioredoxin system and the glutathione system in the regulation of neuronal function by NGF.

The immediate-early genes (IEGs), such as *c-fos,* have been believed to be required for the actions of NGF. In the upstream regulatory region of the *c-fos* gene, CRE is critical for the regulation of *c-fos* transcription in response to a variety of extracellular stimuli that induce neural differentiation *(16,83).* Overexpression of the dominant negative mutant type of thioredoxin blocks NGF-induced activation of a reporter gene containing CRE, but not of that containing SRE. These results indicate that thioredoxin is necessary for NGF signaling through CRE leading to *c-fos* expression *(79).* AP-1 and Ref-1 have been reported to be involved in differentiation *(84,85).* Thioredoxin and Ref-1 regulate the interaction between transcription factors and coactivator *(50)* and the activation of CREB is regulated by thioredoxin *(86).* Therefore, thioredoxin may augment the interaction of CREB with DNA or coactivators to facilitate NGF signaling. The mechanism of involvement of thioredoxin in the NGF signaling pathway should be further elucidated.

NGF has been shown to promote axonal regeneration *(87).* Neural survival and differentiation are influenced by cellular redox conditions *(88).* Endoh et al. reported neurotrophic activity of thioredoxin for cholinergic neurons *(89).* 2-Mercaptoethanol can support the viability and differentiation of fetal mouse brain neurons *(90).* These reports suggest the importance of the redox environment in the regulation of neural survival and differentiation. Overexpression of a dominant negative type of thioredoxin expression vector or an antisense thioredoxin expression vector resulted in suppression of NGF-induced neurite outgrowth in PC12 cells *(79).* The results suggest that thioredoxin is a neurotrophic cofactor that augments the effect of NGF on neuronal differentiation and regeneration.

NGF also acts as a neuronal survival factor *(91).* NGF has been shown to prevent the death of axotomized septal neurons *(92).* NGF withdrawal causes apoptosis in PC12 cells, which is mediated by p38 MAPK and ASK1 *(93–95).* Thioredoxin has been reported to act as an endogenous inhibitor of ASK1 and p38 MAPK *(35,96).* These results indicate that maintenance of the thioredoxin level by NGF plays a role in preventing neuronal death (Fig. 3). A recent report showed that 4-hydroxynoneal contributes to NGF withdrawal-induced neuronal apoptosis *(97).* Since 4-hydroxynoneal causes

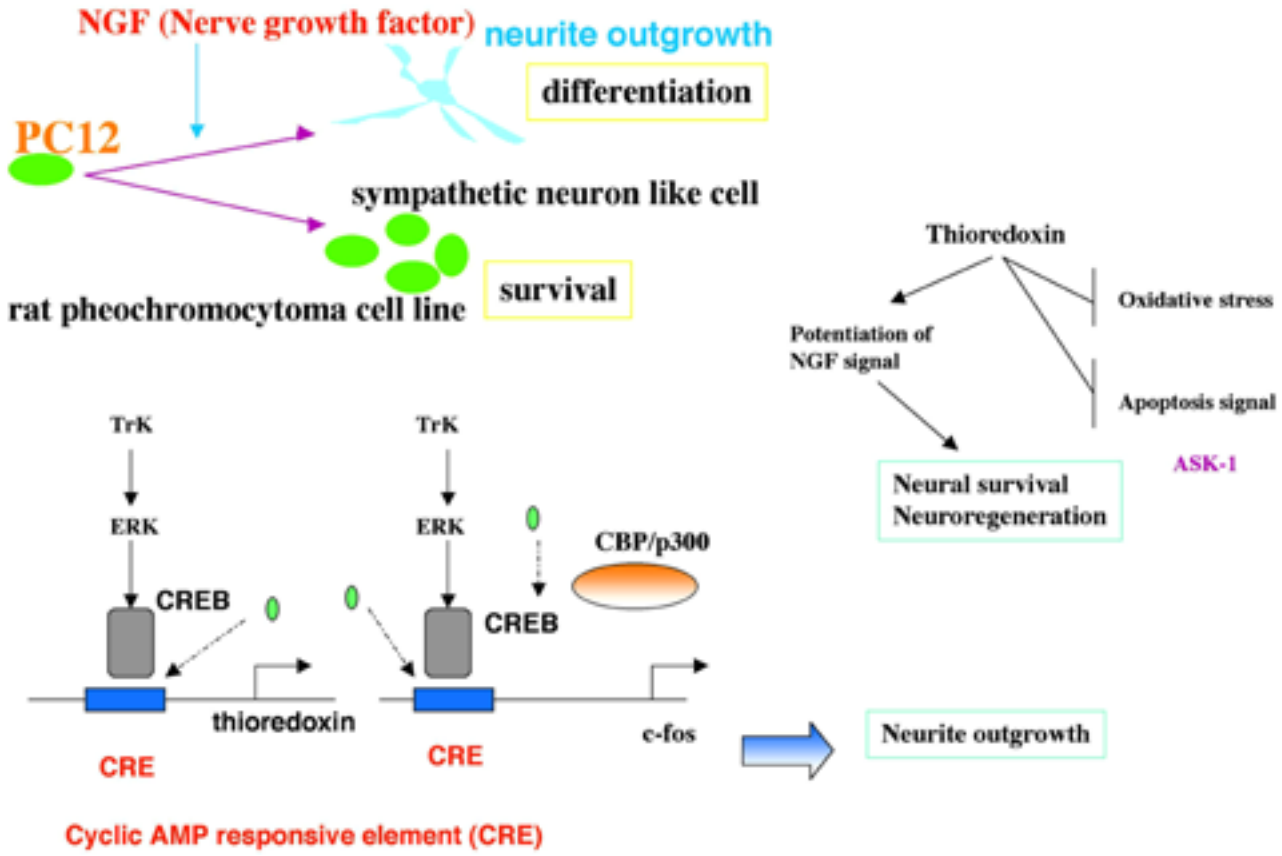

Fig. 3. NGF has profound effects on neurons, including promotion of survival and differentiation via multiple signaling pathways. As for the NGF-induced neurite outgrowth, the signal is initiated by the binding of NGF to TrkA and transduced by activation of the ras and MAPK cascade. NGF treatment in PC12 cells leads to the activation of immediate-early genes such as *c-fos,* through several elements including CRE. NGF activates the transcription of *c-fos* via phosphorylation of CREB. The thioredoxin gene is induced by NGF through the ERK and CRE-CREB cascade. Thioredoxin is necessary for NGF signaling through CRE leading to *c-fos* expression. Thioredoxin may augment the interaction of CREB with DNA or coactivators to facilitate NGF signaling. NGF acts also as a neuronal survival factor. NGF withdrawal causes ASK1-mediated apoptosis in PC12 cells. Maintenance of the thioredoxin level by NGF may play a role in preventing neuronal death. Thioredoxin has a cytoprotective role by enhancing the action of NGF, the regulation of antiapoptotic signaling, or through its antioxidative stress activity.

protein adducts to form, this work indicated the coupling between intracytoplasmic oxidative stress and NGF withdrawal-induced neuronal apoptosis.

It should again be noted that thioredoxin exerts a cytoprotective effect against various oxidative stresses *(69).* Thioredoxin has radical-scavenging activity *(98)* and couples with peroxiredoxins to scavenge hydrogen peroxide. Thioredoxin can protect cells from tumor necrosis factor (TNF) or anti-Fas antibody *(99),* hydrogen peroxide, activated neutrophils *(100),* and ischemic reperfusion injury *(67,68,101).* In the nervous system, various reports have showed a positive role for members of the thioredoxin system. Thioredoxin administra-

tion promoted the survival of glial cells from embryonic mouse cortex and striatum *(102).* Expression of mouse thioredoxin-dependent peroxidase (peroxiredoxin) in PC12 cells prolonged their survival in the absence of NGF and serum *(103).* Overexpression of thioredoxin in transgenic mice attenuates focal cerebral ischemic injury *(104).* Seizure and excitotoxic hippocampal injury induced by kainic acid was significantly reduced in thioredoxin transgenic mice *(105).* Thioredoxin also has a cytoprotective effect on retinal photooxidative damage *(77,106).* Decreased expression of thioredoxin in Alzheimer's disease brain has been reported *(107).* 1-methyl-4-phenylpyridinium ion (MPP+), an active metabolite of 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP), stimulates the production of the superoxide radical in vitro and causes dopaminergic denervation and Parkinsonism in humans. Overexpression of thioredoxin, administration of thioredoxin, or an inducer of thioredoxin attenuates MPP+-induced neurotoxicity in PC12 cells *(108).*

Summary and Future Perspectives

Hosts have multiple defense mechanisms against "neuronal stress" such as apoptosis, cell cycle control, immune responses, and the induction of redox regulating enzymes. Dysregulation of the defense mechanisms leads to untoward effects, including neurodegeneration. Oxidative stress, to a certain extent, is associated with various kinds of noxious stimuli causing "stress" in the nervous system. Although many reports have implicated oxidative stress in various diseases in the nervous system, the precise molecular mechanisms of the pathophysiology and protection remain obscure. Elucidation of the protective mechanism would lead to a better understanding of the neuronal diseases and provide a molecular basis for the development of new approaches to treating neuronal diseases. The role of thioredoxin in the nervous system has been reviewed in this article, focusing on the mechanism of gene regulation and role in NGF signaling. Thioredoxin plays a part in the nervous system not only through its antioxidant action but also through enhancement of the effect of NGF and signal transduction pathways. We believe that a knowledge of the thioredoxin system will be helpful in understanding the pathogenesis of neuronal diseases and feel that attempts to develop thioredoxin inducers are beneficial for neuroregeneration and protection against oxidative stress-associated neural diseases.

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