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Forum News & Views

Does Thioredoxin-1 Prevent Mitochondria- and Endoplasmic Reticulum-Mediated Neurotoxicity of 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine?

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ABSTRACT

We show that 1-methyl-4-phenylpyridinium ion (MPP^+), an active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), induces cytotoxicity via endoplasmic reticulum (ER)- and mitochondria–mediated pathways, and thioredoxin-1 (TRX-1), a redox-active protein, prevents MPTP-induced neurotoxicity. TRX-1 overexpression suppressed reactive oxygen species and the ATP decline caused by MPP^+ in HepG2 cells. MPP^+ activated caspase-12 in PC12 cells and induced cytotoxicity in HeLa-ρ^0 cells lacking mitochondrial DNA, as well as in the parental HeLa-S3 cells. TRX-1–transgenic mice demonstrated significant resistance to caspase-12 activation and the apoptotic decrease of dopaminergic neurons after MPTP administration, compared with wild-type C57BL/6 mice. *Antioxid. Redox Signal.* 9, 603–608.

NEURONAL TOXICITY OF MPTP

Parkinson disease (PD) is a common neurodegenerative disorder whose cardinal features include tremor, slowness of movement, stiffness, and poor balance (13). Most of these disabling symptoms are due to a profound reduction in striatal dopamine content caused by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and their projecting nerve fibers in the striatum (23).

In several mammalian species, the dopaminergic neuronal toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes a severe and irreversible PD-like syndrome (16). It reproduces most of the biochemical and pathologic hallmarks of PD, including the substantial degeneration of dopaminergic neurons. MPTP is metabolized to 1-methyl-4-phenylpyridinium ion (MPP^+) by the enzyme monoamine oxidase B. Subsequently, MPP^+ is taken up by dopaminergic terminals and concentrates in the substantia nigra. MPP^+ induces the inhibition of membrane-bound NADH dehydrogenase in mitochondrial complex I (21), causes ATP depletion (5), and leads to the production of nitric oxide (NO) and superoxide radicals (8). Therefore, it is well characterized that mitochondrial stress plays a crucial role in the MPTP-induced neurotoxicity (25).

Recent reports have shown that endoplasmic reticulum (ER)-mediated stress is also a contributory factor in neuronal death and that it is involved in neurodegenerative disorders including PD (10, 18, 26). However, it has not yet been well clarified that ER stress is involved in MPTP-induced neurotoxicity.

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TRX-1 ATTENAUTES MPP+-INDUCED CYTOTOXICITY UPSTREAM OF THE MITOCHONDIRAL PATHWAY

Thioredoxin-1 (TRX-1) is a small, 12-kDa multifunctional cytosolic protein with a redox-active disulfide/dithiol within its active site sequence, -Cys-Gly-Pro-Cys-. It operates together with NADPH and thioredoxin reductase as a protein disulfide–reducing system (9). TRX-1 is known to have a cytoprotective effect against oxidative stress (20), whereas overexpression of TRX-1 in transgenic mice attenuates focal ischemic brain damage (27). TRX-1 also has been reported to be a neurotrophic factor for central cholinergic neurons (6). We recently reported that TRX-1 plays a critical role in nerve growth factor–mediated signal transduction and neurite outgrowth (3) and has a protective effect in response to MPP^+-induced cytotoxicity in PC12 cells (2).

To investigate the role of TRX-1 against MPP^+ cytotoxicity in other cell types, we studied human hepatoblastoma cell line HepG2 cells, stably transfected with human *TRX-1* or a control pcDNA3 vector (22). Previous reports have shown that MPP^+ cytotoxicity is dependent mainly on the inhibition of complex I in the mitochondria and the subsequent depletion of ATP (21). We observed a decrease in ATP production and an increase in the production of reactive oxygen species (ROS) after MPP^+ treatment of HepG2 cells transfected with the control vector. In contrast, ROS production by MPP^+ was suppressed (Fig. 1A), and the ATP production was not decreased by MPP^+ (Fig. 1B) in *TRX-1*–transfected cells. However, the decrease of ATP production by rotenone, an inhibitor of complex I, was not restored in *TRX-1*–transfected cells, suggesting that TRX-1 suppresses MPP^+-induced cytotoxicity upstream of the mitochondrial pathway.

MPP+ INDUCES CYTOTOXICITY VIA ER-MEDIATED PATHWAY

To investigate further the mechanism of MPP^+ cytotoxicity, we added MPP^+ to the human cervical carcinoma cell line HeLa-S3 and its subclone HeLa-ρ^0, which lacks mitochondrial DNA. MPP^+-induced cell death was observed in HeLa-ρ^0 cells as well as in the HeLa-S3 cell line (Fig. 2). This result suggests that the cytotoxic effect of MPP^+ is at least partly mediated by an extramitochondrial pathway.

Recent studies have suggested the existence of a novel apoptotic pathway in which caspase-12 functions as the initiator in response to a toxic insult to the ER, such as calcium

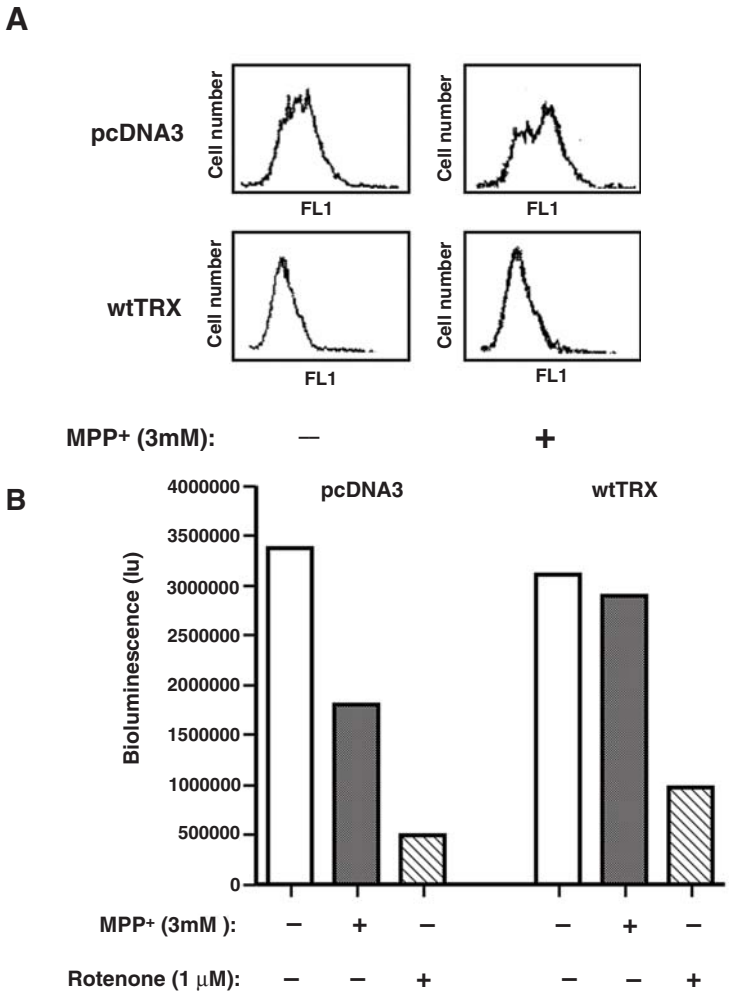

FIG. 1. TRX-1 overexpression suppresses ROS and blocks the decrease in ATP caused by MPP^+. (**A**) HepG2 cells, transfected with *TRX-1* (wtTRX) or the control pcDNA vector, were treated without or with 3 m*M* MPP^+ for 3 h. Intracellular ROS content detected by flow cytometry by using DCFH-DA is demonstrated. The result shown is representative of three independent experiments. (**B**) HepG2 cells transfected with *TRX-1* or the control vector were cultured for 12 h in the absence or presence of 1 μ*M* rotenone or 3 m*M* MPP^+. ATP levels are shown as relative light units (rlu). The result shown is representative of three independent experiments done in duplicate.

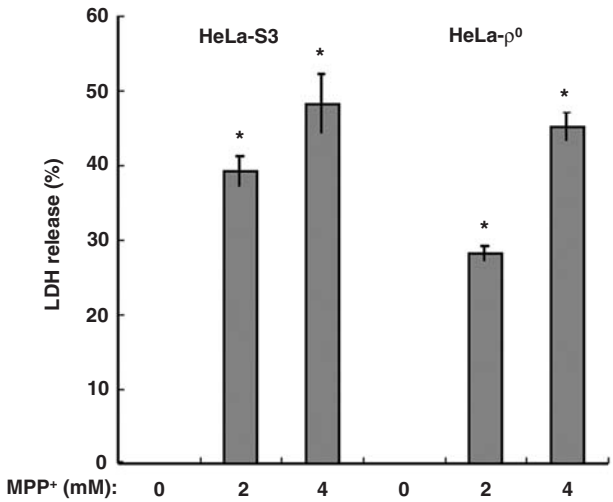

FIG. 2. MPP+ induces cell death in HeLa-S3 and HeLa-ρ0 cells. HeLa-S3 and HeLa-ρ0 cells were cultured with 2 or 4 m*M* MPP^+ for 24 h. Percentage of cell lysis was measured by the LDH-releasing assay. All values are expressed as the mean ± S.D. *The significant difference ($p < 0.05$).

ionophores or thapsigargin, an inhibitor of the ER-specific calcium ATPase (19). Caspase-12 is localized to the ER, where it is processed as a result of excess ER stress (7). To investigate the involvement of ER stress in MPTP-induced neurotoxicity, we examined the extent of caspase-12 activation in PC12 cells after treatment with MPP^+. As shown in Fig. 3, procaspase-12 levels began to decrease 6 h after MPP^+ treatment, and activated caspase-12 was clearly detected 24 h after MPP^+ treatment. These data suggest that MPP^+ induces neurotoxicity via an ER stress-mediated pathway.

TRX-1 TRANSGENIC MICE ARE MORE RESISTANT TO MPTP

We previously reported that transient transfection of *TRX-1* or administration of the recombinant TRX-1 protein suppresses MPP^+-induced neurotoxicity in PC12 cells. In the present study, we compared the neurotoxicity of MPTP in TRX-1 transgenic and wild-type mice. SNpc dopaminergic neurons, defined by staining with tyrosine hydroxylase (TH) and Nissl, were observed in the TRX-1 transgenic mice as well as in the wild-type mice after an injection of saline (Fig. 4A–C). However, after injection of MPTP, TH-stained neurons almost disappeared in the wild-type mice (Fig. 4D and E), whereas a substantial number of neurons were still present in the TRX-1 transgenic mice (Fig. 4F and G). The number of TH-positive cells was significantly higher in TRX-1 transgenic compared with wild-type mice (Fig. 4H).

The percentage of TUNEL-positive, TH-positive cells was significantly higher in wild-type compared with TRX-1 transgenic mice (Fig. 5A, B, and E), indicating that overexpression of TRX-1 suppresses MPTP neurotoxicity *in vivo*. As shown in Fig. 3, MPP^+ induces caspase-12 activation in PC12 cells. We therefore analyzed caspase-12 activation after MPTP treatment and showed that MPTP induced caspase-12 activation in wild-type mice (Fig. 5C), but not in TRX-1 transgenic mice (Fig. 5D). The number of TH-positive cells with active caspase-12 was significantly higher in wild-type compared with TRX-1 transgenic mice (Fig. 5F).

TRX-1 PREVENTS MITOCHONDRIA- AND ER-MEDIATED NEUROTOXICITY OF MPTP

In the present study, we showed that MPP^+ induces cell death of HeLa-ρ0 cells and their parental HeLa-S3 cell line, suggesting that MPP^+ has an extramitochondrial signal pathway. Furthermore, MPP^+ induces the processing and activation of caspase-12 in PC12 cells, and caspase-12 activation also is detected in MPTP-treated mice. Caspase-12–deficient mice are resistant to ER stress–induced apoptosis (19), showing that MPTP induces ER stress both *in vitro* and *in vivo*. ER stress, such as that caused by an accumulation of misfolded proteins in the ER (15), has received growing attention as a cause of pathologically relevant apoptosis, with particular implications for neurodegenerative disorders (1).

We previously reported that TRX-1 transgenic mice are more resistant to a variety of oxidative stresses, including

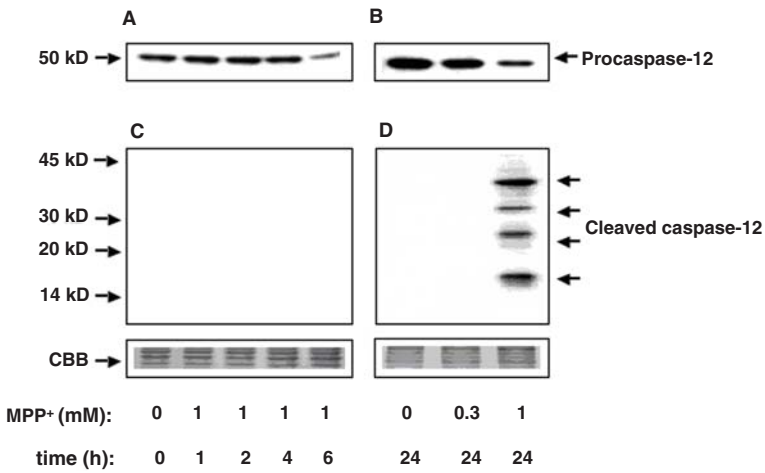

FIG. 3. MPP+ decreases procaspase-12 levels and causes the processing of caspase-12. (**A**) PC12 cells were treated with 1 m*M* MPP^+ for the indicated period, or (**B**) PC12 cells were treated with varying doses of MPP^+ for 24 h. Cells were harvested and subjected to Western blot analysis. All samples were loaded separately and monitored by Coomassie (CBB) staining. Arrows indicate caspase-12 cleavage products.

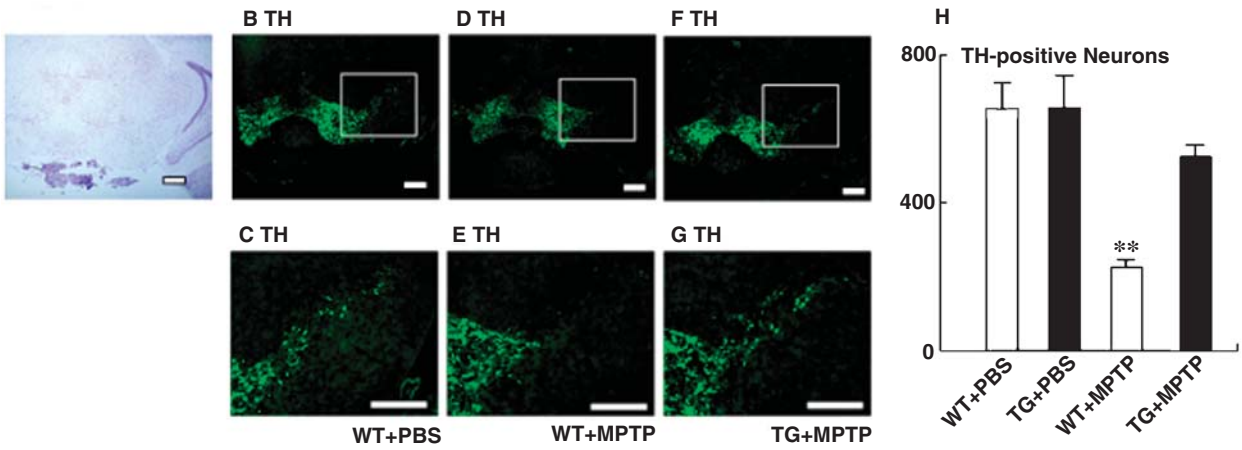

FIG. 4. TRX-1 transgenic mice are more resistant to MPTP. (**A**) Nissl staining shows dopamine neurons of mouse brains. (**B, C**) Tyrosine hydroxylase (TH) staining in wild-type mice treated with PBS for 7 days. (**D, E**) TH staining of wild-type mice treated with MPTP. **F, G**, TH staining in TRX-1 transgenic mice treated with MPTP for 7 days. Boxed areas in **B, D**, and **F** are magnified in **C, E**, and **F**, respectively. The open bar in each panel represents 200 μm. (**H**) The number of TH-positive cells in SNpc are significantly higher (**$p < 0.01$, multiple comparison analysis followed by ANOVA) in TRX-1 transgenic mice treated with MPTP than in wild-type mice receiving the same treatment ($n = 6$). Means ± SD are shown.

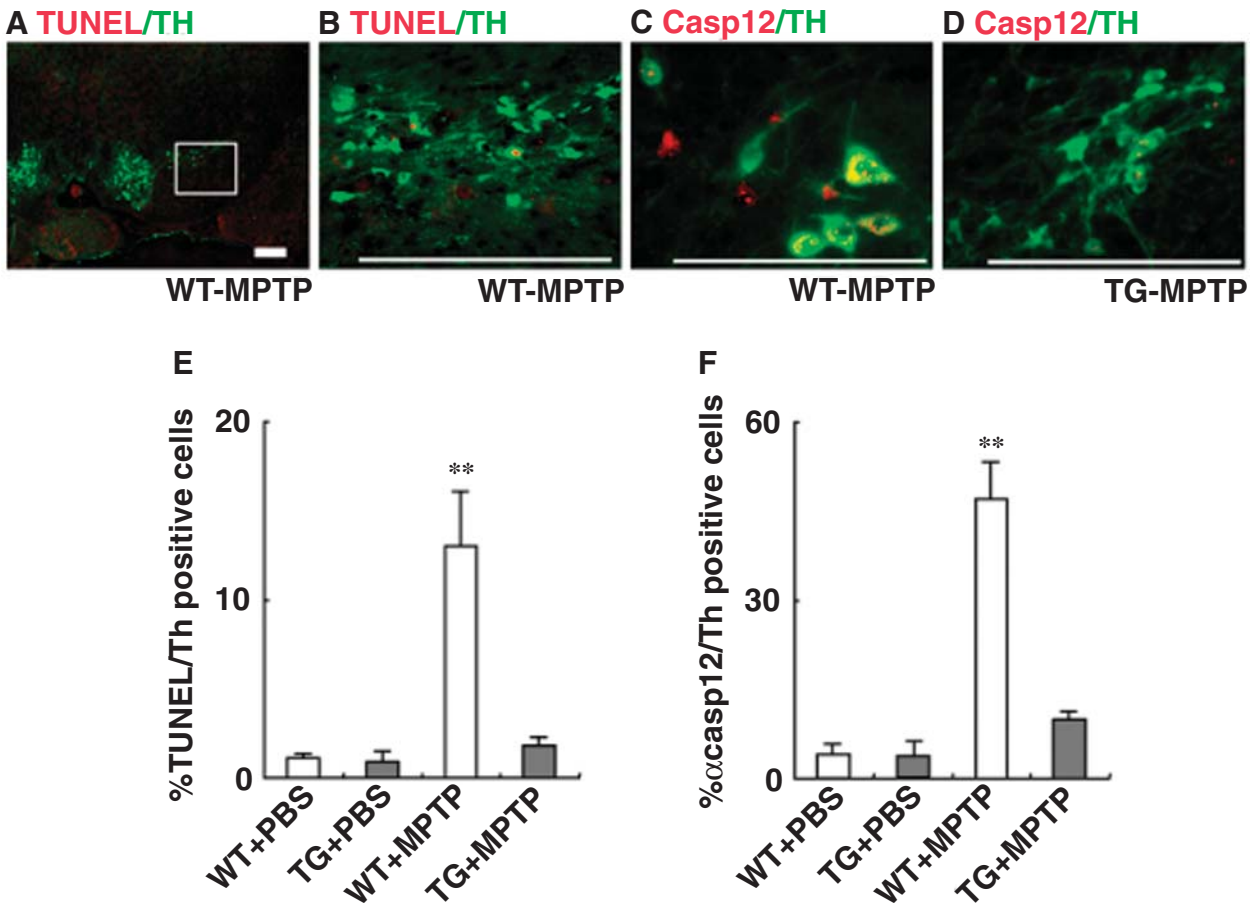

FIG. 5. MPTP-induced apoptosis and caspase-12 activation in SNpc dopaminergic neurons are suppressed in TRX-1 transgenic mice. TH staining is shown in green. TUNEL and activated caspase-12 staining are shown in red. (**A, B**) Adjacent sections prepared from wild-type mice receiving MPTP treatment for 7 days were stained by the TUNEL method or TH immunohistochemical staining, and the images were digitally overlapped. The boxed area in **A** is enlarged in **B.** (**C, D**) Double staining for activated caspase-12 and TH after 7 days of MPTP treatment in wild-type (**C**) and TRX transgenic mice (**D**). The open bar in each panel represents 200 μm. (**E, F**) Percentage of TUNEL-positive (**E**) or activated caspase-12–positive (**F**) cells in TH-positive neurons. Evaluations were performed in wild-type or TRX transgenic mice after 7 days of treatment with either PBS or MPTP ($n = 6$). Means ± SD are shown. **$p < 0.01$ by multiple comparison analysis followed by ANOVA.

cerebral infarction, doxorubicin (Adriamycin)-induced cardiotoxicity, and thioacetamide-induced acute hepatitis (27). In this study, we showed that TRX-1 attenuates the generation of ROS and suppresses the decrease of ATP by MPP^+. We also demonstrated that caspase-12 activation is suppressed in TRX-1 transgenic mice. Thus, the overexpression of TRX-1 attenuates ER-mediated stress and apoptosis by MPTP.

Although TRX-1 is present in the cytosol and the nucleus, several members of the TRX family are localized to the mitochondria and the ER. TRX-2 is a mitochondria-specific member that is involved in mitochondria-mediated apoptosis (28), whereas ER members include protein disulfide isomerase and thioredoxin-related transmembrane protein (TMX), which plays an important role against ER stress (17). TRX-1 blocks MPTP-induced apoptosis upstream of mitochondrial and ER-based events. Several pathways could be considered as a mechanism by which TRX-1 can suppress the MPTP-mediated neuronal cell death in SNpc. First, TRX-1, which is upregulated in pyramidal neurons in TRX-1 transgenic mice (27), had a direct effect on the maintenance of the ER function, even in the presence of MPP^+. Second, TRX-1 may enhance the neuroprotective properties of astrocytes to remove MPP^+ via extraneuronal monoamine transporter (11).

In conclusion, we showed that MPP^+ induces apoptosis via a caspase-12–dependent pathway and inhibits mitochondrial respiration at complex I. In addition to its cytoprotective and neuroprotective functions (2, 15), the overexpression of TRX-1 completely suppresses MPTP neurotoxicity *in vivo*, suggesting its therapeutic potential in the treatment of PD. Recently, we also reported that TRX-1 protects NMDA-induced neurotoxicity (12), and the inducer of TRX-1 suppresses the photooxidative damage in the retina (29). The work is currently in progress for the clinical application of TRX-1 to neurodegenerative disorders including PD.

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ABBREVIATIONS

DCF, fluorescent 2′,7′-dichlorofluorescein; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DMEM, Dulbecco's minimal essential medium; ER, endoplasmic reticulum; FCS, fetal calf serum; HS, horse serum; LDH, lactate dehydrogenase; MPP^+, 1-methyl-4-phenylpyridinium ion; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NO, nitric oxide; PBS, phosphate-buffered saline; PD, Parkinson disease; ROS, reactive oxygen species; SNpc, substantia nigra pars compacta; TH, tyrosine hydroxylase; TMX, thioredoxin-related transmembrane protein; T-PBS, PBS containing 0.05% Tween 20; TRX-1, thioredoxin-1.

APPENDIX

Chemical compounds

MPP^+ and MPTP were obtained from Sigma, St. Louis, MO.

Cells

HeLa-S3 cells were maintained in Dulbecco's minimal essential medium (DMEM; Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS) and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin) at 37°C in a humid atmosphere containing 5% CO_2. PC12 cells were maintained in RPMI1640 medium (Life Technologies) supplemented with 10% heat-inactivated fetal horse serum (HS) and 5% FCS. HeLa-ρ^0 cells were described previously (4). PCR confirmed that these cells are subclones of HeLa-S3 cells that have been cultured with ethidium bromide and lack mitochondrial DNA.

Western blot analysis

Cells were harvested and washed twice with ice-cold phosphate-buffered saline (PBS), and then lysed with a solubilizing solution (20 m*M* Tris-HCI (pH 7.4), 150 m*M* NaCl, 1% NP-40, 1 m*M* EDTA, 1 m*M* PMSF, 1 m*M* EGTA, 1% Triton X-100, 2.5 m*M* sodium pyrophosphate, 1 m*M* Na_3VO_4, 1 m*M* β-glycerolphosphate, and 1 μg/ml leupeptin) on ice for 30 min. The extracts were cleared by centrifugation. Cell lysates were incubated at 95°C for 5 min, then separated by 12% SDS-polyacrylamide gel electrophoresis. The separated proteins (procaspase-12 and activated caspase-12) were transferred to a polyvinylidene difluoride membrane (Millipore Co., Bedford, MA). The membrane was treated with 10% (wt/vol) skim milk in PBS, containing 0.05% Tween 20 (T-PBS) overnight at 4°C, and then incubated with anti–procaspase-12 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-activated caspase-12 polyclonal antibody (7) for 1 h, followed by peroxidase-conjugated anti-rabbit IgG (Amersham Biosciences Corp, NJ) for 1 h. The epitope was visualized with an ECL Western blot detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Lactate dehydrogenase (LDH) release assay

The amount of LDH released from damaged cells was determined from aliquots (100 μl) of the culture medium. Total cellular LDH was obtained by lysing cells with 0.2% Tween-20 in PBS. LDH in 50-μl culture medium or cell lysate samples was measured by using an LDH assay kit (Kyokuto, Tokyo, Japan) according to the manufacturer's instructions. Percentage cell lysis was determined as the ratio of LDH in the medium/total LDH per well.

Intracellular reactive oxygen species (ROS) measurement

The 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA, Molecular Probes, Eugene, OR) was used to measure the amount of intracellular ROS. DCFH-DA is nonfluorescent and permeates into cells where it is modified into fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of ROS. Cells were incubated with 5 m*M* DCFH-DA for 30 min at 37°C. After washing, DCF fluorescence was detected by a flow cytometer (FACSCalibur, Becton Dickinson, San Jose, CA).

Animal experiments

TRX transgenic mice were generated as previously described (27). In these TRX transgenic mice, human TRX was expressed in severalfold systemically under β-actin promoter and more than fourfold of endogenous mouse TRX in the brain. C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). Eight-week-old male TRX transgenic and wild-type C57BL/6 mice were used in the experiments. Four TRX transgenic mice and four wild-type mice received four intraperitoneal injections of MPTP-HCl (20 mg/kg of free base; Sigma) in saline with 2-h intervals on day 0 and were killed after 7 days after the last injection. Control mice received injections of saline only. This protocol was in accordance with the NIH guidelines for use of live animals and was approved by the Institutional Animal Care Committee.

ATP measurement

ATP levels were determined by using the ATP bioluminescence assay kit HS II (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's recommendations. The measurements are shown as relative light units (rlu), which correlate with the amount of ATP detected.

Immunohistochemical analysis and evaluation of cell death

Animals were perfused with PBS and then 4% paraformaldehyde under deep anesthesia, and 10-μm cryostat sections of the brain were obtained by cutting coronally. Sections were processed for cresyl violet staining or immunohistochemistry by using a monoclonal antibody against the dopaminergic neuron marker human tyrosine hydroxylase, which also recognizes the murine protein (TH, 1:5,000, Sigma), as described previously (14). Neuronal survival in SNpc was evaluated as previously described (24). Cell death was assessed by either the TUNEL method by using ApopTag (Intergen Company, New York, NY) or by immunostaining with a rabbit anti-activated caspase-12 antibody (1:400), which was prepared as described previously (7). The number of SNpc neurons positive for either activated caspase-12 or with TUNEL staining was counted in three fields (magnification 40x) by two experimenters without knowledge of the experimental protocol.

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