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Forum News & Views

Does ORP150/HSP12A Protect Dopaminergic Neurons Against MPTP/MPP$^+$-Induced Neurotoxicity?

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ABSTRACT

MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) and its metabolite 1-methyl-4-phenylpyridinium (MPP$^+$) are drugs that are widely used in experimental Parkinson disease (PD) models. What is the significance of ORP150/HSP12A, a molecular chaperone in the endoplasmic reticulum (ER), in the nigrostriatal system? Dopaminergic neuroblastoma SH-SY5Y cells and dopaminergic neurons of the substantia nigra pars compacta (SNpc) were examined. Our observations led to the hypothesis that ORP150 protects against MPTP/MPP$^+$-induced neurotoxicity, and indicate the importance of the ER environment in maintaining the nigrostriatal pathways. *Antioxid. Redox Signal.* 9, 589–595.

PARKINSON DISEASE

Parkinson disease (PD) is a progressive neurodegenerative disease pathologically characterized by the loss of nigrostriatal dopaminergic neurons and the presence of intracellular inclusions, known as Lewy bodies, in the substantia nigra pars compacta (SNpc) (2, 22). Although the etiology of PD is not completely understood, several genetic and environmental factors related to it have been discovered. Mutations in genes such as Parkin, α-synuclein, *ubiquitin* carboxyl terminal hydroxylase-L1 (UCH-L1), DJ-1, PTEN-induced kinase 1 (PINK1), and leucine-rich repeat kinase 2 (LRRK) have been linked to familial forms of PD (21). Some of these genes are functionally associated with the ubiquitin (Ub)–proteasome system (UPS), a potent protein-degradation pathway, suggesting the hypothesis that accumulation of misfolded proteins plays an important role in PD neurodegeneration. In contrast, studies with neurotoxins, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 1-methyl-4-phenylpyridinium (MPP$^+$), 6-hydroxydopamine (6-OHDA), and rotenone, have revealed that the production of reactive oxygen species (ROS) and mitochondrial dysfunction are of crucial importance in the pathogenesis of PD (2).

MPTP/MPP$^+$ AND ER STRESS

MPTP is highly lipophilic and easily crosses the blood–brain barrier. In the brain, MPTP is converted to MPP$^+$ in glial cells and then incorporated into neurons through the dopamine transporter (DAT). MPP$^+$ is believed to be concentrated in mitochondria and to inhibit the multienzyme

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complex I in the mitochondrial electron transport chain, leading to depletion of ATP and production of ROS (2). Recent studies also found that MPTP and other PD-related neurotoxins induced endoplasmic reticulum (ER) stress in both cultured cells and a mouse model (3, 19, 26). ER stress is characterized by accumulation of unfolded proteins in the ER, which occurs when the ER environment is characterized by conditions such as glucose starvation, oxygen deprivation, inhibition of protein modification, disturbance of Ca^{2+} homeostasis, and excessive protein synthesis. Eukaryotic cells respond to ER stress by activating a set of pathways known as the unfolded protein response (UPR). The UPR target genes include molecular chaperones in the ER, subunits of translocation machinery (Sec61 complex), ER-associated protein degradation (ERAD) molecules, and antioxidant genes (5, 20**)**. If the protein load in the ER exceeds its folding capacity, or dome defects exist in the UPR, cells tend to die, typically, with features of apoptosis (ER stress–induced cell death). Because the UPS is also required for ERAD pathway activity, it has been postulated that disturbance of UPS could enhance ER stress and eventually ER stress–induced cell death (4, 7, 16).

ORP150/HSP12A AND NEUROPROTECTION

ORP150 is a molecular chaperone in the ER, the expression of which is regulated by UPR (6, 8). We previously cloned ORP150 cDNA from cultured astrocytes exposed to hypoxia (12) and reported that ORP150 played a central role in the defense of neurons against various types of environmental stress. ORP150 was required for the survival of neurons under conditions of hypoxia/ischemia both *in vitro* and *in vivo* (25). In hippocampal neuronal cultures, ORP150 rescued neurons from glutamate toxicity by stabilizing $[Ca^{2+}]_i$ and thereby suppressing activation of Ca^{2+}-dependent proteinases (4). Adenovirus-mediated expression of ORP150 in the hippocampus suppressed, at least in part, delayed neuronal cell death in Mongolian gerbils (14). In this study, we found that ORP150 was induced in SNpc dopaminergic neurons after MPTP administration into mice, although it was not upregulated in SH-SY5Y cells after MPP^+ treatment. Suppression of ORP150 expression, either by infection with adenovirus carrying the ORP150 antisense cDNA in SH-SY5Y cells or using mice heterozygous for ORP150 deficiency ($ORP150^{+/-}$ mice), resulted in increase in neuronal cell death due to MPP^+- or MPTP-induced neurotoxicity. Our findings suggest the importance of the ER in strategies to maintain the function of nigrostriatal neurons.

Expression of ORP150 in MPP^+-treated cells and MPTP-treated mice

To examine the expression of ORP150 protein in dopaminergic neurons in normal and stressed conditions, human dopaminergic neuroblastoma SH-SY5Y cells were treated with MPP^+ at the indicated concentrations for 24 h (Fig. 1AI) or at 1 m*M* for the indicated durations of time up to 72 h (Fig. 1AII). ORP150 protein was expressed in all

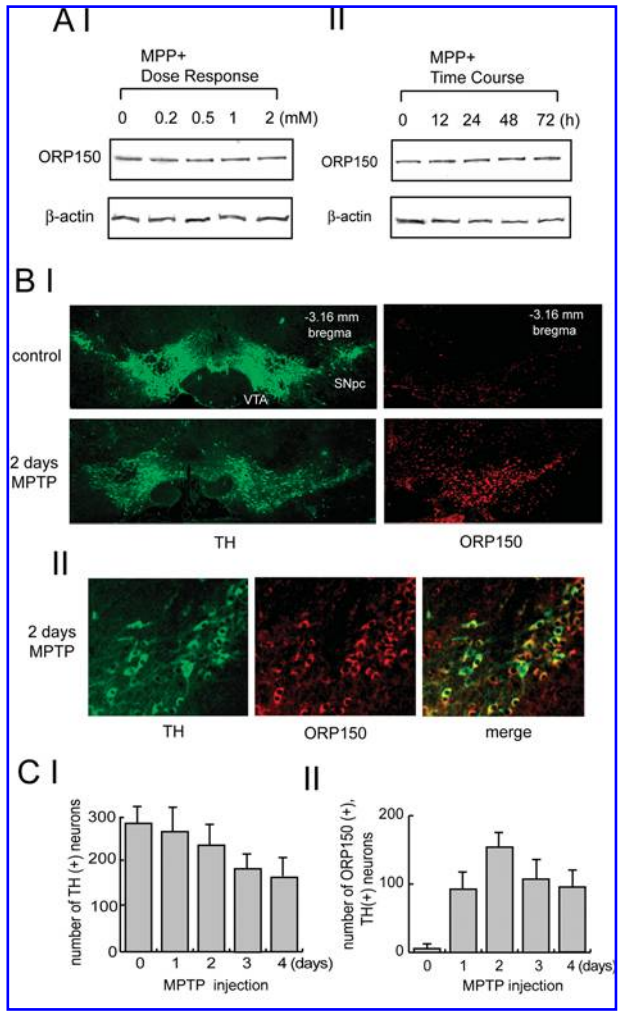

FIG. 1. Expression of ORP150 in dopaminergic neurons. (**A**) SH-SY5Y cells (~5 x 10^5 cells) were exposed to 1-methyl-4-phenylpyridinium (MPP^+; 0–2 m*M* in panel I and 1 m*M* in panel II) for either 24 h (panel I) or the indicated duration (0–72 h in panel II). Cells were harvested, and protein extracts (30 μg) were subjected to Western blot with either anti-ORP150 antibody (**upper panel**) or anti–β-actin antibody (**lower panel**). **B**I, C57BL/c mice were intraperitoneally administered 1-methy l-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; 30 mg/kg, **lower panels**) or vehicle (**upper panel**) daily for 2 days. One day after the last injection, brains were perfusion-fixed, and sections (10 μm) were double-stained with anti–tyrosine hydroxylase (TH) antibody and anti-ORP150 antibody. SNpc, substantia nigra pars compacta; VTA, ventral tegmental area; **B**II, the same sections as in **B**I are shown at higher magnification to demonstrate colocalization of ORP150 and TH antigens. **C**, C57BL/c mice were intraperitoneally administered MPTP or vehicle daily for the indicated number of days as above, and brain sections were double-stained with anti-TH antibody and anti-ORP150 antibody as above. Numbers of TH(+) neurons, or ORP150(+) and TH(+) neurons were counted in SNpc, as described in the text. In each case, $n = 4$, mean ± SD is shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars.)

conditions but not upregulated after MPP^+ treatment (less than 1.2-fold increase compared with nontreated control cells).

In contrast, when MPTP was systemically injected into mice daily for the indicated number of days, the number of TH(+) neurons in SNpc gradually decreased (Fig. 1BI, CI), and the number of ORP150(+) neurons increased, mainly among TH(+) neurons (Fig. 1BI, BII, CII). After injection of MPTP into mice for 2 consecutive days, the number of TH(+) neurons decreased to 80% of that in control (vehicle-injected) mice, whereas the number of ORP150(+) cells increased to 72% of the total number of TH(+) neurons in SNpc (Fig. 1CI, II). When MPTP was injected for longer periods (up to 4 consecutive days), the number of ORP150(+) neurons decreased in a fashion similar to TH(+) neurons (Fig. 1CII).

Effects of adenovirus-mediated suppression of ORP150 expression in SH-SY5Y cells after MPP^+ treatment

Because we previously demonstrated that suppression of ORP150 expression rendered cultured neurons and other types of cells vulnerable to ER stress and hypoxia (14, 18), we examined the effects of adenovirus-mediated suppression of ORP150 expression in SH-SY5Y cells in normal and MPP^+-treated conditions. Infection of adenovirus carrying ORP150 antisense cDNA (Ad/ORP150AS) decreased expression of ORP150 protein to 30% of that in noninfected or LacZ-infected cells at a multiplicity of infection of 50 (moi; Fig. 2AI, II), as previously described (14, 18). Expression of other ER-related molecules such as GRP78 and GRP94 remained unchanged (data not shown), suggesting that the effects of Ad/ORP150AS were ORP150 specific. Exposure of noninfected (data not shown) or LacZ-infected (Fig. 2BII) cells to MPP^+ (1 m*M*) decreased viability to 80% at 48 h and 53% at 72 h, whereas exposure of ORP150AS-infected cells resulted in further decrease in viability to 35% at 48 h and 25% at 72 h (Fig. 2 BI). The increase in death of ORP150AS-infected SH-SY5Y cells was confirmed by LIVE/DEAD cell toxicity assay (Fig. 2C). Enhanced nuclear staining of dead cells and reduced cytosol staining of living cells were apparent in cultures of ORP150AS-infected cells treated with MPP^+ (Fig. 2CII). Infection of adenovirus carrying ORP150

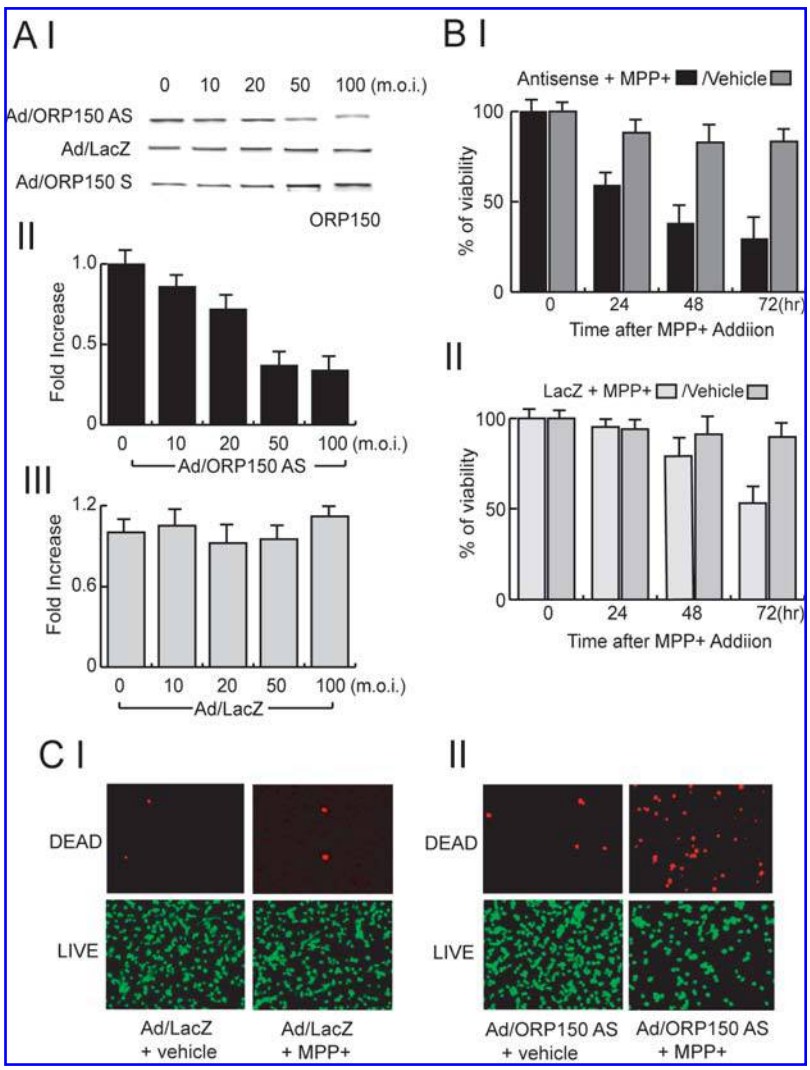

FIG. 2. Effects of ORP150 expression on viability of SH-SY5Y cells after MPP^+ treatment. (**A**) SH-SY5Y cells were infected with adenovirus vector in either ORP150 antisense direction (Ad/ORP150 AS) or β-galactosidase sense direction (Ad/LacZ) at the indicated concentration (0–100 moi) for 16 h. After further incubation in culture medium for 24 h, cells were harvested, and protein extracts (10 μg/lane) were subjected to Western blot with anti-ORP150 antibody. In each case, ORP150 antigen was semiquantitatively assessed with densitometric analysis. Values are fold increase/decrease relative to control condition. In each case, $n = 4$; mean ± SD is shown. (**B**) SH-SY5Y cultures were treated with either Ad/ORP150AS or Ad/LacZ (50 moi in each case) for 40 h. Cultures were then exposed to MPP^+ (1 m*M*) for ≤72 h. Cell viability was measured by MTT assay at the indicated time points (**B**I, II), or LIVE/DEAD cell toxicity assay at 24 h (**C**I, II) after MPP^+ treatment. In (**B**), the MTT value in the absence of adenovirus infection and MPP^+ treatment was set at 100. In each case, $n = 4$; mean ± SD is shown. (**C**) Typical examples of fluorescence images are shown.

sense cDNA (ORP150S) into SH-SY5Y cells did not exhibit deleterious effects on MPP^+ exposure (data not shown).

Effects of suppression of ORP150 expression on MPTP-induced neurotoxicity

To assess the effects of OPR150 expression *in vivo*, we used mice heterozygous for ORP150 deficiency ($ORP150^{+/-}$ mice), as previously described (9). When $ORP150^{+/-}$ was intraperitoneally injected with MPTP for 3 consecutive days, the number of tyrosine hydroxylase (TH)-positive neurons in SNpc decreased to a greater degree (to 30% of that in control mice) than in $ORP150^{+/+}$ littermates (to 62% of that in control mice; Fig. 3A, left panels). Induction of ORP150 protein was suppressed in SNpc dopaminergic neurons in $ORP150^{+/-}$ mice, compared with $ORP150^{+/+}$ mice (Fig. 3A, middle and right panels). Consistent with these findings, the dopamine content in striatum (caudate-putamen) decreased to a greater degree in $ORP150^{+/-}$ mice than in $ORP150^{+/+}$ mice after administration of MPTP for the indicated numbers of days (Fig. 3B). In contrast, noradrenalin content in the same region was not altered in $ORP150^{+/-}$ in mice compared with $ORP150^{+/+}$ mice (Fig. 3C).

Neuronal cell death in SNpc of $ORP150^{+/-}$ mice after MPTP administration

Further to explore the effects of ORP150 suppression in SNpc dopaminergic neurons, immunohistochemical analysis was performed for two independent cell death–related markers, activated caspase 12, a key molecule in ER stress–induced cell death (15), and activated caspase 3, a downstream cell-death effector (Fig. 4). Activation of both caspases was observed to a greater extent in SNpc TH(+) neurons of $ORP150^{+/-}$ mice (15% and 13% of TH(+) neurons, respectively) than in those of $ORP150^{+/+}$ mice [3% and 2% of TH(+) neurons, respectively] after MPTP injection for 4 consecutive days.

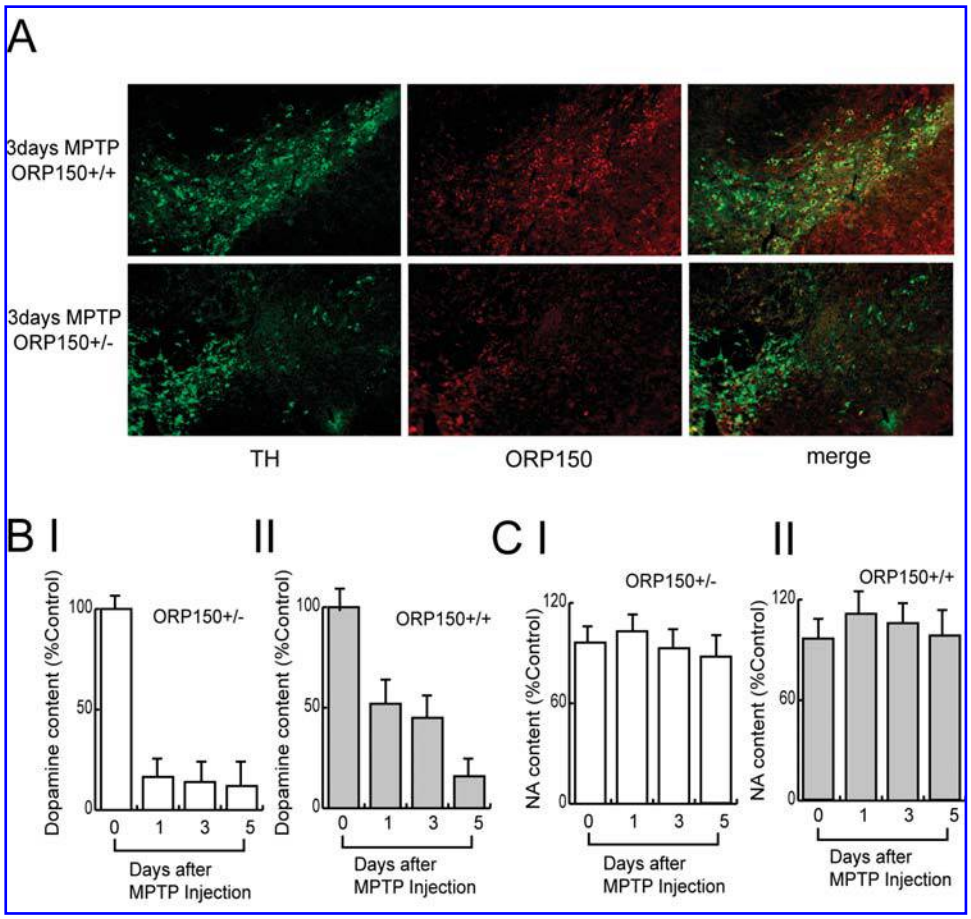

FIG. 3. Effects of suppression of ORP150 expression on MPTP-induced neurotoxicity. (**A**) MPTP (30 mg/kg) was intraperitoneally injected daily for 3 days into $ORP150^{+/-}$ or control $ORP150^{+/+}$ mice. One day after the last injection, brains were perfusion-fixed, and sections (10 μm) were double-stained with anti-TH antibody and anti-ORP150 antibody, as in Fig. 1B. (**B, C**) MPTP (30 mg/kg) was intraperitoneally injected daily for 3 days into $ORP150^{+/-}$ or control $ORP150^{+/+}$ mice as above, and 1 day after the last injection, striatal tissue was homogenized in solution H, as described in the text, and processed for measurement of dopamine (**B**) or noradrenalin (NA; **C**). In each case, $n = 4$; mean ± SD is shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars.)

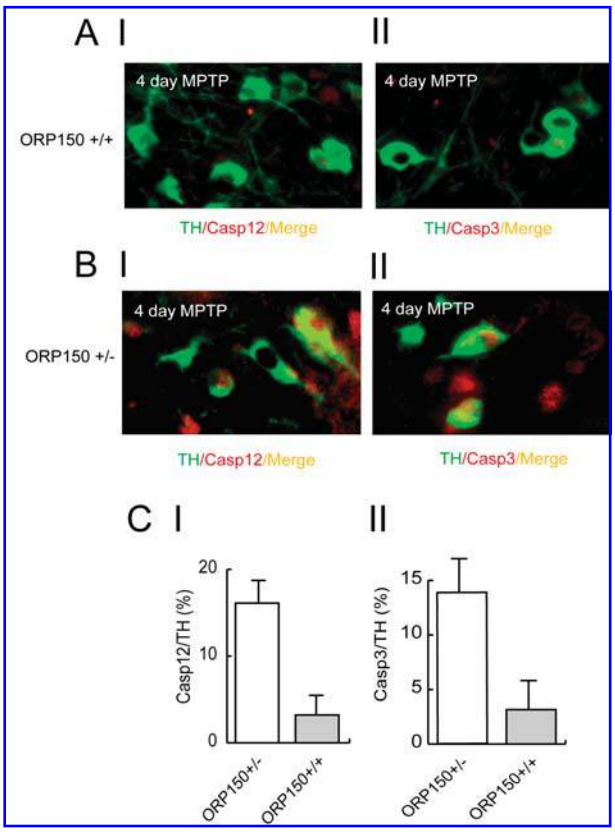

FIG. 4. Activation of cell death markers in ORP150$^{+/-}$ neurons after MPTP administration. MPTP (30 mg/kg) was intraperitoneally injected daily for 4 days into ORP150$^{+/-}$ (**B**) or control ORP150$^{+/+}$ (**A**) mice. One day after the last injection, brains were perfusion-fixed, and brain sections (10 μm) were processed for immunostaining with anti–tyrosine hydroxylase (TH) antibody, and either anti–activated caspase-12 antibody or anti–activated caspase-3 antibody. (**C**) Numbers of caspase 12- or caspase 3-activated neurons among TH(+) SNpc neurons were counted as described in the text and shown in **C**I, II. In each case, $n = 4$; mean ± SD is shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars)

CONCLUSIONS AND OPEN QUESTIONS

In the present study, we found that ORP150 was expressed in both human dopaminergic neuroblastoma SH-SY5Y cells and dopaminergic neurons of SNpc in mice after administration of MPTP. Although ORP150 protein was not upregulated in SH-SY5Y cells exposed to MPP$^+$, suppression of ORP150 expression in SH-SY5Y cells by infection with adenovirus carrying ORP150 antisense cDNA resulted in increase in neuronal cell death after MPP$^+$ exposure, suggesting that MPP$^+$ plays important roles in dopaminergic neurons. *In vivo*, ORP150 was induced in mice injected with MPTP for 2 consecutive days, consistent with our recent finding of enhanced immunoreactivity of GRP78/GRP94 after MPTP administration in mice (26). Analysis using mice heterozygous for ORP150 deficiency (ORP150$^{+/-}$ mice) revealed that both the number of TH-positive neurons in SNpc and the dopamine content in striatum decreased more markedly in ORP150$^{+/-}$ mice than in ORP150$^{+/+}$ mice after administration of MPTP. Caspase 12, a cell-death marker specific to ER stress, and caspase 3, a downstream cell-death effector, were both activated in SNpc dopaminergic neurons of ORP150$^{+/-}$ mice after MPTP injection. These observations suggest the importance of maintaining the ER environment in dopaminergic neurons exposed to neurotoxins such as MPP$^+$ and MPTP, and the crucial role of ORP150 in these processes.

The mechanisms underlying the enhancement of UPR-target gene expression in mice after MPTP injection and the protection of dopaminergic neurons by ORP150 are currently unknown. It is believed that MPTP decreases energy supply by inhibiting mitochondrial complex I activity and enhances the production of reactive oxygen species (ROS). MPTP also decreases intracellular glutathione (GSH) levels, which, in turn, alters the redox status of intracellular organelles, including the ER (23, 24). These events could perturb ER function and lead to ER stress directly or indirectly through reduction of cellular activity. Many of the molecular events in the ER, such as protein folding and protein oxidation, are ATP or redox dependent, and the ERAD system, which is required for degradation of proteins in the ER, also is ATP dependent (20, 27). The failure of upregulation of ORP150 protein expression in SH-SY5Y cells in response to MPP$^+$ treatment suggests that the deleterious effects of MPP$^+$ in the ER may be relatively mild, compared with those of other PD model drugs such as 6-OHDA in cultured cells, as previously described (1, 3). However, the findings that ORP150 was induced in mice injected with MPTP, and that suppression of ORP150 expression either by overexpression of ORP150 antisense cDNA in cultured cells or by using ORP150$^{+/-}$ mice enhanced the vulnerability of dopaminergic neurons to MPP$^+$ or MPTP, emphasized that maintenance of the ER and regulation of UPR play important roles in dopaminergic neurons. It is possible that environmental changes outside of neurons, including activation of glial cells, also participate in maintenance of the ER circumstances in neurons *in vivo*.

In conclusion, we found that ORP150 was induced in SNpc dopaminergic neurons after MPTP administration into mice, and that suppression of ORP150 expression resulted in increase of neuronal cell death after exposure to MPP$^+$ or MPTP. Our findings suggest the importance of the ER environment in maintaining the nigrostriatal pathways.

ABBREVIATIONS

ER, endoplasmic reticulum; GRP, glucose-regulated protein; HSP, heat-shock protein; MPP$^+$, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; 6-OHDA, 6-hydroxydopamine; PD, Parkinson disease; ROS, reactive oxygen species; SNpc, substantia nigra pars compacta; TH, tyrosine hydroxylase; UPR, unfolded protein response; UPS, ubiquitin–proteasome system; VTA, ventral tegmental area.

APPENDIX

1. Cell cultures, neurotoxin treatment, and adenovirus infection

Human dopaminergic neuroblastoma SH-SY5Y cells were maintained in DMEM with 15% FBS. The cells were treated with MPP^+ iodide (Sigma, St Louis, MO) for 24 h at 0–2 m*M* or for the indicated durations of time at 1 m*M*. Adenovirus vectors containing ORP150 sense or antisense cDNA (Ad/ORP150S and Ad/ORP150AS, respectively) were constructed as previously described (18). Control adenovirus carrying β-galactosidase (Ad/LacZ) was obtained from Riken Bioresource Center (Tsukuba, Japan). Infection was performed by incubating cells with Ad(s) at the indicated multiplicity of infection (moi) for 16 h. After further incubation in culture medium for 24 h, cells were harvested for Western blotting or challenged with MPP^+ (1 m*M*) for the indicated durations. Cell viability was measured by MTT assay (Nacalai Tesque, Osaka, Japan) or LIVE/DEAD cell toxicity assay (Molecular Probes, Eugene, OR), as previously described (4, 26).

2. Cell lysis and Western blotting

For analysis of the expression of ORP150, SH-SY5Y cells were harvested at the indicated times and solubilized in buffer containing 1% NP40, 0.1% SDS, and 0.2% deoxycholate. Protein extracts were then subjected to Western blotting with anti-ORP150 antibody (12) and anti–β-actin antibody (Sigma). Sites of primary antibody binding were visualized using alkaline phosphatase–conjugated secondary antibodies.

3. Animal experiments and histologic analysis

Animal experimental protocols were approved by the Committee on Animal Experimentation of Kanazawa University (Takara-machi Campus). Mice heterozygous for ORP150 deficiency ($ORP150^{+/-}$ mice) were prepared as previously described (9) and backcrossed into the C57BL/6 strain for 10 generations. All animal experiments were thus performed with $ORP150^{+/-}$ mice and their $ORP150^{+/+}$ littermates of C57BL/6 strain (male, 8–12 weeks old). MPTP (30 mg/kg) was intraperitoneally injected daily for the indicated number of days. One day after the last injection, brains were removed from mice after perfusion with 4% paraformaldehyde. Coronal brain sections (10 μm) were cut on a cryostat and processed for immunostaining with anti-ORP150 antibody, anti-tyrosine hydroxylase (TH) antibody (Chemicon International, Temecula, CA), anti–activated caspase-12 antibody (10), and anti–activated caspase-3 antibody (Genzyme, Cambridge, MA). FITC- or Cy3-conjugated secondary antibodies were used for visualization of immunolabeling.

4. Dopamine contents in striatum

Striatal tissue was homogenized in solution H (0.4 *M* $HClO_4$ containing 4 m*M* $Na_2S_2O_5$, 4 m*M* diethylenetriaminepentaacetic acid, and 5 m*M* 1,4-dithiothreitol). The dopamine and noradrenalin contents in the samples were then determined in a fully automated HPLC-fluorometric system (Model HLC-725CA Catecholamine Analyzer, Tosoh, Tokyo, Japan) using a diphenylethylenediamine condensation method (13).

5. Quantitative data analysis

Laser densitometric analysis was performed for semiquantification of results of Western blotting, as previously described (Hori *et al*., 2004). The numbers of TH(+), ORP150(+), caspase 3(+), and caspase 12(+) neurons in the SNpc were manually counted in two representative sections (bregma, –3.16 and –3.64 mm), as previously described (11) after acquiring digital images using a CCD camera (Hamamatsu Photonics, Shizuoka, Japan).

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