Proteasome Inhibition Induces Glutathione Synthesis and Protects Cells from Oxidative Stress

RELEVANCE TO PARKINSON DISEASE*

Received for publication, April 18, 2006, and in revised form, December 6, 2006. Published, JBC Papers in Press, December 6, 2006, DOI 10.1074/jbc.M603712200

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The cause of selective dopaminergic neuronal degeneration in Parkinson disease has still not been resolved, but it has been hypothesized that oxidative stress and the ubiquitin-proteasome system are important in the pathogenesis. In this report, we investigated the effect of proteasome inhibition on oxidative stress-induced cytotoxicity in PC12 cells, an in vitro model of Parkinson disease. Treatment with proteasome inhibitors provided significant protection against toxicity by 6-hydroxydopamine and H2O2 in a concentration-dependent manner. The measurement of intracellular reactive oxygen species using 2′,7′-dichlorofluorescin diacetate demonstrated that lactacystin, a proteasome inhibitor, significantly reduced 6-hydroxydopamine- and H2O2-induced reactive oxygen species production. Proteasome inhibitors elevated the amount of glutathione and phosphorylated p38 mitogen-activated protein kinase (MAPK) prior to glutathione elevation. The treatment with lactacystin induced the nuclear translocation of NF-E2-related factor 2 (Nrf2) and increased the level of mRNA for γ-glutamylcysteine synthetase, a rate-limiting enzyme in glutathione synthesis. Furthermore, SB203580, an inhibitor of p38 MAPK, abolished glutathione elevation and cytoprotection by lactacystin. These data suggest that proteasome inhibition afforded cytoprotection against oxidative stress by the elevation of glutathione content, and its elevation was mediated by p38 MAPK phosphorylation.

Parkinson disease (PD)‡ is characterized by the selective loss of dopaminergic neurons and by the appearance of Lewy bodies in the midbrain. Among the various causes suspected in the pathogenesis of PD, oxidative stress and the ubiquitin-proteasome system (UPS) have been thought to play important roles (1, 2).

The involvement of oxidative stress is supported by postmortem studies showing increased lipid peroxidation (3), oxidative DNA damage (4), and protein carbonylation (5, 6). Endogenous catecholamines may participate in neurodegeneration because sympathetic norepinephrine neurons as well as mesencephalic dopaminergic neurons are degenerated (7, 8). Since dopamine is metabolized by monoamine oxidase B and easily auto-oxidized to produce harmful reactive oxygen species (ROS), this neurotransmitter contributes, as dopaminergic neuron-specific oxidative stress, to the selective loss of dopaminergic neurons.

In the midbrain of patients with PD, the activities of glutathione peroxidase and catalase were reduced, and the amount of glutathione was decreased (9–11). These observations suggested that antioxidant defense systems are collapsed in the dopaminergic neurons of patients with this disease. Several previous reports suggested that the reduced form of GSH is the most abundant cellular antioxidant and plays a pivotal role in antioxidant defense systems (12, 13). Our previous study demonstrated that the depletion of GSH content increased susceptibility to nitric oxide (14). Furthermore, GSH provided potent neuroprotection against catecholaminergic neurotoxins, such as 6-hydroxydopamine and dopamine (15, 16).

Recently, another important hypothesis concerned with the UPS has been developed. The UPS is a major mechanism responsible for the degradation of short-lived, damaged, and misfolded proteins (17). Autosomal recessive juvenile parkinsonism is caused by loss-of-function mutations of the gene encoding parkin (18), a ubiquitin-protein isopeptide ligase (E3) of the UPS (19). In postmortem brains with sporadic PD, a decrease in proteasome activity has been found (20). Although it is suggested that impairment of the UPS has been implicated in the pathogenesis of PD, several groups have reported that proteasome inhibition is also able to provide neuroprotection (21, 22). Proteasome inhibition increases the levels of molecular chaperones including heat shock proteins, and enhances cell

* This work was supported by grants-in-aid for Scientific Research from the Japan Society for the Promotion of Science and from the Ministry of Education, Culture, Sports, Science and Technology of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: PD, Parkinson disease; AMC, 7-amino-4-methylcoumarin; ARE, antioxidant-responsive element; DCF-DA, 2′,7′-dichlorofluorescein diacetate; DMEM, Dulbecco’s modified Eagle’s medium; DTNB, 5,5′-di-thiobis-(2-nitrobenzoic acid); ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; γ-GCS, γ-glutamylcysteine synthetase; GLCLC, glutamate-L-cysteine ligase catalytic; HO-1, heme oxygenase-1; MG-132, benzyloxycarbonyl-Leu-Leu-leucinal; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, N-acetyl-L-cysteine; NF-κB, nuclear factor-κ B; NGF, nerve growth factor; Nrf2, NF-E2-related factor 2; 6-OHDA, 6-hydroxydopamine; PSI, proteasome inhibitor I; ROS, reactive oxygen species; UPS, ubiquitin-proteasome system.
resistance to various insults (23, 24). It is known that nuclear factor-κB (NF-κB) translocates to the nucleus, where it binds to specific promoter sequences, initiating the transcription of NF-κB-dependent genes, many of which are mediators of inflammatory response (25). In dopaminergic neurons of patients with PD, nuclear translocation of NF-κB is enhanced (26), and proteasome inhibition suppresses the activities of NF-κB by stabilizing the inhibitory protein IκB (27). Moreover, we demonstrated that proteasome inhibition accelerated the appearance of α-synuclein-positive inclusion bodies and provided neuroprotection (28, 29).

The relationship, however, between proteasome activity and antioxidant effects has not been clarified. Previous studies indicated that undifferentiated PC12 cells have been shown to synthesize, store, release, and metabolize dopamine as well as differentiated PC12 cells (30, 31). Therefore, undifferentiated PC12 cells in this study have important features that are shared with dopaminergic neurons such as synthesis and storing dopamine and are suitable for a dopaminergic neuronal model. Furthermore, the dopaminergic cells develop features of neurons upon treatment with nerve growth factor (NGF). Differentiated PC12 cells by NGF possess many important neurochemical and signal transduction processes similar to dopaminergic neurons (32). In this study, we particularly focused on the antioxidant effect of glutathione and investigated the effect of proteasome inhibition on oxidative stress-induced cytotoxicity in differentiated and non-differentiated PC12 cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). 6-Hydroxydopamine hydrochloride and N-acetyl-L-cysteine were purchased from Sigma. 5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB), NADPH, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Glutathione (reduced form) and hydrogen peroxide were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Lactacystin, benzoyloxycarbonyl-Leu-Leu-leucinal (MG-132), benzyloxycarbonyl-Ile-Pro-OH, and hydrogen peroxide were from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). 6-Hydroxydopamine hydrochloride and N-acetyl-L-cysteine were purchased from Sigma. 5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB), NADPH, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Glutathione (reduced form) and hydrogen peroxide were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Lactacystin, benzoyloxycarbonyl-Leu-Leu-leucinal (MG-132), benzyloxycarbonyl-Ile-Pro-OH, and hydrogen peroxide were from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). 6-Hydroxydopamine hydrochloride and N-acetyl-L-cysteine were purchased from Sigma. 5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB), NADPH, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Glutathione (reduced form) and hydrogen peroxide were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Lactacystin, benzoyloxycarbonyl-Leu-Leu-leucinal (MG-132), benzyloxycarbonyl-Ile-Pro-OH, and hydrogen peroxide were from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). 6-Hydroxydopamine hydrochloride and N-acetyl-L-cysteine were purchased from Sigma. 5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB), NADPH, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Glutathione (reduced form) and hydrogen peroxide were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Lactacystin, benzoyloxycarbonyl-Leu-Leu-leucinal (MG-132), benzyloxycarbonyl-Ile-Pro-OH, and hydrogen peroxide were from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). 6-Hydroxydopamine hydrochloride and N-acetyl-L-cysteine were purchased from Sigma. 5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB), NADPH, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Nacalai Tesque, Inc. (Kyoto, Japan).

**PC12 Cell Cultures**—Rat adrenal pheochromocytoma PC12 cells were maintained in DMEM supplemented with 5% fetal calf serum and 10% horse serum. Cell cultures were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO2. PC12 cells were seeded in 48-well culture plates for MTT assay, in 35-mm dishes for glutathione analysis and Western blotting, and in 8-well culture slides for ROS evaluation at a density of 4 × 104 cells/cm2. Cells were grown for 24 h and then exposed to drugs. The EC50 was determined by regression analysis according to the following formula. Cell survival (percentage) = 100 × EC50H/(EC50 + [Drug]) (where [Drug] is the drug concentrations and H is the Hill slope) The analysis was performed using GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego, CA).

**Induction of Differentiation of PC12 Cells**—In part of the experiment, PC12 cells were differentiated by NGF. PC12 cells were seeded in poly-L-lysine-coated 48-well culture plates at a density of 4 × 103 cells/cm2. PC12 cells were treated with 50 ng/ml NGF for 7 days and then exposed to drugs.

**Evaluation of Viability**—The cell viability of PC12 cells was determined by MTT assay. The protocol of the MTT assay was described previously (33). Briefly, the activity of dehydrogenase, which reduces the MTT tetrazolium salt to MTT formazan, was measured by spectrophotometry. The culture medium was replaced with serum-free DMEM containing 0.5 mg/ml MTT tetrazolium salt and incubated at 37 °C for 30 min. The medium was removed, and the cells were scraped and solubilized into isopropyl alcohol solution. Aliquots were transferred to a 96-well plate, and absorbance was measured at 595 nm. The viability of the cultures was expressed as a percentage of the absorbance measured in control cells.

**Evaluation of ROS**—The average level of intracellular oxygen radicals was measured using DCF-DA dye. The fluorescence of cell-permeable DCF-DA significantly increases after oxidation. PC12 cells were preincubated in phosphate-buffered saline containing 30 μM DCF-DA for 20 min and washed with drug-free DMEM. Cells were exposed to 6-OHDA and H2O2 for 30 min, and fluorescence was detected and photographed with an Olympus IX 81 photomicroscope (Olympus Optical, Tokyo, Japan). ROS formation in the cells was evaluated by the fluorescence intensity of 80–120 randomly selected cells.

**Glutathione Analysis**—Glutathione concentration was determined colorimetrically according to how it was oxidized by DTNB (34). PC12 cells were washed with Tris-buffered saline on ice and deproteinized with 10% trichloroacetic acid. The samples were centrifuged, and the supernatants were mixed with 1 m phosphate-buffered saline, 0.5 mM EDTA, 0.4 mM NADPH. When total glutathione (oxidized and reduced glutathione) concentration was determined, glutathione reductase was added and incubated for 10 min, whereas when only reduced glutathione concentration was determined, glutathione reductase was not added. Subsequently, the reaction was initiated by adding 10 mM DTNB, and the absorbance was measured at 450 nm. Oxidized glutathione concentration was obtained by subtracting the reduced from the total concentration.

**Western Blot Analysis**—PC12 cells were washed with Tris-buffered saline on ice, harvested using a cell scraper, and lysed in 20 mM Tris buffer (pH 7) containing 25 mM β-glycerophosphate, 2 mM EGTA, 1% Triton X-100, 1 mM vanadate, 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 2 mM dithiothreitol. Lysates were centrifuged at 15,000 rpm for 30 min, and the supernatants were denatured by boiling. An aliquot of proteins was loaded on a sodium dodecyl sulfate-polyacrylamide gel, separated electrophoretically, and transferred to a polyvinylidene fluoride microporous membrane (Millipore Corp., Bedford, MA). After blocking with non-fat milk, the membrane was incubated with the primary antibody (anti-phospho-p38 MAPK (Cell Signaling Technology, Inc., Beverly, MA), anti-p38 MAPK (Santa Cruz Biotech-
Antioxidant Effect by Proteasome Inhibition

FIGURE 1. Effects of proteasome inhibitors on 6-OHDA- and H2O2-induced cytotoxicity. A, concentration-response curves of 6-OHDA-induced and H2O2-induced cytotoxicity. PC12 cells were exposed to 30–1000 μM 6-OHDA or H2O2 for 24 h. Cell viability was assessed by MTT assay. Open circles represent cell viability after exposures to 6-OHDA, and closed circles represent cell viability after H2O2 exposures. Curve fitting was performed according to the regression model described under “Experimental Procedures.” B, effect of lactacystin on 6-OHDA- and H2O2-induced toxicity. PC12 cells were pretreated with lactacystin for 24 h and exposed to 6-OHDA or H2O2 for 24 h. C and D, effects of MG-132 and PSI on 6-OHDA- and H2O2-induced toxicity. PC12 cells were pretreated with MG-132 and PSI for 24 h and exposed to toxin for 24 h. **, p < 0.01 when compared with control. ##, p < 0.01 when compared with toxin alone.

Reverse Transcription-PCR—Total RNA was extracted from PC12 cells using ISOGEN as recommended by the manufacturer (Nippon Gene, Tokyo, Japan). RNA was reverse-transcribed in a reaction mixture containing SuperScript II RNaseH-reverse transcriptase (Invitrogen) and oligo dT primers, and then cDNAs were obtained after the reaction ribonuclease H (Invitrogen). The primer sequences were glutamate-L-cysteine ligase catalytic (GLCLC) subunit forward 5’-CCCTTCGGACACGGACGTGGT-3’, GLCLC reverse 5’-TAAGACGGCATCTCGCTCTT-3’, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward 5’-AGTGAAGCCAGATGCGCTT-3’, and GAPDH reverse 5’-GCAAGGTCATCCATGACAAC-3’. GAPDH was used as an internal standard. The amplification protocol involved denaturation at 94 °C for 1 min, primer annealing at 60 °C for 1 min, and extension at 72 °C for 1.5 min. This cycle was repeated 25 (GLCLC) or 20 (GAPDH) times. PCR products were subjected to 1.5% agarose gel electrophoresis and visualized with 0.1% ethidium bromide.

Proteasome Activity Assay—PC12 cells were washed with Tris-buffered saline on ice and resuspended in a buffer containing 25 mM Hespes and 0.5 mM EDTA. Cells were lysed by brief sonication, added to N-succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (AMC) (Affinity Research Product, Exeter, UK), a fluorogenic proteasome substrate, and incubated at 37 °C for 30 min. Levels of released AMC were measured using an excitation wavelength of 380 nm and an emission wavelength of 460 nm with an automatic multiwell plate reader. The relative activity was standardized by protein concentration, determined using the Bio-Rad protein assay kit.

Statistics—The statistical significance of the difference between three or more groups of individual data were analyzed by one-way analysis of variance and post hoc multiple comparison using Dunnett’s test. Statistical significance was defined as p < 0.05. Data are expressed as means ± S.E. of the mean (S.E.).

RESULTS

6-OHDA- and H2O2-induced Cytotoxicity Is Blocked by Proteasome Inhibitors—PC12 cells were exposed to 6-OHDA or H2O2 for 24 h. Exposure to these drugs decreased the viability in a dose-dependent manner (Fig. 1A). The EC50 was calculated as 105.8 μM (95% confidential intervals, 97.2–114.0 μM) and 194.9 μM (184.0–205.9 μM) for 6-OHDA and hydrogen perox-
ide, respectively. Taking into consideration rapid degradation and attenuation of the cytotoxicity, 150 μM 6-OHDA and 200 μM H2O2 are taken as optimum conditions for further experiments. Lactacystin, an irreversible proteasome inhibitor, which was administrated from 24 h before toxic exposure, significantly blocked the cytotoxicity induced by 6-OHDA and H2O2 in a concentration-dependent manner (Fig. 1B). As well as lactacystin, other proteasome inhibitors, MG-132 and PSI, also provided cytoprotection against these toxins (Fig. 1, C and D). Six-hour treatment with lactacystin induced a significant decrease in proteasome activity, but 24-h treatment did not (Table 1).

Table 1: Effect of lactacystin on proteasome activity

| Treatment            | Proteasome activity (%) of control |
|----------------------|-----------------------------------|
|                      | 6 h                               | 24 h                              |
| Control              | 100.0 ± 3.8                       | 100.0 ± 5.9                       |
| Lactacystin (0.1 μM) | 98.0 ± 6.5                        | 94.3 ± 3.2                        |
| Lactacystin (0.3 μM) | 70.3 ± 5.1**                      | 84.9 ± 7.5                        |
| Lactacystin (1 μM)   | 49.7 ± 2.7**                      | 77.2 ± 6.1                        |

Proteasome Inhibition Reduces 6-OHDA- and H2O2-induced ROS Production—ROS generation is thought to be involved in cytotoxicity by 6-OHDA because 6-OHDA is easily oxidized by molecular oxygen to produce harmful ROS such as superoxide radical, hydrogen peroxide, and hydroxyl radical (35). To evaluate whether proteasome inhibitors suppress ROS production, the amount of ROS formation was measured using DCF-DA. For the purpose of detecting ROS generation, the cells needed to be exposed to 6-OHDA or H2O2 within 30 min because background fluorescence by DCF-DA autoxidation gradually elevated. Therefore, the concentrations of 6-OHDA and H2O2 were set at 300 μM for ROS generation study, respectively, higher than those in the experiments for the cell survival study. Lactacystin provided protection against 300 μM 6-OHDA or H2O2 as well as against 150 μM 6-OHDA or 200 μM H2O2 (data not shown). Exposure to 300 μM 6-OHDA and H2O2 elevated the fluorescence intensity of DCF-DA, indicating the elevation of intracellular ROS formation, and pretreatment of lactacystin significantly suppressed the elevation (Fig. 2).

6-OHDA- and H2O2-induced Cytotoxicity Is Blocked by the Elevation of Intracellular Glutathione—To clarify the contribution of intracellular glutathione to ROS suppression, we examined the effect of N-acetyl-l-cysteine (NAC), which is a precursor of glutathione and elevates glutathione contents (12). As shown in Fig. 3A, glutathione content was significantly elevated by treatment with NAC for 24 h pretreatment that provided significant protection against cytotoxicity caused by 6-OHDA and H2O2 exposure (Fig. 3B).

Proteasome Inhibitors Elevated Glutathione Content—As ROS-mediated toxicity is abolished by the elevation of glutathione content, we investigated the involvement of glutathione in the cytoprotection of proteasome inhibitors. Exposure to 1 μM lactacystin significantly increased the amount of reduced GSH, and GSH elevation peaked at about 24 h after exposure. The amount of GSSG was not increased within 12 h and was elevated only after 24 h (Fig. 4A). In addition, treatment with lactacystin for 24 h elevated the glutathione content in a concentration-dependent manner (Fig. 4B). Other proteasome inhibitors, such as MG-132 and PSI, also increased the glutathione content (Fig. 4C).

The Elevation of Glutathione Content Requires de Novo Synthesis and the Phosphorylation of p38 MAPK—We examined the mechanism of glutathione elevation by proteasome inhibitors. To determine whether de novo protein synthesis is required for the elevation of glutathione content, cycloheximide, an inhibitor of cytoplasmic translation, was co-administrated with lactacystin. Cycloheximide significantly suppressed the elevation of glutathione content induced by lactacystin (Table 2).
Antioxidant Effect by Proteasome Inhibition

To reveal the phosphorylation pathways involved in glutathione synthesis, several phosphorylation inhibitors were tested. The elevation of glutathione content by lactacystin was diminished by neither bisindolylmaleimide I, a protein kinase C inhibitor, wortmannin, a phosphoinositide 3-kinase inhibitor, nor SP600125, a c-Jun N-terminal kinase (JNK) inhibitor. However, co-administration of SB203580, a p38 mitogen-activated protein kinase (MAPK) inhibitor, inhibited the elevation of glutathione content (Table 2).

Cytoprotection by Proteasome Inhibition Is Mediated by the Phosphorylation of p38 MAPK—To confirm the relationship between p38 MAPK phosphorylation and glutathione elevation, the activation of p38 MAPK was determined by Western blots using a specific phosphorylated p38 MAPK antibody. After treatment with lactacystin, phosphorylated p38 MAPK was transiently detectable around the 6-h time point (Fig. 5A). Furthermore, cytoprotection by lactacystin was significantly reversed by co-treatment with SB203580 (Fig. 5B).

Inhibition of Proteasome Induces Nuclear Translocation of Nrf2 and Expression of GLCLC, the Catalytic Subunit for γ-GCS—To determine the molecular details of glutathione elevation, we focused on γ-glutamylcysteine synthetase (γ-GCS), a rate-limiting enzyme in glutathione synthesis, and Nrf2, a transcription factor of γ-GCS. γ-GCS is also known as glutamate cysteine ligase. After treatment with lactacystin, Nrf2 was transiently translocated to the nucleus around the 6- or 9-h time point (Fig. 6A). Furthermore, GLCLC subunit mRNA expression was significantly increased after 12-h treatment with lactacystin, and these events were abolished by co-administration with SB203580 (Fig. 6, A–C).

In Differentiated PC12 Cells, 6-OHDA-induced Cytotoxicity Is Blocked by Proteasome Inhibitor—We examined the cytoprotection of lactacystin in differentiated PC12 cells because dif-
Differentiated PC12 cells by NGF are used as a model of neurons (36). The administration of lactacystin for 24 h before toxin exposure significantly blocked 6-OHDA-induced toxicity in a concentration-dependent manner (Fig. 7). Phase-contrast microscopic analysis showed that exposure to 6-OHDA reduced the number of surviving cells (Fig. 7, A–C), and this toxic effect was reversed by lactacystin (Fig. 7D). As well as the MTT assay, the lactate dehydrogenase release assay demonstrated that lactacystin inhibited 6-OHDA-induced cytotoxicity (data not shown). In addition, treatment with lactacystin for 24 h elevated the glutathione content in a concentration-dependent manner (Fig. 7E).

**DISCUSSION**

In this study, exposure to proteasome inhibitors elicited the elevation of glutathione content, suppressed ROS production, and protected against oxidative stress-induced cytotoxicity. NAC, which is a precursor of glutathione and elevates glutathione content, provided significant protection against cytotoxicity caused by 6-OHDA and H₂O₂ exposure (Fig. 3). According to these results, we suggest that the elevation of glutathione by proteasome inhibition contributes to cytoprotection. In support of this hypothesis, proteasome inhibitors provided cytoprotection in a concentration and temporal profile consistent with the elevation of glutathione. Treatment with lactacystin for longer than 48 h reversed the glutathione content to the basal level, and in this condition, cytoprotection by lactacystin was hardly observed (data not shown). This was further supported by data that the co-administration of SB203580, a p38 MAPK inhibitor, blocked the elevation of glutathione content and cytoprotection by lactacystin (Table 2, Fig. 5B).

For cytoprotection against ROS, cells have a variety of antioxidant defense systems against oxidative stress. Several studies have suggested that UPS is involved in the regulation of antioxidants, such as catalase, heme oxygenase-1 (HO-1), and copper/zinc-superoxide dismutase (37–39). Although catalase plays important roles in ROS scavenging, there was a discrepancy between cytoprotection by lactacystin and the up-regulation of catalase activity. The up-regulation of catalase required 24-h exposure of 1 μM lactacystin and did not elevate catalase at lower concentrations. However, treatment with 0.3 and 1 μM lactacystin revealed cytoprotection, as shown in Fig. 1B. Furthermore, SB203580 did not abrogate its up-regulation (data not shown). In addition to catalase, HO-1, copper/zinc-superoxide dismutase, and manganese-superoxide dismutase conferred cytoprotection against oxidative injury and provided a vital function in maintaining tissue homeostasis. In our preliminary study, immunoblotting analysis revealed that the amounts of HO-1, copper/zinc-superoxide dismutase, and manganese-superoxide dismutase were not changed by proteasome inhibition in PC12 cells. Taken together, these data suggested that the elevation
Antioxidant Effect by Proteasome Inhibition

In this study, the amount of GSH was increased by treatment with 1 μM lactacystin, but that of GSSG was not increased except after 24 h (Fig. 4A). In addition, the elevation of glutathione content induced by the proteasome inhibitor was attenuated by co-administration with cycloheximide (Table 2). One molecule of GSSG was reduced by glutathione reductase and converted to two molecules of GSH (12). As the amount of GSSG was not decreased, it is suggested that the amount of GSH was increased not by the up-regulation of glutathione reductase activity but by the novel synthesis of γ-GCS, a rate-limiting enzyme in GSH synthesis. In fact, cycloheximide significantly suppressed the elevation of glutathione content (Table 2). The transcriptional induction of γ-GCS is regulated by antioxidant responsive element (ARE), which is found in the 5’-flanking sequences of gcs catalytic and regulatory subunit genes (40). ARE-dependent γ-gcs gene expression is regulated, in part, by the “cap’n’collar” family of basic leucine zipper transcription factor Nrf2 (41, 42). Previous studies have demonstrated that Nrf2 is degraded by proteasomes, and proteasome inhibition induces the accumulation of Nrf2 and ARE activation, resulting in γ-gcs catalytic gene expression (43–45). Corresponding to these reports, we showed that Nrf2 translocated to the nucleus and that γ-GCS mRNA expression was significantly increased after exposure to lactacystin (Fig. 6, A–C).

Recently, it has been reported that the phosphorylation of Nrf2 plays an important role in transcriptional activation through ARE (46, 47); recent studies revealed that the phosphorylation of Nrf2 triggers its nuclear translocation to potentiate the cellular response against oxidative stress. Nrf2 phosphorylation is involved in several cytosolic protein kinases including protein kinase C, phosphoinositide 3-kinase, and MAPKs (48–51). As shown in Table 2, the elevation of glutathione did not involve protein kinase C, phosphoinositide 3-kinase, ERK, and JNK, whereas Figs. 5 and 6 suggest that p38 MAPK is involved in the elevation of glutathione content. Although the involvement of protein kinase in Nrf2 phosphorylation remains controversial and further research will be required, the kinds of kinases involved in ARE-mediated gene expression differ in a stimulus- and cell type-specific manner.

Although we demonstrated that proteasome inhibition induced the activation of p38 MAPK, the detailed mechanism of p38 MAPK phosphorylation has not been clarified well. The accumulation of protein, which is degraded rapidly by proteasome, acts as a positive regulator of the p38 MAPK pathway (52). A previous report suggested that p38 MAPK phosphorylation results in the activation of transcription factors that may be involved in the induction of γ-GCS (53). In agreement with this early report, the results of our study demonstrate that activation of the p38 MAPK pathway is necessary for the elevation of glutathione.

Phosphorylation of p38 MAPK, translocation of Nrf2 to the nucleus, γ-GCS induction, and elevation of glutathione were transiently observed after treatment with lactacystin (Figs. 4A, 5A, and 6). In aqueous solution, lactacystin easily undergoes spontaneous hydrolysis to yield clasto-lactacystin β-lactone, an active inhibitor of proteasomes (54). β-Lactone has high membrane permeability, and in addition to proteasome inhibition, it can be conjugated with GSH. β-Lactone is hydrolyzed to inactive dihydroxy acid by binding to the catalytic site of the proteasomes and reacting with GSH to form lactacystine, an inactive GSH conjugate (55). Therefore, increased glutathione by lactacystin may result in negative feedback inhibition of β-lactone, and lactacystin may no longer be effective in the cells. In fact, 6-h treatment with lactacystin induced a significant decrease in proteasome activity, but 24-h treatment did not (Table 1).

As shown in Fig. 7, we examined the cytoprotection of lactacystin in differentiated PC12 cells. PC12 cells develop features of neurons upon treatment with NGF. In differentiated PC12 cells, treatment with lactacystin elevated the glutathione content and provided protective effect. Furthermore, neuroprotective effect of lactacystin against 6-OHDA and H$_2$O$_2$ toxicity was ascertained in primary mesencephalic dopaminergic neurons (data not shown). Thus, in both neuronal and non-neuronal...
cells, the common pathway may be involved in cytoprotection by proteasome inhibitor. Differentiated PC12 cells by NGF seemed to be more resistant to 6-OHDA-induced cytotoxicity than undifferentiated cells (compare Figs. 1B and 7D). However, several reports demonstrated that NGF exerts anti-apoptotic and trophic actions, and NGF protects against 6-OHDA-induced oxidative stress (56). Because NGF withdrawal induced apoptosis in differentiated PC12 cells, the cells were exposed to drugs in NGF-containing medium. Therefore, it is not necessarily indicated that neuronal cells are insensitive to toxins.

Exposure to 6-OHDA and H2O2 elevated intracellular ROS formation (Fig. 2). The elevation of ROS production by 6-OHDA was less than that by H2O2. Although the precise formation (Fig. 2). The elevation of ROS production by apoptosis in differentiated PC12 cells, the cells were exposed to ever, several reports demonstrated that NGF exerts ant-apoptosis (57). In PC12 cells, besides the production of ROS, p38 MAPK was mediated by its oxidized product, (59).

Considering that proteasome inhibition elevates the amount of glutathione, which is declined in PD patients and alleviates cytotoxicity, proteasome inhibition does not necessarily enhance the progression of PD. Although the overall inhibition of proteasome activity causes cell death (60, 61), partial suppression of the activity may be neuroprotective. In conclusion, this study revealed that UPS plays an important role in the regulation of glutathione.

Acknowledgments—We thank our colleagues for helpful discussion.

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