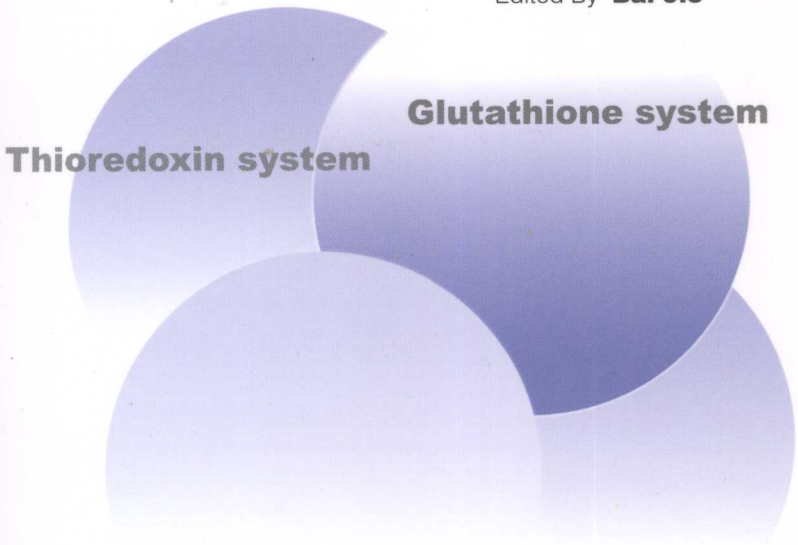
Relation between Thioredoxin and Cell Signaling and Clinical Application

Edited By **Bai Jie**



Yunnan Publishing Group Corporation
Yunnan Science and Technology Press

昆明理工大学学科建设资金资助项目

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Yunnan Publishing Group Corporation
Yunnan Science and Technology Press
· Kunming ·

图书在版编目（CIP）数据

硫氧还蛋白以信号和临床应用的关系 = Relation between Thioredoxin and Cell Signaling and Clinical Application：英文/白洁编著．—昆明：云南科技出版社，2007.3
ISBN 978-7-5416-2561-9

Ⅰ．硫… Ⅱ．白… Ⅲ．硫氧还蛋白-临床应用-英文
Ⅳ．Q51 R4

中国版本图书馆 CIP 数据核字（2007）第 040279 号

云南出版集团公司
云南科技出版社出版发行
（昆明市环城西路 609 号云南新闻出版大楼 邮政编码：650034）
云南省地质矿产勘查开发局印刷厂印刷 全国新华书店经销
开本：850mm×1168mm 1/32 印张：4 字数：110 千字
2007 年 3 月第 1 版 2007 年 3 月第 1 次印刷
印数：1～1000 定价：15.00 元

Preface

Thioredoxin is a small protein which plays important roles in the redox system in cell. After it is found, it has been studied by many researchers. Its structure and its functions have been well known.

Human thioredoxin was first cloned by Professor Junji Yodoi. I have chance to study at Professor Junji Yodoi's lab. I have got involved in their research. Because I am interested in neuroscience field. I concentrated my study on the role of thioredoxin in the NGF signal pathway and the role of thioredoxin in preventing and therapy the Parkinson's disease.

It is my pleasure to organize my work here to give the people who do the same work as reference. Also you are welcome to give me the advice and suggestion.

At the same time, let me express my appreciation to my supervisors, my colleges, my family, and all the friends who supported me.

Content

Chapter 1 Introduction ..... (1)
Chapter 2 Critical Roles of Thioredoxin in Nerve Growth Factor-Mediated Signal Transduction and Neurite Outgrowth in PC12 Cells ..... (5)
1 Cell Lines and Culture ..... (7)
2 Plasmids ..... (8)
3 Western Blot Analysis ..... (10)
4 Northern Blot Analysis ..... (10)
5 Electrophoretic Mobility Shift Assay ..... (11)
6 Transfection and Luciferase Assay ..... (11)
7 Immunofluorescence Cell Staining ..... (12)
8 Identification of the NGF-responsive Region in the TRX Promoter ..... (14)
9 TRX Expression was Suppressed by PD98059 ..... (14)
10 NGF-induced Nuclear Translocation of TRX was Blocked by PD98059 ..... (16)
11 Overexpression of Mutant TRX (C32S/C35S) or TRX Antisense Vector Blocked NGF-induced Neurite Outgrowth of PC12 cells ..... (17)
12 Overexpression of Mutant TRX (C32S/C35S) Blocked CRE-mediated C-*fos* Induction by NGF ..... (20)

Chapter 3 Thioredoxin Suppresses 1-methyl-4-phenylpyridinium-induced Neurotoxicity in Rat PC12 Cells ..... (33)
1 Abstract ..... (33)
2 TRX and Parkinson disease ..... (34)
3 Acknowledgements ..... (41)
Chapter 4 Degradation of Cyclin D1 in 1-methyl-4-phenylpyridinium Ion (MPP^+) -induced Cell Cycle Arrest ..... (45)
1 Reagents and Cells ..... (47)
2 Establishment of HeLa ρ^0 Cells ..... (47)
3 Confirmation of Mitochondrial DNA (mtDNA) ..... (48)
4 Western Blot Analysis ..... (49)
5 Cell Cycle Analysis by Flow Cytometry ..... (49)
6 Northern Blot Analysis ..... (50)
7 MPP^+ Suppresses the Cell Growth of P53 – deficient MG63 Cells ..... (50)
8 MPP^+ Induces G_1 Arrest in MG63 Cells ..... (50)
9 MPP^+ Down-regulates Cyclin D1 in a Post-transcriptional Mechanism ..... (53)
10 MPP^+ Induces Cyclin D1 Down-regulation Even in Cells Lacking mtDNA ..... (53)
11 PI3K-Akt-GSK-3β Pathway Regulates MPP^+-mediated Cyclin D1 Proteolysis ..... (55)

Chapter 5 Thioredoxin-1 Prevents Mitochondria- and Endoplasmic Reticulum-mediated Neurotoxicity of 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydroryridine ..... (62)
1 Abstract ..... (62)
2 Introduction ..... (63)
3 Materials and Methods ..... (65)
4 Results ..... (68)
5 Discussion ..... (75)
Chapter 6 Thioredoxin as a Neurotrophic Cofactor and an Important Regulator of Neuroprotection ..... (82)
1 NGF Signal ..... (83)
2 The Thioredoxin System ..... (84)
3 Regulatory Mechanism of Thioredoxin Gene Expression ..... (89)
4 Thioredoxin in the Nervous System ..... (93)
5 Summary and Future Perspectives ..... (96)
Chapter 7 Thioredoxin Clinical Application ..... (115)

Chapter 1

Introduction

Oxidative stress evokes various cellular events, including activation of transcription factors, apoptosis and cell cycle arrest. The TRX system in concert with the GSH and glutaredoxin system constitutes a cellular reducing environment. TRX is a small protein with two redox inactive center (Cys-Gly-Pro-Cys) and operates together with NADPH and TRX reductase as an efficient reducing system of exposed protein disulfides. TRX is present in many different prokaryotes and eukaryotes and appears to be present in all living cells.

TRX is induced by various stresses, including virus infection, mitogens, X-ray and UV irradiation[1], hydroperoxide, and ischemic reperfusion[2]. TRX translocates from cytoplasm into the nucleus on oxidative stress. In the 5'-upstream region of the TRX gene, there exist binding sites such as AP-1, CRE, ARE and SP-1.

Transcription factors are important sensing and signaling components of oxidative signaling. Redox factor (Ref-1) activity is in turn modulated by various redox-active compounds, including TRX. The DNA-binding activity of activator protein 1 (AP-1) is modified by a DNA repair enzyme, redox factor 1 (Ref-1), which is identical to a DNA repair enzyme, AP endonuclease. the re-

quirement of cysteine residues in the TRX catalytic center for the potentiation of AP-1 activity[3]. TRX plays dual and opposing roles in the regulation of NF-kappaB. In the cytoplasm, it interferes with the signals to IkappaB kinases and blocks the degradation of IkappaB. In the nucleus, however, TRX enhances NF-kappaB transcriptional activities by enhancing its ability to bind DNA[4]. TRX augmented the DNA binding activity of p53 and also further potentiated Ref-1-enhanced p53 activity. TRX enhanced p53-dependent expression of p21 and further intensified Ref-1-mediated p53 activation[5].

There are several TRX family reported. They have different roles in different signal pathway. Thioredoxin-2 (Trx-2) is a mitochondria-specific member of the thioredoxin superfamily. Mitochondria have a crucial role in the signal transduction for apoptosis. Trx-2 and cytochrome c are co-immunoprecipitated in an *in vitro* assay. These results suggest that mitochondrial Trx-2 is essential for cell viability, playing a crucial role in the scavenging ROS in mitochondria and regulating the mitochondrial apoptosis signaling pathway[6]. The thioredoxin related transmembrane protein (TMX) protein possesses an N-terminal signal peptide followed by one thioredoxin (Trx) -like domain with a unique active site sequence, Cys-Pro-Ala-Cys, and a potential transmembrane domain. The TMX transcript is widely expressed in normal human tissues, TMX is predominantly localized in the endoplasmic reticulum (ER). When TMX was expressed in HEK293 cells, it significantly suppressed the apoptosis induced by brefeldin A, an inhibitor of ER-Golgi transport[7].

TRX binding protein was reported. The association of TRX with thioredoxin-binding protein-2/vitamin D (3) up-regulated protein1 (TBP-2/VDUP1) was observed *in vitro* and *in vivo*. TBP-2/VDUP1 bound to reduced TRX but not to oxidized TRX nor to mutant TRX, in which two redox active cysteine residues are substituted by serine. These results suggested that TBP-2/VDUP1 serves as a negative regulator of the biological function and expression of TRX. The TRX-TBP-2/VDUP1 interaction may be an important redox regulatory mechanism in cellular processes, including differentiation of myeloid and macrophage lineages. [8] Trx associated with the N-terminal portion of ASK1 *in vitro* and *in vivo*. The evidence that Trx is a negative regulator of ASK1 suggests possible mechanisms for redox regulation of the apoptosis signal transduction pathway as well as the effects of antioxidants against cytokine- and stress-induced apoptosis. [9]

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Chapter 2

Critical Roles of Thioredoxin in Nerve Growth Factor-Mediated Signal Transduction and Neurite Outgrowth in PC12 Cells

Thioredoxin (TRX) has a role in a variety of biological processes, including cytoprotection and the activation of transcription factors. Nerve growth factor (NGF) is a major survival factor of sympathetic neurons and promotes neurite outgrowth in rat pheochromocytoma PC12 cells. In this study, we showed that NGF induces TRX expression at protein and mRNA levels. NGF activated the TRX gene through a regulatory region positioned from −263 to −217 bp, containing the cAMP-responsive element (CRE). Insertion of a mutation in the CRE in this region abolished the response to NGF. NGF induced binding of CRE-binding protein to the CRE of the TRX promoter in an electrophoretic mobility shift assay. NGF also induced nuclear translocation of TRX. 2'-Amino-3'-methoxyflavone, an inhibitor of mitogen-activated protein kinase kinase, which is a known inhibitor of NGF-dependent differentiation in PC12 cells, suppressed the NGF-dependent expression and nuclear translocation of TRX. Overexpression of mutant TRX (32S/35S) or TRX antisense vector blocked the neurite outgrowth of PC12 cells by NGF.

Overexpression of mutant TRX (C32S/C35S) suppressed the NGF-dependent activation of the CRE-mediated c-*fos* reporter gene. These results suggest that TRX plays a critical regulatory role in NGF-mediated signal transduction and outgrowth in PC12 cells.

Neural survival and differentiation are influenced by the cellular redox condition[1]. Thioredoxin (TRX) is a small 12 kDa multifunctional protein having a redox-active disulfide/dithiol within its active site sequence, -Cys-Gly-Pro-Cys-, and operates together with NADPH and thioredoxin reductase as a protein disulfide-reducing system[2]. Several reports have shown that TRX-dependent redox regulation is closely involved in the signal transduction mediated by activator protein-1 (AP-1), nuclear factor- κB, p53, apoptosis-signaling kinase 1 (ASK1), and p38 mitogen-activated protein kinase[3~6]. TRX is widely distributed and induced by various stresses[7~8]. TRX expression is also elevated by hemin, an inducer of differentiation in K562 erythroleukemia cells[9], or cAMP analogues in retinal pigment epithelial cells[10]. In the regulatory region of the TRX gene, there are several promoter-specific transcription factor 1 binding motifs, an antioxidant-responsive element, and a putative cAMP-responsive element (CRE). In neuronal tissues, TRX is induced in astroglia after ischemia[11] and in motor neurons after nerve injury[12]. TRX is known to have a cytoprotective effect against oxidative stress[13] and neuroprotective activity[14]. Furthermore, overexpression of TRX in transgenic mice attenuates focal ischemic brain damage[15]. TRX was also reported as a neurotrophic factor for central cholinergic neurons and has neurotrophic activity[16], although the molecular basis of this effect has not been eluci-

dated.

Nerve growth factor (NGF) and the other members of the neurotrophin family, such as brain-derived neurotrophic factor, have profound effects on neurons, including the promotion of survival and differentiation[17]. NGF has been reported as a potential therapeutic agent in neurodegenerative disorders linked to aging, such as Alzheimer's disease[18]. The current understanding of these mechanisms depends mostly on studies of NGF action on the pheochromocytoma cell line PC12[19]. On exposure to NGF, PC12 cells differentiate into sympathetic neuron-like cells. The signal is initiated by the binding of NGF to its high-affinity receptor TrkA on the plasma membrane [20] and transduced by activation of *ras* and the MAPK cascade[21]. 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 NGF action[22]. NGF activates the c-*fos* gene through several elements, including the serum response element[23] and CRE[24~25].

The aim of the present study is to investigate the possible roles of TRX as a neurotrophic cofactor in NGF-dependent outgrowth of PC12 cells and the NGF-mediated signal transduction pathway. We report here that TRX plays an important role in the NGF-mediated signal transduction and neurite outgrowth in PC12 cells.

1 Cell Lines and Culture

NGF, polyethylenimine (PEI), and 2'-amino-3'-methoxyflavone (PD98059) were purchased from Sigma (St. Louis, MO). Ho-

echst 33324 was purchased from Molecular Probes (Eugene, OR). Cells of the rat pheochromocytoma tumor cell line PC12 were maintained in RPMI 1640 medium (Invitrogen, Grand Island, NY) with 10% heat-inactivated horse serum and 5% heat-inactivated fetal calf serum (FCS) supplemented with antibiotics (100 IU/mL penicillin and 100 μg/mL streptomycin) at 37°C in a humid atmosphere containing 5% CO_2.

2 Plasmids

The pTrx-chloramphenicol acetyltransferase (CAT) plasmids were constructed as described previously[26]. The *Hind*III-*Bam*HI inserts from the pTrxCAT vectors were subcloned into pBluescript II KS (+) (pTRXblue vectors). The pTRX (-1148) -luciferase (Luc), pTRX (-1062) -Luc, pTRX (-352) -Luc, and pTRX (-263) -Luc vectors were constructed by ligating the *Kpn*I-*Bam*HI fragments of the pTRXblue vectors into the *Kpn*I-*Bgl*II sites of the pGL3 basic vector (Promega, Madison, WI). The *Apa*I-*Pvu*II insert of the pTRX (-263) -Luc vector was excised, filled in, and self-ligated to produce the pTRX (-217) -Luc vector. The pGL3-c-fos (-40, +42) and pGL3-c-fos (-99, +42) luc vectors were constructed by subcloning an *Mlu*I-*Hind*III fragment of the Fos-40 luc[27] and the pFDE-luc vectors into the *Mlu*I-*Hind*III site of the pGL3 basic vector (Promega). SRE3-luc was constructed as described previously[27]. pFDE-luc was constructed by subcloning a *Bam*HI-*Hind*III fragment of FDE-CAT[28] into the *Bgl*II-*Hind*III site of the pGL2 basic vector (Promega). PTrxCREwt-Luc

and pTrxCREmt-Luc vectors were constructed by inserting CRE wild-type (wt) or CRE mutant (mt) oligonucleotides into the *Xho*I-*Puv*II site of the pTRX (-1148)-Luc, respectively. The pCDSRα-TRX, pCDSR-TRX (C32S/C35S), and pcDNA3TRX (32S/35S) vectors were constructed as described previously[3],[29]. The *Bam*HI inserts from pCDSRδ-TRX and pCDSRδ-TRXm[30],[3] were subcloned into the *Bam*HI site of pBluescript II KS [pBS-wtTRX, pBS-antisense, and pBS-double mutant (dm) TRX]. pBI-enhanced green fluorescent protein (EGFP)-wtTRX, pBI-EGFP-antisenseTRX, and pBI-EGFP-dmTRX (32S/35S) were constructed by ligating the *Eco*RV-*Xba*I fragments of the pBS-wtTRX, pBS-antisense, and pBS-dmTRX vectors into the *Pvu*II-*Nhe*I sites of the pBI-EGFP vector (Clontech, Palo Alto, CA), respectively. All the constructs were controlled by direct nucleotide sequencing using a Thermo Sequenase II dye terminator cycle sequencing kit (Amersham Biosciences, Arlington Heights, IL). The pRL-TK vector was purchased from Promega. pcDNA3 was purchased from Invitrogen. The oligonucleotides used for construction of vectors and the electrophoretic mobility shift assay (EMSA) were as follows: CREwt, forward, 5'-CGCCTCCCACCGTCACGGGCAGTGC-3'; and reverse, 5'-TCGAGCACTGCGCGTGACGGTGGGAGGCGGTAC-3'; and CREmt, forward, 5'-CGCCTCCCACTATCACGGGCAGTGC-3'; and reverse, 5'-TCGAGCACTGCGCGTGATAGTGGGAGGCGGTAC-3'.

3 Western Blot Analysis

Cells were collected and washed twice with ice-cold PBS, and then lysed with a solubilizing solution (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 0.1 mM PMSF, 8 μg/mL aprotinin, and 2 μg/mL leupeptin) on ice for 30 min. The extracts were cleared by centrifugation. Cell lysates were kept at 95°C for 5 min and then separated by 15% SDS-PAGE. The separated proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was treated with 10% (w/v) skim milk in PBS containing 0.05% Tween 20 overnight, and incubated with anti-mouse TRX rabbit polyclonal antibody[31] (dilution, 1∶1000; Redox Bioscience, Inc.) for 1 h, followed by peroxidase-conjugated anti-rabbit IgG (dilution, 1∶5000; Amersham Biosciences) for 1 h. The epitope was visualized with an ECL Western blot detection kit (Amersham Biosciences). We reported previously that this anti-mouse antibody cross-reacts with rat TRX[32~33].

4 Northern Blot Analysis

Total RNA was extracted using TRIzol reagent according to the manufacturer's instructions[34]. Twenty micrograms of total RNA were electrophoresed and transferred to maximum-strength Nytran nylon (Schleicher & Schuell, Keene, NH) with a Turbo-Blotter system (Schleicher & Schuell). The filter was hybridized with a mouse