Compromising σ -1 Receptors at the Endoplasmic Reticulum Render Cytotoxicity to Physiologically Relevant Concentrations of Dopamine in a Nuclear Factor- κ B/Bcl-2-Dependent Mechanism: Potential Relevance to Parkinson's Disease

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ABSTRACT

The endoplasmic reticulum (ER) chaperone σ -1 receptor (Sig-1R) is cytoprotective against ER stress-induced apoptosis. The level of Sig-1Rs in the brain was reported to be lower in early parkinsonian patients. Because dopamine (DA) toxicity is well known to be involved in the etiology of Parkinson's disease, we tested in this study whether a relationship might exist between Sig-1Rs and DA-induced cytotoxicity in a cellular model by using Chinese hamster ovary (CHO) cells. DA in physiological concentrations (e.g., lower than 10 μ M) does not cause apoptosis. However, the same concentrations of DA cause apoptosis in Sig-1R knockdown CHO cells. In search of a mechanistic explanation, we found that unfolded protein response is not involved. Rather, the level of protective protein Bcl-2 is

critically involved in this DA/Sig-1R knockdown-induced apoptosis. Specifically, the DA/Sig-1R knockdown causes a synergistic proteasomal conversion of nuclear factor κ B (NF- κ B) p105 to the active form of p50, which is known to down-regulate the transcription of Bcl-2. It is noteworthy that the DA/Sig-1R knockdown-induced apoptosis is blocked by the overexpression of Bcl-2. Our results therefore indicate that DA is involved in the activation of NF- κ B and suggest that endogenous Sig-1Rs are tonically inhibiting the proteasomal conversion/activation of NF- κ B caused by physiologically relevant concentrations of DA that would otherwise cause apoptosis. Thus, Sig-1Rs and associated ligands may represent new therapeutic targets for the treatment of parkinsonism.

Introduction

The overproduction of reactive oxygen species (ROS) is a common underlying mechanism of many neurodegenerative diseases, because ROS can damage many cellular components including proteins (Slemmer et al., 2008). Parkinson's disease (PD) is one of the progressive neurodegenerative diseases of the nigrostriatal dopaminergic system, and its etiology is hypothesized to relate to the auto-oxidation of endogenous dopamine (DA) (Barzilai et al., 2001). ROS are produced mostly during the oxygen-using cellular metabolic processes, such as oxidative phosphorylation in the mito-

chondria. Neurotoxins may cause dysfunction of mitochondria and thus parkinsonian symptoms. It has been traditionally believed that DA-induced mitochondrial dysfunction plays a central role in the pathophysiology of Parkinson's disease. However, less well known is the role of the endoplasmic reticulum (ER), specifically the ER-derived ROS, in neurodegeneration. Emerging data suggest that oxidations in the ER, including that caused by disulfide bond formation or small-molecule metabolisms, may also contribute to the oxidative stress and cytotoxicity that lead to neurodegenerative disease (Harding et al., 2003). Nevertheless, neurological consequences of the ROS generated at the ER have not been extensively examined but are just beginning to be unveiled.

The ER is a major locus for the synthesis of proteins. Under physiological conditions, the overall protein level in the ER is maintained by a balance between protein synthesis and deg-

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ABBREVIATIONS: ROS, reactive oxygen species; AA, ascorbic acid; ANOVA, analysis of variance; ATF6, activating transcription factor 6; CHO, Chinese hamster ovary; CHX, cycloheximide; DA, dopamine; DCFH₂-DA, 2',7'-dichlorofluorescein diacetate; EM, emission; EOR, endoplasmic reticulum overload response; ER, endoplasmic reticulum; PERK, protein kinase R-like ER kinase; ERK, extracellular signal-regulated kinase; Ex, excitation; NF- κ B, nuclear factor κ B; PBS, phosphate-buffered saline; PD, Parkinson's disease; RT-PCR, reverse transcription-polymerase chain reaction; siCon, siControl; Sig-1R, σ -1 receptor; siRNA, small interfering RNA; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; UPR, unfolded protein response; XBP1, X-box binding protein 1.

radation. When the balance is perturbed, signaling pathways [the unfolded protein response (UPR) or the ER overload response (EOR)] are activated to regulate cell survival/death signaling (Pahl and Baeuerle, 1997; Wu and Kafuman, 2006). The UPR activates three ER sensor proteins, ATF6, PERK and serine/threonine-protein kinase/endonuclease, to increase the folding capacity by transcriptionally up-regulating ER molecular chaperones or suppressing global protein synthesis to prevent the accumulation of misfolded proteins in the ER (Wu and Kafuman, 2006). On the other hand, the EOR activates NF- κ B to mount a rapid and coordinated induction of proinflammatory genes (Pahl and Baeuerle, 1997). Thus, the ER Ca^{2+} depletion or blockade of ER-Golgi secretory pathway that causes accumulation of proteins in the ER can cause the activation of NF- κ B. However, the precise mechanism whereby ER-related NF- κ B activation controls cell survival/death is not well clarified.

The Sig-1R is a nonopioid receptor that resides specifically at the endoplasmic reticulum-mitochondrion interface and has been shown to be protective against neurodegeneration (Cormaci et al., 2007; Hayashi et al., 2010). However, the exact mechanism whereby Sig-1Rs protect against neurodegeneration is not totally understood. It is known that oxidative stressors can trigger ER stress (Holtz and O'Malley, 2003; Dukes et al., 2008). It is noteworthy that ER stress promotes the up-regulation of Sig-1Rs (Hayashi and Su, 2007). In alignment with this notion, we have demonstrated that knockdown of Sig-1R potentiates apoptosis induced not only by ER stress but also by free radicals (Hayashi and Su, 2007; Meunier and Hayashi, 2010). Moreover, Sig-1Rs were up-regulated in the substantia nigra and ventral tegmental area of methamphetamine-administered rats (Hayashi et al., 2010). Methamphetamine is well known to cause DA-related oxidative damage in neurons. It is noteworthy that a positron emission tomography study demonstrated that Sig-1Rs are down-regulated in the brains of patients in the early stage of Parkinson's disease (Mishina et al., 2005). Collectively, these findings suggest that the Sig-1R may represent a critical cellular element against DA-induced oxidative stress and associated neuronal apoptosis. Here, by using a cellular model, we found that Sig-1Rs at the ER equip CHO cells against apoptosis, which would otherwise be exerted by the physiological concentration of dopamine. Sig-1Rs do so by attenuating the activation of transcription factor nuclear factor κ B (NF- κ B) to increase the antiapoptotic protein Bcl-2. In addition, we found that this cellular protective action of Sig-1Rs is not related to the UPR, but may instead be associated with the EOR. We have chosen to use CHO cells in this study. CHO cells are known to contain no DA receptors or transporters and thus can be used as a model to study the apoptosis in the soma of neurons without interferences from neuronal circuitries.

Materials and Methods

Cell Cultures and Treatment. CHO cell cultures were maintained in a 175-cm² flask (Falcon; BD Biosciences Discovery Labware, Bedford, MA) with 10% fetal calf serum-containing α -minimum essential medium. Cells were cultured at 37°C with 5% CO₂ under saturating humidity. The medium was changed every 1 to 2 days. For drug treatment experiments, cells were seeded in 24-well plates (Falcon; BD Biosciences Discovery Labware). All drugs were dissolved in distilled H₂O.

Western Blotting. After treatments with drugs, cells were placed on ice, rapidly washed once with ice-cold phosphate-buffered saline (PBS), and harvested in PBS with a rubber policeman. Cell suspensions were centrifuged at 800g for 10 min at 4°C. The supernatants were discarded, and the pellets were dissolved with 2× Laemmli sample buffer (100 mM Tris-HCl, pH 6.8, 20% glycerol, and 4% SDS) followed by boiling for 3 min with β -mercaptoethanol and bromophenol blue.

Proteins (15–100 μ g) were resolved by SDS-polyacrylamide gel electrophoresis. Gels were electroblotted onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA) in Towbein buffer (25 mM Tris-base and 192 mM glycine) without methanol. Membranes were blocked with 10% nonfat dry milk (Bio-Rad Laboratories) in Tris-buffered saline plus 0.05% Tween 20 for 1 h at room temperature. Membranes were incubated overnight at 4°C with an antibody in Tris-buffered saline plus 0.05% Tween 20 and probed with the secondary anti-rabbit or anti-mouse IgG conjugated with horseradish peroxidase for 1 h at room temperature (1:1000 dilution). Protein bands were visualized with a SuperSignal West Femto reagent (Thermo Fisher Scientific, Waltham, MA) with a Kodak Image Station 440 CF (Carestream Health, Rochester, NY).

Specific antibodies were purchased as follows: phosphorylated PERK, ERK, Bcl-2, and NF- κ B p105/50 subunit from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The polyclonal anti-Sig-1R antibody was produced by immunizing rabbits with synthetic antigenic peptides. The antigenic peptide corresponds to the second hydrophilic domain of the rat Sig-1R (52-CLDHELAFSRRLIVELRLH-69). Anti-ATF6 antibody was provided by Dr. K. Mori (Kyoto University, Kyoto, Japan). Lipofectamine 2000 and Hoechst 33342 were purchased from Invitrogen (Carlsbad, CA). Dopamine, tunicamycin, cycloheximide (CHX), ascorbic acid, and lactacystin were from Sigma-Aldrich (St. Louis, MO). Oridonin was from EMD Biosciences (San Diego, CA), thapsigargin was from Calbiochem (La Jolla, CA), and dithiothreitol was from MP Biomedicals (Solon, OH).

Analysis of Protein Levels. Western blotting digital images were analyzed by using Kodak 1D Image Analysis Software. The background intensity was subtracted from the total intensity for each band. Sig-1R protein levels were normalized to ERK by dividing the intensities of Sig-1R bands by those from the total ERK. Total ERK was detected by reprobing the same membrane.

ROS Analysis. Superoxide levels were measured by using the fluorescent dye dihydroethidium. Cells were suspended at a concentration of 10×10^5 cells by RPMI medium 1640 containing 1 mM dihydroethidium. Then, cells were seeded to 96-well plates and incubated for 15 min. Dihydroethidium fluorescence was measured over 90 min by using a Victor plate reader (PerkinElmer Life and Analytical Sciences, Waltham, MA) set at Ex of 485 nm and Em of 530 nm after administration of dopamine. To analyze intracellular ROS formation, 2',7'-dichlorofluorescein diacetate (DCFH₂-DA) was used. After DA (10 μ M) exposure for different periods of times to cells, 500 μ M DCFH₂-DA was applied and incubated for 30 min at 37°C. After washing the cells by PBS, cells were lysed with 0.5% Triton X-100. Dihydroethidium fluorescence was measured over 90 min by using a Victor plate reader (PerkinElmer Life and Analytical Sciences) set at Ex of 485 nm and Em of 530 nm.

Vector Transfection. The cDNA encoding small interfering RNA (siRNA) was constructed in the pSIREN vector (Clontech, Mountain View, CA) as described previously (Hayashi and Su, 2004). The Bcl-2 pCIneo vector was kindly donated by Drs. Xingming Deng and W. Stratford May Jr. (University of Florida Shands Cancer Center, Gainesville, FL). Cells were transfected 2 days before experiments by applying the DNA/Lipofectamine-2000 mixture (1 μ g/2 μ l) to the culture medium. Concentrations of vectors in medium were 1 μ g/ml for Bcl-2 and 1 μ g/ml for Sig-1R siRNA/control siRNA. After 6 h, transfected cells were harvested and spread on a new plate.

Analysis of Apoptosis. CHO cells were incubated with 1 μ l/ml Hoechst 33342 and/or 20 μ l/ml trypan blue solution for 15 min at

room temperature in the culture medium. Annexin V staining (annexin V conjugates for apoptosis detection; Invitrogen) and TUNEL staining (Apo-BrdU In Situ DNA Fragmentation Assay Kit; MBL International, Woburn, MA) were carried out according to the manufacturer's instructions. Microscopic images were collected by using a Zeiss 40 $\times$ microscope (Carl Zeiss Inc., Thornwood, NY) with the X-Cite 120 Fluorescence Illumination system (Lumen Dynamics, Mississauga, Ontario, Canada) and iVision (BioVision Technologies, Mountain View, CA). Fluorescence filter sets were: Ex = 470 nm and Em = 525 nm for Hoechst 33342 and TUNEL staining or Ex = 550 nm and Em = 605 nm for annexin V staining.

XBP1 Splicing Assay. Total RNA was prepared by using the NucleoSpin RNA II kit (Macherey-Nagel, Bethlehem, PA). The reverse transcription-polymerase chain reaction (RT-PCR) for XBP1 mRNA was performed by using the titanium one-step RT-PCR kit (Clontech) with 0.2 μ g of total RNA and 50 μ l of the reaction mixture under the following thermal cycle: 50°C for 60 min, 94°C for 5 min, 25 cycles of 94°C (30 s), 57°C (30 s), 68°C (60 s), followed by 68°C for 2 min and 4°C (GeneAmp PCR System 9700; Applied Biosystems, Foster City, CA). XBP1 mRNA was amplified by primers 5'-AAACA-GAGTAGCAGCGCAGAC-3' and 5'-GGGATCTCTAAGACTAGAGGC-3'. PCR products were analyzed by 2% agarose electrophoresis followed by imaging with Kodak Image Station 440CF under UV light.

Statistical Analysis. All of the quantifications for Western blotting and RT-PCR were performed by using Kodak 1D Image Analysis Software. Data were submitted to statistical analyses using Prism 3.0cx software (GraphPad Software Inc., San Diego, CA). Data are presented as percentage of control with S.E.M. The level of statistical significance was $p < 0.05$. For data with multiple groups, one-way analysis of variance (ANOVA) followed by Turkey test and/or two-way ANOVA followed by Bonferroni post hoc test were used.

Results

Effects of Dopamine on Apoptosis and Necrosis.

Within 6 h after addition into the cell cultures, DA (1–1000 μ M) did not cause apoptosis or necrosis in CHO cells, except at 1000 μ M DA tended to increase apoptosis. At the 16-h treatment time point, however, DA enhanced apoptosis at 100 to 1000 μ M and increased necrosis at 1000 μ M (Fig. 1, A and B). Sig-1R siRNAs caused a knockdown of approximately 80% of Sig-1Rs (Fig. 1C). In Sig-1R knockdown cells, DA, even at 10 μ M, could cause a significant enhancement of apoptosis both at the 6- and 16-h time points. However, necrosis was not seen unless the concentration of DA exceeded 500 μ M (Fig. 1C). Furthermore, DA (10 μ M; 6 h) could significantly increase the percentage of annexin V-positive cells in Sig-1R knockdown cells but not in control cells (Fig. 1D). Likewise, dopamine (10 μ M; 16 h) increased the percentage of TUNEL-positive cells in Sig-1R knockdown CHO cells (Fig. 1D). These results strongly indicate that Sig-1Rs exert cytoprotective effects against DA-induced cellular damage. The potential relation between DA and Sig-1Rs in this regard is examined in the following sections.

Dopamine Regulates Sig-1R Expression. DA at 10 to 500 μ M significantly increased the expression of Sig-1Rs and the ER chaperone protein (Figs. 1C and 2A). The Sig-1Rs were progressively increased over 3 h (Fig. 2A). DA (1–100 μ M) significantly increased ROS (Fig. 2B). We reported previously that the knockdown of Sig-1Rs increased the accumulation of ROS (Meunier and Hayashi, 2010), and we found here again that the basal level of intracellular ROS in Sig-1R knockdown cells was higher than that seen in wild-type cells (Fig. 2C). DA (10 μ M) significantly increased the intracellular ROS level in both wild-type and Sig-1R knockdown CHO

cells. We found that the increased expression of Sig-1Rs caused by DA (10 μ M; for 1 h) was completely blocked by pretreatment of cells with 0.56 to 5.6 mM ascorbic acid applied 10 min before DA (Fig. 2C) and the DA effect could be reduced by actinomycin D (data not shown). DA may thus increase Sig-1R gene transcription via an oxidative stress-related mechanism. These results also suggest that the up-regulation of Sig-1R at the ER may play a role against DA-induced cytotoxicity.

UPR Is Not Elicited by Physiological Concentrations of Dopamine. It is known that the overexpression of Sig-1Rs regulates UPR signaling, whereas the knockdown of Sig-1Rs enhances apoptosis induced by ER stress (Hayashi and Su, 2007). Because oxidative stress could be an ER stressor, the apoptosis triggered by DA in Sig-1R knockdown cells may relate to the UPR. Here, we examined the UPR activation caused by DA. DA, at 10 μ M (1 h) that is comparable with the concentration in the synaptic region (Kebabian and Greengard, 1971), did not affect the activation of ATF6 or PERK even in Sig-1R knockdown cells (Fig. 3A). Furthermore, DA did not induce the splicing of XBP1 mRNA, a downstream effector of serine/threonine-protein kinase/endonuclease signaling even in Sig-1R knockdown cells (Fig. 3B). Therefore, DA-induced apoptosis in Sig-1R knockdown cells is unrelated to the UPR.

Knockdown of Sig-1Rs Reduces Bcl-2 Levels by Activating NF- κ B. We reported previously that the knockdown of Sig-1Rs caused a ROS-dependent up-regulation of NF- κ B followed by a down-regulation of Bcl-2 (Meunier and Hayashi, 2010). We speculated therefore that the NF- κ B/Bcl-2 pathway may relate to the DA-induced apoptosis in Sig-1R knockdown cells.

We found that either the knockdown of Sig-1Rs or DA treatment (10 μ M; 6 h) could independently up-regulate p105, the precursor of the NF- κ B complex (Fig. 4A). However, the use of DA in Sig-1R knockdown cells (Fig. 4A) did not cause an anticipated synergistic increase of p105, indicating that up-regulation of p105 is maximized by either the knockdown of Sig-1Rs or DA treatment. Knockdown of Sig-1R also significantly increased the level of p50, the active component of the NF- κ B complex (Fig. 4B). Those results support our previous results that endogenous Sig-1Rs attenuate the ROS-induced activation of NF- κ B. We found that p50 levels were only slightly increased by DA (10 μ M; 6 h) in wild-type cells but were significantly enhanced by DA in Sig-1R-knockdown cells. Those results suggest that endogenous Sig-1Rs may tonically inhibit the conversion of p105 to p50 and thus counteract the p105-to-p50 converting action of DA.

Because our previous study indicated that Sig-1Rs regulate the transcriptional expression of Bcl2 via the activation of NF- κ B in a ROS-dependent manner (Meunier and Hayashi, 2010), we examined next whether DA may affect the Bcl2 expression via the Sig-1R-ROS-NF- κ B pathway. The knockdown of Sig-1Rs or the DA treatment (10 μ M; 6 h) caused a down-regulation of Bcl-2. When DA was used in Sig-1R-knockdown cells, the Bcl-2 level was decreased in a negatively additive manner (Fig. 4C). To address the mechanism whereby DA caused the down-regulation of Bcl-2 in Sig-1R knockdown cells, we used oridonin to disrupt the interaction between NF- κ B and DNA (Ikezoe et al., 2005). Consistent with our previous results, oridonin itself promoted a slight up-regulation of Bcl-2 in both control and

Fig. 1. Enhanced DA-induced cell death in Sig-1R knockdown CHO cells. **A**, DA-induced chromatin condensation. CHO cells were labeled with Hoechst 33342 (1:1000) 16 h after administration of DA (1–1000 μM). **B**, quantification of the induction of apoptosis/necrosis-induced by DA. Hoechst 33342 (1:1000) (left) or trypan blue (1:100) (right) were applied 6 or 16 h after administration of DA. One-way ANOVA of effects of dopamine treatments on cell death were: $F_{5,42} = 10.67$ (6 h on apoptosis), 14.79 (16 h on apoptosis), and 43.71 (16 h on trypan blue), $p < 0.001$. **, $p < 0.01$ and ***, $p < 0.001$ compared with nontreatment. The percentage of apoptotic cells was counted in eight fields (>10 cells/field) and is reported as mean $\pm$ S.E.M.; $n = 8$. **C**, effects of knockdown of Sig-1R on 10 μM DA-induced apoptosis in CHO cells. Two-way ANOVA of effects of siRNA transfection on cell death were: $F_{5,84} = 6.09$ (6 h on apoptosis), 14.48 (16 h on apoptosis), 5.84 (6 h on trypan blue), and 5.20 (16 h on trypan blue), $p < 0.001$; $F_{4,29} = 9.578$, $p = 0.0003$. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with siRNA control (Bonferroni post hoc test). Sig-1Rs were knocked down by transfecting vectors in CHO cells as described under *Materials and Methods*. Total cell lysates (15 $\mu\text{g}/\text{lane}$) were analyzed by Western blotting. As control, the pSIREN vector carrying an inactive siRNA (control siRNA) or the empty pCR3.0 vector was transfected. Veh, vehicle; $n = 8$. **D**, confirmation of 10 μM DA-induced apoptosis by annexin V and TUNEL staining. Tm, tinicamycin (1 mM). One-way ANOVA of effects of DA treatments on cell death were: $F_{2,21} = 15.86$, $p = 0.0002$ (for annexin V) and $F_{2,21} = 17.88$, $p = 0.0005$ (for TUNEL). ***, $p < 0.001$ compared with nontreatment; $n = 6$.

Sig-1R knockdown cells (Fig. 4C). We found that the action of DA potentiating the down-regulation of Bcl-2 in Sig-1R knockdown cells was completely blocked by oridonin, indicating that the DA-induced down-regulation of Bcl-2 depends on NF- κB signaling (Fig. 4C).

As indicated above, the DA (10 μM)-induced apoptosis was observed in Sig-1R knockdown cells (Fig. 1C). We found that

the overexpression of Bcl-2 was able to block DA (10 μM)-induced apoptosis in Sig-1R knockdown cells (Fig. 4D). These results suggest that the Sig-1R-NF- κB -Bcl-2 pathway plays a crucial role against DA-induced apoptosis.

Sig-1Rs Regulate the Activation of NF- κB . At least two pathways are proposed to regulate the expression of p50 (e.g., cotranslational and post-translational mechanism) (Pereira

Fig. 2. DA effect on Sig-1R expression is mediated by ROS. **A**, effects of DA on expression of Sig-1Rs. One-way ANOVA of effects of DA treatment on Sig-1R levels were: $F_{4,25} = 3.683$, $p = 0.0148$. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with nontreatment. Total cell lysates (30 μ g/lane) were analyzed by Western blotting. ERK is for the loading controls. Data represent means $\pm$ S.E.M. of six to eight independent experiments. **B**, detection of superoxide generated by DA (1–100 μ M) using dihydroethidium fluorescence. One-way ANOVA of effects of DA treatments on cell death were: $F_{8,88} = 1.94$ (for 1 μ M DA), 4.17 (for 10 μ M DA), and 3.12 (for 100 μ M DA), $p < 0.0001$. *, $p < 0.05$ and ***, $p < 0.001$ compared with before treatment; $n = 12$. **C**, measurement of ROS generated by 10 μ M DA using DCFH₂-DA in Sig-1R knockdown CHO cells. The y-axis represents arbitrary units of fluorescence after background subtraction. Student's *t* test indicates significant difference of ROS levels between wild-type and Sig-1R knockdown CHO cells ($p < 0.01$). One-way ANOVA of effects of DA on ROS levels were: $F_{4,55} = 6.757$, $p = 0.0002$ in control cells and $F_{4,55} = 13.89$, $p < 0.0001$ in Sig-1R knockdown cells. **, $P < 0.05$ compared with siCont; ###, $P < 0.001$ compared with time 0; $n = 6$. **D**, effects of ascorbic acid on the up-regulation of Sig-1Rs induced by 10 μ M DA. Ascorbic acid (AA) was applied to the culture medium 10 min before DA (10 μ M, 1 h). One-way ANOVA of effects of ascorbic acid treatments on DA-induced up-regulation of Sig-1Rs were: $F_{3,52} = 8.176$, $p < 0.0001$. **, $p < 0.01$ compared with nontreatment. ###, $P < 0.001$ compared with dopamine alone; $n = 6$.

and Oakley, 2008). The cotranslational expression of p50 is regulated by the conversion of p105 to p50 during the translation of p105, thus this conversion largely depends on the activity of protein synthesis at the ER and could be blocked by cycloheximide. The post-translational mechanism is regulated by the conversion of a priori existing matured p105 into p50 that in part depends on the level of accumulated ROS in the cell. The proteasomal degradation of p105, however, plays a role in either of the conversion pathways (Skaug et al., 2009). To elucidate which pathway is affected by Sig-1R and/or DA (10 μ M), we first examined the overall stability of existing p105 and p50 in the CHO cell by 1-h pretreatment with CHX in DA-challenged cells. p50 completely disappeared 6 h after the CHX treatment (data not shown), indicating that the protein turnover of p50 is considerably rapid. Therefore, p50 could not be detected beyond 6 h

after the DA treatment in this paradigm. P105, however, showed only marginal reductions in the presence of CHX for more than 7 h, indicating that p105 is relatively stable in CHO cells (Fig. 5A). Given those results, we assessed the proteasomal conversion of p105 to p50 by measuring the remaining level of p105.

Please note that because CHX inhibits the translational synthesis of new p105, the “cotranslational mechanism” in the conversion of p105 to p50 is excluded from the system and only the post-translational mechanism exists in the system. We found that in the absence of DA the degradation kinetics of p105 were similar between control and Sig-1Rs knockdown cells (Fig. 5B). However, because the basal level of p105 was much higher in Sig-1R knockdown cells, the total number of p105 converted to p50/time is probably higher in Sig-1R knockdown cells. This result is consistent with what we see

Fig. 3. Effect of DA on UPR in wild-type and Sig-1R knockdown CHO cells. **A**, effects of dopamine on the activation of ATF6 and PERK on wild-type and Sig-1R siRNA-treated CHO cells. CHO cells transfected with control or Sig-1R siRNA were treated with DA (10 μ M) for the indicated periods of time. Total cell lysates (80 μ g/lane) were analyzed by Western blotting. ERK is for the loading controls. The appearance of p50 indicates the activation of ATF6, whereas that of the phosphorylation form indicates activation of PERK. DTT, dithiothreitol; Tg, thapsigargin. **B**, effects of DA on the splicing of XBP1 mRNA in wild-type and Sig-1R knockdown CHO cells. Total RNA was extracted, and XBP-1 transcripts were amplified by RT-PCR. Un, unspliced; S, spliced.

in Fig. 4B in which the basal level of p50 is higher in Sig-1R knockdown cells (Fig. 4B, bottom). DA, on the other hand, was found to dramatically increase the rate of p105 degradation in Sig-1R knockdown cells but not control cells (Fig. 5B). The results with DA suggest that DA apparently activates the “post-translational mechanism” in the conversion of p105 to p50 only when cells are low in Sig-1Rs.

It is known that the activation of NF- κ B, i.e., the conversion of p105 to p50, involves the proteasomal degradation of p105. In fact, we found that the proteasome inhibitor lactacystin not only largely increased the stability or the basal level of p105 but also completely abolished the DA's effect in potentiating the degradation of p105 in Sig-1R knockdown cells (Fig. 5C). These results indicate that the molecular mechanism whereby Sig-1Rs protect CHO cells against DA-induced decrease in the gene expression of Bcl-2 involves the inhibition of proteasomal degradation of p105.

Discussion

A growing body of evidence has demonstrated that DA exerts its toxic effect through the production of ROS either in vivo or in vitro. In the present study, we found that physiological concentrations of DA (1–10 μ M) significantly produced ROS in our system and should therefore be cytotoxic. However, neurons survive under those concentrations of DA. Therefore, neurons may possess endogenous counteracting factors that protect neurons from DA-induced ROS. In this

study, we used CHO cells as a model system to address this issue and found that DA at physiological concentrations (10 μ M) could induce apoptosis when CHO cells were depleted of Sig-1Rs. Thus the Sig-1R is one of the endogenous substrates that counteracts the cytotoxicity of DA. Although we used CHO cells in this study, our findings may have an important bearing on the dopaminergic system in the brain. For example, under normal physiological conditions when the extracellular DA concentration is relatively low because the DA is taken up by the DA transporter or is degraded by DA-degrading enzymes our results suggest that the physiological levels of Sig-1Rs may counteract the toxicity of DA. However, when the extracellular level of DA is increased in the brain, for instance, because of the use of methamphetamine or cocaine, our results would predict that in response to the insult caused by the increased concentration of DA Sig-1Rs would increase (see Fig. 2B) to counteract DA-induced toxicity. Because Sig-1Rs are ER proteins existing mainly in the soma of neurons, our results suggest that the Sig-1Rs protect the soma of neurons against extracellular DA toxicity. Relevant to those suggestions, it is interesting to note that Sig-1Rs are increased in the substantia nigra of rats that have been exposed to methamphetamine (Hayashi et al., 2010). Thus, Sig-1Rs may protect the substantia nigra against DA surge caused either by drugs or pathological conditions.

We found in this study that Bcl-2 plays a critical role in the survival of CHO cells against DA-induced apoptosis and the Sig-1Rs' main action against the cytotoxic action of DA is to counteract DA's action in the activation of NF- κ B, which suppresses the gene transcription of Bcl-2. Inasmuch as Sig-1Rs are ER proteins, we speculate that this action of Sig-1Rs take place most probably at the ER. Although DA-induced ROS or damages are often associated with mitochondria-related biochemical events, we infer that the DA effect seen in this study, i.e., within the context of Bcl-2 and its regulation by Sig-1Rs, is ER-related because DA at the studied concentration of 10 μ M did not affect the procaspase-3 level in mitochondria (our unpublished data).

It is interesting to note that the cellular protective action against DA afforded by Sig-1Rs is unrelated to the UPR. We have recently shown that Sig-1Rs attenuate the activation of NF- κ B in a ROS-related manner (Meunier and Hayashi, 2010). In the present study, we found that DA up-regulates Sig-1Rs via the production of ROS. It is possible therefore that Sig-1Rs up-regulated by DA represent a defense mechanism whereby cells fight against DA-induced damage by responding to the presence of DA in the cell by producing more Sig-1Rs. Because the effects of DA or Sig-1Rs in our study do not relate to UPR but are related to the NF- κ B signaling and NF- κ B signaling is related to the so-called EOR (Pahl and Baeuerle, 1997; Kuang et al., 2005) that is also induced by ER stress, we surmise that the cytoprotective effect of Sig-1Rs against DA seen here most likely is related to the activation of EOR.

DA toxicity is related to PD. As mentioned in the beginning of *Discussion*, our results in this study suggest that Sig-1Rs may protect against DA-induced cytotoxicity and thus likely against PD. The Sig-1Rs' action may involve the EOR as shown in this study. Although the UPR has been implicated in PD (Dauer and Przedborski, 2003; Smith et al., 2005; Hoozemans et al., 2007), our current results suggest that the EOR may be involved as well. It is noteworthy that NF- κ B,

Fig. 4. Sig-1Rs attenuating the activation of NF- κ B to maintain the expression of Bcl-2 to protect cells against DA-induced cytotoxicity. A and B, effects of DA (10 μ M for 6 h) and Sig-1R knockdown on the levels of p105 (A) and p50 (B) subunits of the NF- κ B complex. CHO cells transfected with control or Sig-1R siRNA were treated with DA (10 μ M) for the indicated periods of time. Total cell lysates (50 μ g/lane) were analyzed by Western blotting. Levels of p50 or p105 analyzed by Western blotting were normalized to ERK and are shown as percentage of control in the graph. One-way ANOVA of effects of DA treatments on NF- κ B levels were: $F_{3,15} = 4.345$ (for p105) and 19.22 (for p50), $p = 0.014$ and < 0.0001 , respectively. *, $p < 0.05$ compared with siCon. ##, $p < 0.05$ compared with nontreatment; $n = 5$. C, involvement of the NF- κ B pathway in down-regulation of Bcl-2 levels in Sig-1R knockdown cells by dopamine. Total cell lysates (30 μ g/lane) were analyzed by Western blotting. Six hours after transfection with siRNA control vector or Sig-1R knockdown vector, cells were cultured with oridonin (1 μ g/ml) for the following 18 h. One-way ANOVA of effects of oridonin treatments on cell death were: $F_{2,18} = 5.965$ (for siCon), $p = 0.0103$ and 21.78 (for siSig-1R), $p < 0.0001$. *, $p < 0.05$ compared with siControl. ##, $p < 0.01$ and ###, $p < 0.001$ compared with DA alone. Two-way ANOVA of effects of siRNA transfection on cell death were: $F_{3,48} = 8.35$, $p = 0.0012$. *, $p < 0.05$ compared with control siRNA (Bonferroni post hoc test; $n = 7$). D, effects of overexpression of Bcl-2 on the apoptosis-induced by combined effects of DA and knockdown of Sig-1Rs. Twenty-four hours after transfection with siRNA and/or Bcl-2 expression vectors, CHO cells were treated with DA (10 μ M) for 6 h. One-way ANOVA of effects of overexpression of Bcl-2 on dopamine-induced apoptosis in Sig-1R knockdown cell were: $F_{3,28} = 11.25$, $p < 0.0001$. ***, $p < 0.001$ compared with siControl. ###, $p < 0.001$ compared with nonoverexpression. The percentages of apoptotic cells were counted in eight fields (> 10 cells/field) and are reported as mean $\pm$ S.E.M.; $n = 7$.

which is EOR-related, is activated within the substantia nigra of patients with PD and PD animal models (Ghosh et al., 2007; Karunakaran and Ravindranath, 2009). Sig-1Rs are reported to be decreased in the putamen, a terminal

region of the nigrostriatal dopaminergic pathway, in patients in the early stage of Parkinson's disease (Mishina et al., 2008; Toyohara et al., 2009). Therefore, a lowered level of the ER chaperone Sig-1R in the brain may cause increases of

Fig. 5. Sig-1R inhibits the conversion of NF- κ B by regulating the proteosomal degradation of p105. **A**, evaluation of the conversion of NF- κ B in Sig-1R knockdown CHO cells by DA (10 μ M). Effect of CHX (1 mM) on the levels of p105 in Sig-1R knockdown cells. Forty-eight hours after transfection with siRNA vectors, CHO cells were treated with DA (10 μ M) for 6 h. CHX (1 mM) was applied into the medium 1 h before DA. One-way ANOVA of effects of CHX on expression of p105 were: $F_{3,24} = 14.42$, $p < 0.0001$. **, $p < 0.05$ and ***, $p < 0.001$ compared with nontreatment; $n = 8$. **B**, kinetics of p105 degradation in CHO cells transfected with control or Sig-1R knockdown with or without treatment of DA (10 μ M). Forty-eight hours after transfection, CHO cells were treated with CHX (1 mM) for different periods to prevent protein synthesis. Levels of p105 were measured by Western blotting. The protein level at each time point is shown as percentage of time 0 h of the respective groups; $n = 8$. **C**, effects of lactacystin of p105 levels in Sig-1R knockdown CHO cells with or without treatment of DA. Forty-eight hours after transfection with siRNA vectors, CHO cells were treated with DA (10 μ M) for 6 h. CHX and lactacystin were pretreated with DA for 1 h. One-way ANOVA of effects of lactacystin treatments on Sig-1R knockdown cells were: $F_{3,24} = 28.61$, $p < 0.0001$, respectively. ###, $p < 0.001$; ##, $p < 0.01$; and *, $p < 0.05$ compared with nontreatment; $n = 8$. **D**, schematic drawing of the related pathways.

misfolded and aggregated proteins and also the activation of EOR that eventually may lead to the neuropathology of PD.

In conclusion, although DA is cytotoxic to cells, cells are equipped with endogenous counteracting factors like Sig-1Rs to respond to the damaging effect caused by DA. Compromising Sig-1Rs may thus render cytotoxicity to the physiological concentration of DA because nonfunctional Sig-1Rs will lead to the activation of NF- κ B that in turn would decrease the gene expression of the antiapoptotic protein Bcl-2 and perhaps thus lead to central nervous system diseases.

Authorship Contributions

Participated in research design: Mori, Hayashi, and Su.

Conducted experiments: Mori and Hayashi.

Performed data analysis: Mori, Hayashi, and Su.

Wrote or contributed to the writing of the manuscript: Mori, Hayashi, and Su.

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