Korean J Physiol Pharmacol
Vol 7: 33-37, February, 2003

Overexpressed Mitochondrial Thioredoxin Protects PC12 Cells from Hydrogen Peroxide and Serum-deprivation

Yun-Song Lee and Seung-A Yu

Division of Pharmacology, Department of Molecular & Cellular Biology, Sungkyunkwan University School of Medicine, Suwon 440-746, Korea

Oxidative damage to mitochondria is a critical mechanism in necrotic or apoptotic cell death induced by many kinds of toxic chemicals. Thioredoxin (Trx) family proteins are known to play protective roles in organisms under oxidative stress through redox reaction by using reducing equivalents of cysteines at a conserved active site, Cys-X-X-Cys. Whereas biological and physiological properties of Trx1 are well characterized, significance of mitochondrial thioredoxin (Trx2) is not well known. Therefore, we addressed physiological role of Trx2 in PC12 cells under oxidative stress. In PC12 cells, transiently overexpressed Trx2 significantly reduced cell death induced by hydrogen peroxide, whereas mutant Trx2, having serine residues instead of two cysteine residues at the active site did not. In addition, stably expressed Trx2 protected PC12 cells from serum deprivation. These results suggest that Trx2 may play defensive roles in PC12 cells by reducing oxidative stress to mitochondria.

Key Words: Mitochondrial thioredoxin (Trx2), Oxidative stress, Serum deprivation, PC12 cells, Cell death

INTRODUCTION

Oxidative stress caused by reactive oxygen species (ROS) has been implicated in the pathogenesis of several kinds of neurodegenerative diseases, such as Alzheimer's disease and Parkison's disease (Simonian et al, 1996). Mitochondria are considered to be important endogenous sources of ROS and to play pivotal roles in cell death. Impaired mitochondrial energy production by ROS induces apoptosis, and perturbed mitochondrial redox status is also known to be related to apoptosis through mitochondria-mediated pathway (Green & Reed, 1998; Nicholls & Budd, 2000).

Thioredoxin (Trx) is a family of small proteins that contain a conserved redox active center, Cys-Pro-Gly-Cys (Powis, 2001). The reversible redox reaction in the active site enables Trx to transfer electron to protein disulfide substrates in the presence of thioredoxin reductase (TrxR) and NADPH. Although Trx was originally identified as an electron donor of ribonucleotide reductase, a wide range of other biological functions have been characterized, including ROS scavenging (Mitsui et al, 1992), growth stimulation (Wakasugi et al, 1990), regulation of activities of transcriptional factors (Hirota et al, 1999) and inhibition of apoptosis (Saitoh et al, 1998).

Thioredoxin 2 (Trx2) is a mitochondrial Trx that has the same functionally active site, Cys-Pro-Gly-Cys (Spyrou et al, 1997). It can reduce insulin *in vitro*, suggesting that Trx2 shares biological properties with Trx1, a cytosolic homologue. The cloning of mitochondrial TrxR (Miranda-Vizuete et al, 1999; Lee et al, 1999) indicates that a complete system of thioredoxin exists in mitochondria. This mitochondrial system may play potentially important physiological roles, particularly in protection against oxidant stress. Even though pleiotrophic functions of Trx1 have been revealed, physiological roles of Trx2 have been poorly studied. In this study, to better understand roles of Trx2, protective effect of Trx2 against oxidant stress was evaluated in Trx2-overexpressing PC12 cells.

Corresponding to: Yun-Song Lee, Division of Pharmacology, Department of Molecular & Cellular Biology, Sungkyunkwan University School of Medicine, Suwon 440-746, Korea. (Tel) +82-31-299-6190, (Fax) +82-31-299-6209, (E-mail) yslee@yurim.skku.ac.kr

METHODS

Cell culture

PC12 cells were cultured in RPMI containing 10% horse serum, 5% fetal calf serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin in a humidified atmosphere of 5% CO_2 and 95% room air. For serum deprivation, the cells in dishes were washed three times with serum-free RPMI. All reagents used in cell culture were from Life Technologies.

Construction of Trx2 vector

To make Trx2 constructs for transfection, total RNAs were isolated from PC12 cells and cDNAs were synthesized with a reverse transcription kit (Roche). Trx2 gene from the start

ABBREVIATIONS: Trx, Thioredoxin; TrxR, Thioredoxin reductase; ROS, Reactive oxygen species.

to the stop codon was amplified by PCR with the forward primer (Trx2F; 5' CG***A AGC TT***T CTG CAT CCC TCT GCT CAC ACT 3', ***HindIII site***) and the reverse primer (Trx2R; 5' GC***T CTA GA***C TCT TCC CTG CTT GTC AGC CAA 3', ***XbaI site***) as described previously with slight modification (Spyrou et al, 1997). The PCR product was cloned to TA vector (Invitrogen) and then to pcDNA3.0 (pcDNA-Trx2). To construct the mutant Trx2 (mTrx2) plasmid coding two serine residues instead of two cysteine residues at the active site (-***Ser***-Pro-Gly-***Ser***-), site-directed mutagenesis was performed. Authenticity of the sequence was confirmed by automatic sequencing (Applied Biosystems). The mutant PCR product was cloned to pcDNA3.0 (pcDNA-mTrx2). The amplified plasmids were purified using an endotoxin-free plasmid purification kit (Stratagene) for transfection.

Transfection of vectors, establishment of stable cells and determination of cell viability

The plasmids were transfected to PC12 cells with Lipofectamine 2000 reagents, using the manufacturer's instructions (Life Technologies). In brief, 0.8 mg each of pcDNA3.0, pcDNA-Trx2 or pcDNA-mTrx2 was transfected to 2.5×10^5 cells in the wells of a 24 well plate together with 3.2 μg of pEGFP-N3 (Clontech) to verify transfection. Noxious stimuli were then applied 1 day after transfection. Viability of transfected cells to show green fluorescence (pEGFP-N3) was determined by trypan blue dye exclusion.

To obtain stable cells, transfected cells were screened with 400 μg/ml of G418 (Life Technologies) for 3 weeks and collected for further studies. Cell viability in 96 well plates (1×10^4 cells/well) was determined with MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide) reduction assay by the manufacturer's instruction (Promega; Mosmann, 1983).

Protein expression and purification

To confirm the absence of the H_2O_2-scavenging activity of the mutant Trx2 protein, the coding region beyond the putative mitochondrial targeting sequence up to the stop codon was amplified by PCR using either pcDNA-Trx2 or pcDNA-mTrx2 as a template with two primers (the forward primer, 5' ACC ACC AGA GTC ***CAT ATG*** ACA ACC TTT AAC GTC 3', ***NdeI site***; and the reverse primer 5' CTG ***GTC GAC*** TCC CTG CTT GTC AGC CAA TTA GC 3', ***SalI site***). Each amplified PCR product was cloned to pET28a (Novagen). Expression of the 6 X His-tagged Trx2 protein was induced by 0.5 mM IPTG at 37°C for 5 hrs, and it was purified by nickel nitriloacetic acid affinity column chromatography by the manufacturer's instruction (Novagen). Traces of impurity were removed by Superdex 75 gel filtration column chromatography (10×300 mm, Pharmacia Biotech) and their absence was confirmed by 15% SDS-PAGE gel with Coomassie staining.

Production of polyclonal antibody

Polyclonal anti-rat Trx2 antibody was raised in rabbit as described previously (Spyrou et al, 1997). In brief, rabbits were immunized six times with purified recombinant Trx2, and antibody was purified by protein A-Sepharose (Amersham).

Assay for scavenging activity of Trx2

H_2O_2 scavenging activity of Trx2 and mutant Trx2 was determined by NADPH oxidation in the presence of TrxR1. Reactions were started by the addition of 1 mM H_2O_2 to 1 ml of a reaction mixture containing 20 μg of Trx2 or mutant Trx2, 1 U of bovine liver TrxR1 (American Diagnostica), 100 mM Tris-HCl (pH 7.5), 2 mM EDTA and 0.2 mM NADPH at 37°C. Oxidation of NADPH was monitored at 340 nm with UV-VIS spectrophotometer (Hewlett-Packard).

Statistical evaluations

The results represent the mean±standard error of 6 individual experiments in each group. Statistical analyses were performed using analysis of variance (ANOVA) followed by Dunnett's test or unpaired Student's t-test.

RESULTS

Effect of replacement of two cysteine residues in the active site

To validate the absence of reducing activity of mutant Trx2, the activities to scavenge H_2O_2 were determined using the recombinant proteins. Since the Trx1-TrxR1 system has been reported to scavenge H_2O_2 at the expense of NADPH (Mitsui et al, 1992) and Trx1 is known to transfer reducing equivalent from NADPH to Trx2 (Spyrou et al, 1997), the H_2O_2 scavenging activity of Trx2 was determined by NADPH oxidation. As shown in Fig. 1, recombinant wild-type Trx2 protein dramatically augmented the decrease of absorbance due to the oxidation of NADPH from -1.46×10^{-3} AU/min to -3.43×10^{-3} AU/min, when 1 mM H_2O_2 was added. In contrast, mutant Trx2 protein having serine residues instead of two cysteine residues at the active site did not induce NADPH oxidation, indicating that the two cysteine residues at the active site in Trx2 is critical for scavenging H_2O_2.

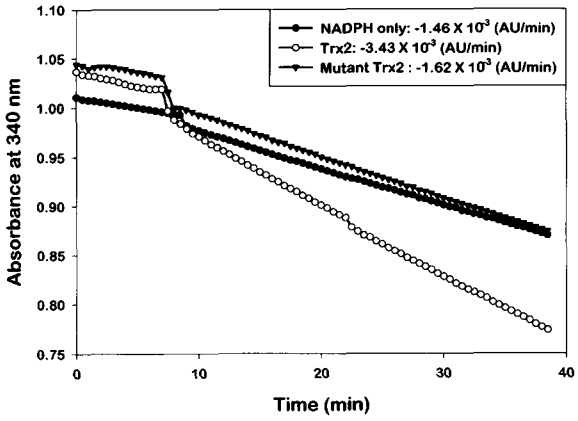

Fig. 1. Hydrogen peroxide scavenging by Trx2. NADPH oxidation due to decomposition of H_2O_2 was monitored at 340 nm in the absence or in the presence of recombinant Trx2 or mutant Trx2. The values in the inset indicate the net rates of absorbance decrease at 340 nm per minute after the addition of H_2O_2.

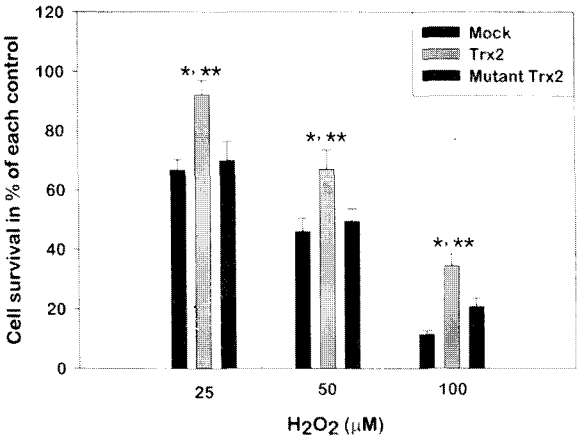

Fig. 2. Effect of Trx2 on cell viability in hydrogen peroxide-treated PC12 cells. One day after transfection with pcDNA3.0 (Mock), pcDNA-Trx2 (Trx2) and pcDNA-mTrx2 (Mutant Trx2), H_2O_2 was added to culture media. After 1 day, survival of PC12 cells was determined by inverted fluorescence microscopy. Number of the live cells to exclude trypan blue and show green fluorescence (GFP) was counted. Relative survival rates are shown by the means and standard errors. Asterisks indicate statistical significance ($P<0.05$) in 6 separate experiments in each group (*, Trx2 vs. mock; **, Trx2 vs. mutant Trx2).

Effect of Trx2 on survival of PC 12 cells challenged with H_2O_2

To determine protective effect of Trx2 against oxidative stress, PC12 cells were challenged with H_2O_2 after transient transfection with pcDNA3.0, pcDNA-Trx2 or pcDNA-mTrx2 together with pEGFP-N3 (Fig. 2). One day after the H_2O_2 treatment, the number of live cells that expressed GFP and excluded trypan blue was counted in 5 fields per well using an inverted fluorescence microscope ($\times 200$ magnification, Zeiss). After treated with 25, 50 and 100 μM of H_2O_2, the survival rate of PC12 cells harboring pCDNA3.0 (Mock) was reduced to 60%, 50% and 10%, respectively. In contrast, the survival after 25, 50 and 100 μM of H_2O_2 treatment was significantly improved in PC12 cells transfected with pcDNA-Trx2. However, little difference was observed among the mock, Trx2 and mutant Trx2 groups at higher concentrations (500 μM or 1 mM) of H_2O_2 (data not shown). As expected from the H_2O_2-scavenging data, mutant Trx2 had no protective effect upon the survival of the PC12 cells.

Expression of transfected Trx2

Expression of transfected trx2 gene was determined by Western blot of total lysate of stably transfected PC12 cells. Overexpressed Trx2 was observed at around 14 kDa in the immunoblot of whole cell lysates of pcDNA-Trx2 PC12 cells (50 μg proteins). However, there was no signal at around 18 kDa, which is the estimated molecular weight of untruncated Trx2 (Fig. 3a). This shows that the N-terminal peptide (58 amino acids) harboring a putative mitochondrial translocation signal was removed by a mitochondrial peptidase, as reported previously (Spyrou et al, 1997). Since stable cells transfected with pcDNA-mTrx2 were not obtained in this experiment, expression of mutant Trx2 could not be checked.

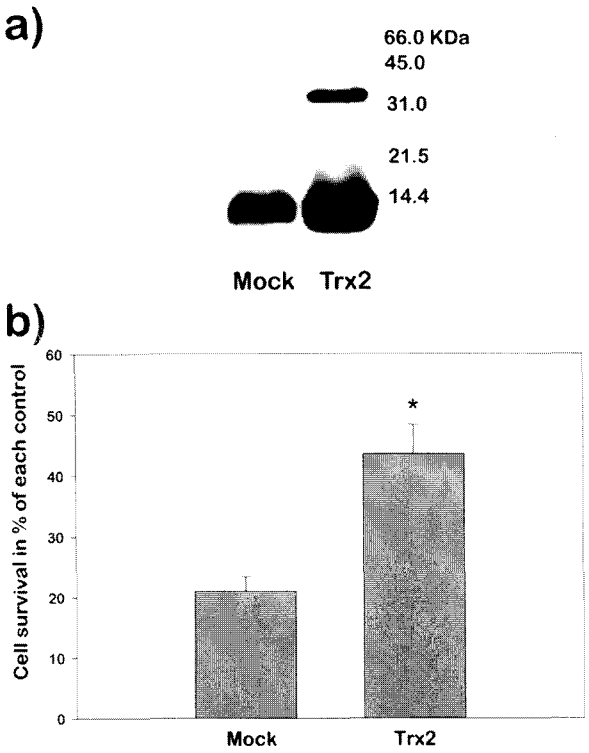

Fig. 3. Effect of Trx2 on the survival of serum-deprived PC12 cells. (a) Immunoblot of whole cell lysate (50 μg of proteins) of PC12 cells stably transfected with pcDNA3.0 or pcDNA-Trx2. (b) Stably transfected PC12 cells were deprived of serum for 1 day. Viablity of the cells in 96 well plates (1×10^4 cells/well) was determined by MTT reduction assay, as described in *Methods*. Asterisk indicates statistical significance ($P<0.05$) in 6 separate experiments in each group.

Protection of PC 12 cells by Trx2 from serum deprivation

To address whether Trx2 protects PC12 cells from other stresses, PC12 cells stably transfected with pcDNA3.0 or pcDNA-Trx2 were deprived of serum for 1 day. In mock-transfected cells, 20% of PC 12 cells survived 1 day after serum deprivation, however, survival rate significantly increased to 40% in Trx2-transfected PC 12 cells (Fig. 3b).

DISCUSSION

In this study, we demonstrated that overexpressed Trx2 protected PC12 cells from H_2O_2. Recombinant wild-type Trx2 scavenged hydrogen peroxide, whereas mutant Trx2 did not. Thus, it is highly likely that Trx2 protects PC12 cells from hydrogen peroxide using this scavenging activity, and mutant Trx2 which lacks in scavenging activity, could not. Similarly, it was reported that overexpressed human Trx2 inhibited apoptosis of osteosarcoma cells induced by t-butyl hydroperoxide (Chen et al, 2002). In the above study, mitochondrial translocation signal peptide in human

Trx2 was removed, and only the truncated Trx2 was detected in mitochondria. In this experiment, even though the location of Trx2 expression was not determined by immunocytochemistry, we observed only the truncated Trx2 in cell lysates by immunoblotting. Together with the above mentioned report of Chen et al. (2002), our present result suggests that the overexpressed Trx2 may be translocated to and truncated in mitochondria, where Trx2 plays its protective roles.

In addition to protection against H_2O_2, Trx2 protected PC12 cells from serum deprivation, which caused oxidative stress in cells by forming peroxynitrite by the elevated levels of nitric oxide (NO) and superoxide (Estevez et al, 1998). As Trx1 is shown to have a protective effect upon NO-mediated cell injury in THP1 human leukemic cells (Ferret et al, 2000) and Trx2 shares the same functional site (Spyrou et al, 1997), it is postulated that Trx2 may protect PC12 cells by scavenging reactive oxygen or nitrogen species formed from NO/superoxide during serum withdrawal conditions. This possibility was supported by a recent report that Trx2deficient cells underwent mitochondria-dependent apoptosis, showing an accumulation of intracellular ROS (Tanaka et al, 2002).

Another possible protective mechanism involves the contribution of Trx2 to the maintenance of the mitochondrial redox status against apoptotic stimuli, such as lower concentrations of hydrogen peroxide or serum withdrawal. The perturbation of mitochondrial redox status leading to collapse of mitochondrial potential is known to be closely related to mitochondria-mediated apoptotic pathway (Green & Reed, 1998; Nicholls & Budd, 2000). In the case of neuronal apoptosis induced by serum withdrawal, decreased mitochondrial potential and increased mitochondrial calcium concentrations were observed even without detection of ROS (Waida et al, 1998). In addition, cytochrome c, an important mediator of apoptosis, was released prior to the generation of ROS in excitotoxic neuronal death (Luetjens et al, 2000). Thus, the prevention of collapse of mitochondrial potential by maintaining redox potential will tend to protect the cells from apoptosis. In this context, Trx2 may contribute to maintenance of the mitochondrial redox potential under oxidative stress. This notion is supported by a recent study that overexpressed human Trx2 regulated mitochondrial potential through possible interaction with specific components of mitochondrial respiratory chain (Damdimopoulos et al, 2002).

Alternatively, Trx2 may affect functions of the key proteins involved in cell survival/death in the vicinity of its location in a similar manner that Trx1 inhibited apoptosis signal-regulating kinase 1 (Saitoh et al, 1998). Trx2 was reported to be located in the inner mitochondrial membrane (Rybnikova et al, 2000), where Bcl-2 and cytochrome c are also located. This potential regulation by Trx2 is supported by a report that cytochrome c was co-immunoprecipitated with Trx2 *in vitro* (Tanaka et al, 2002). However, detailed regulatory mechanisms remains to be elucidated, especially *in vivo*.

Trx2 is prominently expressed in the areas of the brain that showed the most intensive free radical production (Rybnikova et al, 2000), and hypoxia/reoxygenation causes a significant increase of Trx2 expression in cortical neurons (Yamagata et al, 2000). These reports together with our present results suggest that Trx2 may play a physiological role in protecting cells from oxidative stresses, similar to manganese superoxide dismutase (MnSOD) (Keller et al, 1998). Since a recent report showed changes in Trx1 and TrxR1 levels in the Alzheimer's disease brain (Lovell et al, 2000), studies of Trx2 expression in pathological brains will be helpful in understanding its significance in the pathogenesis of human diseases including neurodegenerative diseases.

ACKNOWLEDGEMENT

This work was supported by Medical Research Grant of Sungkyunkwan University.

REFERENCES

Chen Y, Cai J, Murphy TJ, Jones DP. Overexpressed human mitochondrial thioredoxin confers resistance to oxidant-induced apoptosis in human osteosarcoma cells. *J Biol Chem* 277: 33242 −33248, 2002

Damdimopoulos AE, Miranda-Vizuete A, Pelto-Huikko M, Gustafsson JA, Spyrou G. Human mitochondrial thioredoxin. Involvement in mitochondrial membrane potential and cell death. *J Biol Chem* 277: 33249−33257, 2002

Estevez AG, Spear N, Manuel SM, Radi R, Henderson CE, Barbeito L, Beckman JS. Nitric oxide and superoxide contribute to motor neuron apoptosis induced by trophic factor deprivation. *J Neurosci* 18: 923−931, 1998

Ferret PJ, Soum E, Negre O, Wollman EE, Fradelizi D. Protective effect of thioredoxin upon NO-mediated cell injury in THP1 monocytic human cells. *Biochem J* 346: 759−765, 2000

Green DR, Reed JC. Mitochondria and apoptosis. *Science* 281: 1309 −1312, 1998

Hirota K, Murata M, Sachi Y, Nakamura H, Takeuchi J, Mori K, Yodoi J. Distinct roles of thioredoxin in the cytoplasm and in the nucleus. A two-step mechanism of redox regulation of transcription factor NF-kappaB. *J Biol Chem* 274: 27891−27897, 1999

Hori K, Katayama M, Sato N, Ishii K, Waga S, Yodoi J. Neuroprotection by glial cells through adult T cell leukemia-derived factor/human thioredoxin (ADF/TRX). *Brain Res* 652: 304−310, 1994

Keller JN, Kindy MS, Holtsberg FW, St. Clair DK, Yen HC, Germeyer A, Steiner SM, Bruce-Keller AJ, Hutchins JB, Mattson MP. Mitochondrial manganese superoxide dismutase prevents neural apoptosis and reduces ischemic brain injury: suppression of peroxynitrite production, lipid peroxidation, and mitochondrial dysfunction. *J Neurosci* 18: 687−697, 1998

Lee SR, Kim JR, Kwon KS, Yoon HW, Levine RL, Ginsburg A, Rhee SG. Molecular cloning and characterization of a mitochondrial selenocysteine-containing thioredoxin reductase from rat liver. *J Biol Chem* 274: 4722−4734, 1999

Lovell MA, Xie C, Gabbita SP, Markesbery WR. Decreased thioredoxin and increased thioredoxin reductase levels in Alzheimer's disease brain. *Free Radic Biol Med* 28: 418−427, 2000

Luetjens CM, Bui NT, Sengpiel B, Munstermann G, Poppe M, Krohn AJ, Bauerbach E, Krieglstein J, Prehn JH. Delayed mitochondrial dysfunction in excitotoxic neuron death: cytochrome c release and a secondary increase in superoxide production. *J Neurosci* 20: 5715−5723, 2000

Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Meth* 65: 55−63, 1983

Miranda-Vizuete A, Damdimopoulos AE, Pedrajas JR, Gustafsson JA, Spyrou G. Human mitochondrial thioredoxin reductase cDNA cloning, expression and genomic organization. *Eur J Biochem* 261: 405−12, 1999

Mitsui A, Hirakawa T, Yodoi J. Reactive oxygen-reducing and protein-refolding activities of adult T cell leukemia-derived

factor/human thioredoxin. *Biochem Biophys Res Commun* 186: 1220–1226, 1992
Nicholls DG, Budd SL. Mitochondria and neuronal survival. *Physiol Rev* 80: 315–360, 2000
Powis G, Montfort WR. Properties and biological activities of thioredoxins. *Annu Rev Pharmacol Toxicol* 41: 261–95, 2001
Rybnikova E, Damdimopoulos AE, Gustafsson JA, Spyrou G, Pelto-Huikko M. Expression of novel antioxidant thioredoxin-2 in the rat brain. *Eur J Neurosci* 12: 1669–1678, 2000
Saitoh M, Nishitoh H, Fujii M, Takeda K, Tobiume K, Sawada Y, Kawabata M, Miyazono K, Ichijo H. Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *EMBO J* 17: 2596–2606, 1998
Simonian NA, Coyle JT. Oxidative stress in neurodegenerative diseases. Annu Rev Pharmacol Toxicol 36: 83–106, 1996
Spyrou G, Enmark E, Miranda-Vizuete A, Gustafsson J. Cloning and expression of a novel mammalian thioredoxin. *J Biol Chem* 272: 2936–2941, 1997
Takagi Y, Mitsui A, Nishiyama A, Nozaki K, Sono H, Gon Y, Hashimoto N, Yodoi J. Overexpression of thioredoxin in transgenic mice attenuates focal ischemic brain damage. *Proc Natl Acad Sci USA* 96: 4131–4136, 1999
Tanaka T, Hosoi F, Yamaguchi-Iwai Y, Nakamura H, Masutani H, Ueda S, Nishiyama A, Takeda S, Wada H, Spyrou G, Yodoi J. Thioredoxin-2 (TRX-2) is an essential gene regulating mitochondria-dependent apoptosis. *EMBO J* 21: 1695–1703, 2002
Wadia JS, Chalmers-Redman RM, Ju WJ, Carlile GW, Phillips JL, Fraser AD, Tatton WG. Mitochondrial membrane potential and nuclear changes in apoptosis caused by serum and nerve growth factor withdrawal: time course and modification by (-)-deprenyl. *J Neurosci* 18: 932–947, 1998
Wakasugi N, Tagaya Y, Wakasugi H, Mitsui A, Maeda M, Yodoi J, Tursz T. Adult T-cell leukemia-derived factor/thioredoxin, produced by both human T-lymphotropic virus type I- and Epstein-Barr virus-transformed lymphocytes, acts as an autocrine growth factor and synergizes with interleukin 1 and interleukin 2. *Proc Natl Acad Sci USA* 87: 8282–8286, 1990
Yamagata K, Tagami M, Ikeda K, Yamori Y, Nara Y. Altered gene expressions during hypoxia and reoxygenation in cortical neurons isolated from stroke-prone spontaneously hypertensive rats. *Neurosci Lett* 284: 131–134, 2000