Journal of Pharmacological Sciences 129 (2015) 150–159

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Journal of Pharmacological Sciences

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Full paper

Compensatory role of the Nrf2–ARE pathway against paraquat toxicity: Relevance of 26S proteasome activity

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ARTICLE INFO

Article history:
Received 24 June 2015
Received in revised form
3 September 2015
Accepted 24 September 2015
Available online 9 October 2015

Keywords:
Nrf2–ARE pathway
Oxidative stress
Paraquat
Parkinson disease
Proteasome activity

ABSTRACT

Oxidative stress and the ubiquitin–proteasome system play a key role in the pathogenesis of Parkinson disease. Although the herbicide paraquat is an environmental factor that is involved in the etiology of Parkinson disease, the role of 26S proteasome in paraquat toxicity remains to be determined. Using PC12 cells overexpressing a fluorescent protein fused to the proteasome degradation signal, we report here that paraquat yielded an inhibitory effect on 26S proteasome activity without an obvious decline in 20S proteasome activity. Relative low concentrations of proteasome inhibitors caused the accumulation of nuclear factor erythroid 2-related factor 2 (Nrf2), which is targeted to the ubiquitin–proteasome system, and activated the antioxidant response element (ARE)-dependent transcription. Paraquat also upregulated the protein level of Nrf2 without increased expression of Nrf2 mRNA, and activated the Nrf2–ARE pathway. Consequently, paraquat induced expression of Nrf2-dependent ARE-driven genes, such as γ-glutamylcysteine synthetase, catalase, and hemeoxygenase-1. Knockdown of Nrf2 or inhibition of γ-glutamylcysteine synthetase and catalase exacerbated paraquat-induced toxicity, whereas suppression of hemeoxygenase-1 did not. These data indicate that the compensatory activation of the Nrf2–ARE pathway via inhibition of 26S proteasome serves as part of a cellular defense mechanism to protect against paraquat toxicity.

1. Introduction

Parkinson disease (PD) is a common neurodegenerative disorder that is characterized by a relatively selective loss of dopaminergic neurons and by the appearance of Lewy bodies in the substantia nigra. Although the cause of neuronal loss remains unknown, oxidative stress and the ubiquitin–proteasome system (UPS) are thought to play important roles in this process (1, 2). In the midbrain of patients with PD, the activities of peroxidase and catalase are reduced, and the amount of glutathione is decreased (3, 4). These observations indicate that antioxidant defense systems collapse in the dopaminergic neurons of patients with PD. The expression of antioxidant enzymes is primarily controlled by the nuclear factor erythroid 2-related factor 2 (Nrf2)–antioxidant response element (ARE) pathway. In response to oxidative stress, Nrf2 is translocated to the nucleus and binds to ARE, resulting in the transcriptional activation of phase 2 detoxifying and antioxidant enzymes (5). Ramsey *et al.* (6) reported the nuclear localization of Nrf2 in the remaining dopaminergic neurons of patients with PD.

Some forms of familial PD are caused by mutations in components related to UPS, which highlights the potential importance of UPS in PD (7, 8). Importantly, in the substantia nigra of patients with sporadic PD, proteasomal function is reduced by 30%–40%, and the levels of the α-subunit of the proteasome and PA700 proteasome activator are reduced (9, 10). Severe impairment of UPS

Abbreviations: ARE, antioxidant response element; BSO, L-buthionine-[*S*,*R*]-sulfoximine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; γ-GCS, γ-glutamylcysteine synthetase; GSH, reduced glutathione; HO-1, hemeoxygenase-1; LDH, lactate dehydrogenase; NQO-1, NAD(P)H, quinone oxidoreductase-1; NGF, nerve growth factor; Nrf2, nuclear factor erythroid 2-related factor 2; PD, Parkinson disease; siRNA, small interfering RNA; UPS, ubiquitin-proteasome system.

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Peer review under responsibility of Japanese Pharmacological Society.

http://dx.doi.org/10.1016/j.jphs.2015.09.003

reportedly causes an increase in ubiquitin- and α-synuclein-positive aggregation, along with dopaminergic neuronal death (11, 12). However, UPS is responsible for the degradation of short-lived proteins as well as misfolded proteins. For example, the transcription factor Nrf2 is degraded by UPS under normal conditions (13); therefore, proteasomal inhibition induces the accumulation of Nrf2, the translocation of Nrf2 into nuclei, and the activation of ARE-driven transcription of antioxidant genes (14, 15). Previous studies demonstrated that mild inhibition of proteasome activity affords protection against toxin-induced cell death, at least in part, via the activation of the Nrf2–ARE pathway (15–18). Because proteasomal inhibition activates both neuroprotective and pro-apoptotic response pathways (19), the role of UPS impairment in the cell-death process remains controversial.

Human epidemiological studies indicate an association between exposure to pesticides and an increased risk for PD (20). Some pesticides excert inhibitory effects on 26S proteasome activity without affecting 20S proteasome activity (21). Evidence from epidemiological and experimental studies (22, 23) strongly suggest the existence of link between paraquat, a widely used herbicide, and sporadic PD. However, the effect of paraquat on 26S proteasome activity remains to be determined because of the lack of cellular uptake in the previous study (21). Our previous report has demonstrated that paraquat was incorporated into PC12 cells via the dopamine transporter (24). Furthermore, exposure of PC12 cells to paraquat (50 μM) caused delayed toxicity from 36 h onward through endogenous dopamine-mediated oxidative stress. Therefore, we used the concentration of paraquat as an experimental model of dopaminergic neuronal death in PD. In the present study, we used PC12 cells overexpressing a green fluorescent protein that fused to the proteasome degradation signal to examine the effect of paraquat on 26S proteasome activity and the role of 26S proteasome activity in paraquat-induced toxicity.

2. Materials and methods

2.1. Materials

Paraquat dichloride and bilirubin were purchased from Nacalai Tesque (Kyoto, Japan). Benzyloxycarbonyl-Leu-Leu-leucinal (MG132), lactacystin, and 3-amino-1,2,4-triazole were obtained from Calbiochem (San Diego, CA, USA). α-Methyl-DL-*p*-tyrosine methyl ester hydrochloride, ascorbic acid, and L-buthionine-[*S*,*R*]-sulfoximine (BSO) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Nerve growth factor (NGF) was from Promega Co. (Madison, WI, USA).

2.2. Cell culture

Rat adrenal pheochromocytoma PC12 cells were maintained as described previously (24). NGF-induced differentiation was conducted as described previously (25).

2.3. Evaluation of cell viability

Cell viability was determined using the lactate dehydrogenase (LDH) release assay. Briefly, 10 μL of culture supernatant was mixed with 90 μL of the LDH substrate mixture (174 mM lactate, 1.5 mM nicotinamide adenine dinucleotide, 815 μM nitroblue tetrazolium, and 13.3 U/mL diaphorase (Oriental Yeast Co., Tokyo, Japan) in 50 mM Tris-HCl; pH 8.5). After incubation for 20 min at room temperature, absorbance was measured at 570 nm. Cell viability was calculated as 100% minus the ratio of the activity of the released LDH to total activity; LDH was released by exposure to paraquat (300 μM) for 48 h.

2.4. Determination of cellular ubiquitin-dependent 26S proteasome activity

PC12 cells were transfected with the Proteasome Sensor Vector (pZsProSensor-1, Takara Bio, Shiga, Japan) using Lipofectamine 2000 (Life technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Stably transfected cell lines were generated via selection with G418 (400 μg/mL). Treated cells were trypsinized for 5 min and suspended as individual cells before the flow cytometric analysis. Cellular fluorescence was measured on a FACScan (Becton–Dickinson, Rutherford, NJ, USA) flow cytometer.

2.5. 20S proteasome activity assay in cell lysates

20S proteasome activity was measured using Suc-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Enzo Life Sciences, Exeter, UK), a fluorogenic proteasome substrate, as described previously (15).

2.6. Measurement of ARE-dependent transcriptional activity

PC12 cells were stably transfected with an ARE-luciferase construct and designated ARE reporter PC12 cells as described previously (26). In the reporter gene assays, firefly luciferase activity was measured in cell lysates using a luminometer by the ONE-Glo™ Luciferase Assay System (Promega).

2.7. Small interfering RNA (siRNA) sequences and transfection

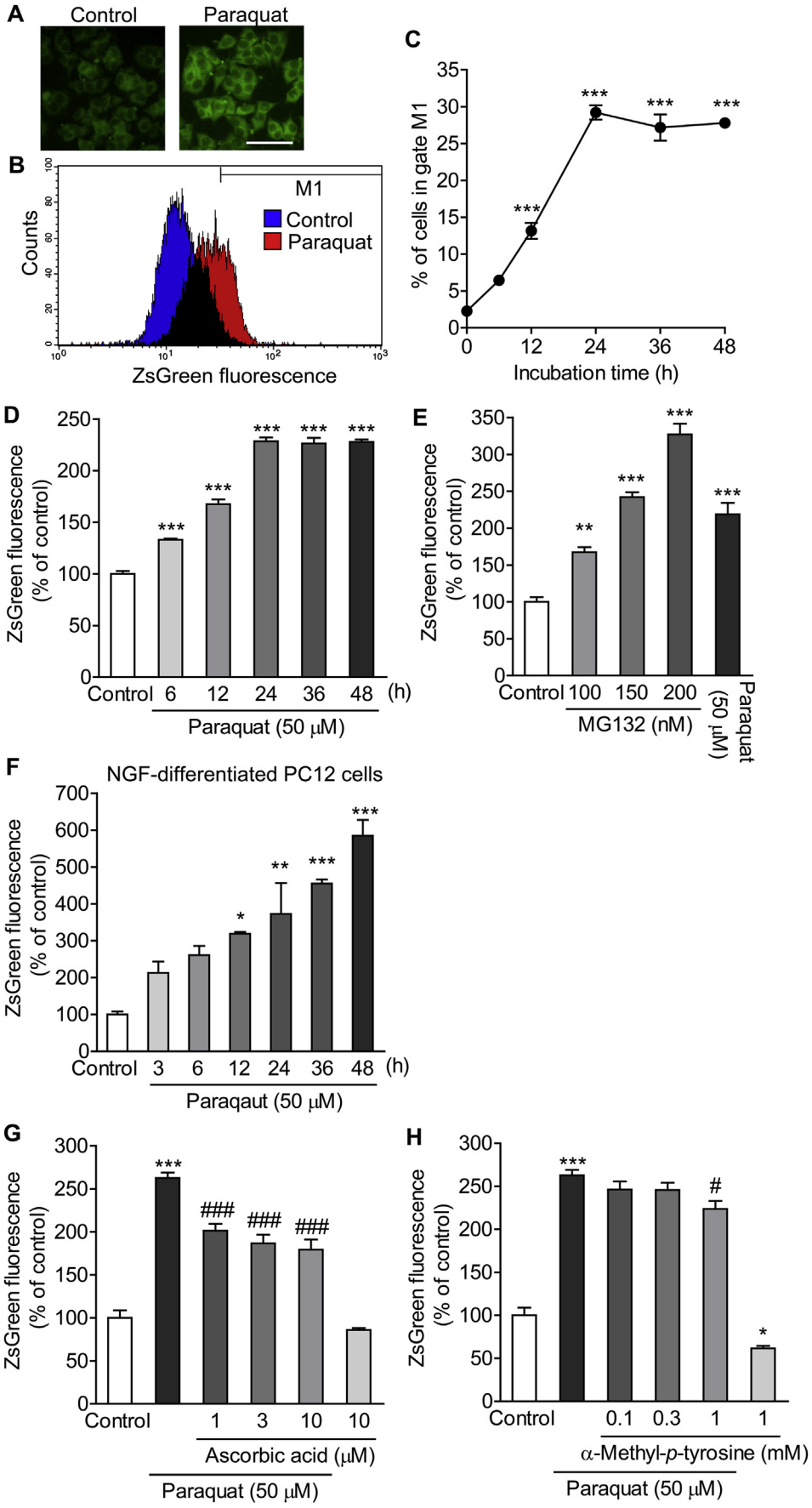
Stealth™ RNAi siRNAs were purchased from Life Technologies. The siRNA sequence targeting *Nrf2* was 5′-UUUACACAGGGACGAUCACAGCCC-3′ and that targeting *Hemeoxygenase-1* (*Ho-1*) was 5′-AUGGCAUAAAUUCCCACUGCCACGG-3′. Stealth™ RNAi Negative Control Medium GC Duplex #2 (Life Technologies) was used as the negative-control siRNA. PC12 cells were transfected with each siRNA using Lipofectamine 2000 according to the manufacturer's protocol. The medium was changed at 9 h after transfection and cultures were treated with drugs from 24 h after transfection.

2.8. Real-time PCR analysis

Total RNA was extracted and reverse transcribed. Real-time PCR was performed using SYBR® Premix Ex Taq™ II (Takara Bio). The following primer sequences were used: *Nrf2* forward, 5′-GAGACGGCCATGACTGAT-3′; *Nrf2* reverse, 5′-GTGAGGGGATCGATGAGTAA-3′, γ-*Glutamylcysteine synthetase* (γ-*Gcs*) forward, 5′-CCTTCTGGCACAGCACGTTG-3′; γ-*Gcs* reverse, 5′-TAAGACGGCATCTCGCTCCT-3′; *Ho-1* forward, 5′-TGCTCGCATGAACACTCTG-3′; *Ho-1* reverse, 5′-TCCTCTGTCAGCAGTGCC-3′; *Glyceraldehyde 3-phosphate dehydrogenase* (*Gapdh*) forward, 5′-AGTGTAGCCCAGGATGCCCTT-3′; and *Gapdh* reverse, 5′-GCCAAGGTCATCCATGACAAC-3′. The mRNA levels of the tested genes were quantified using standard curves generated by serially diluted reference samples. The relative levels of target mRNAs were analyzed by normalizing to GAPDH mRNA expression level.

2.9. Western blotting

Western blot analysis using cell lysates was performed as described previously (24). Briefly, whole-cell lysates were separated on an SDS–polyacrylamide gel, followed by transfer to a polyvinylidene fluoride membrane (Millipore). The membranes were probed with primary antibodies [anti-Nrf2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-HO-1 (Stressgen, Victoria, Canada), anti-β-actin (Sigma), and anti-GAPDH (Ambion, Austin, TX, USA)] and a horseradish peroxidase-conjugated secondary antibody (GE Healthcare, Little Chalfont, UK) for 1 h. The


A
Control
Paraquat
B
M1
Control
Paraquat
Counts
ZsGreen fluorescence
C
% of cells in gate M1
Incubation time (h)
D
ZsGreen fluorescence (% of control)
Control
Paraquat (50 μM)
(h)
E
ZsGreen fluorescence (% of control)
Control
MG132 (nM)
Paraquat (50 μM)
F
NGF-differentiated PC12 cells
ZsGreen fluorescence (% of control)
Control
Paraqaut (50 μM)
(h)
G
ZsGreen fluorescence (% of control)
Control
Ascorbic acid (μM)
Paraquat (50 μM)
H
ZsGreen fluorescence (% of control)
Control
α-Methyl-p-tyrosine (mM)
Paraquat (50 μM)

membrane-bound secondary antibody was detected using an enhanced chemiluminescence detection system (GE Healthcare). The band intensities were analyzed using the ImageJ 1.33u software (National Institute for Health, Bethesda, MD, USA).

2.10. Measurement of intracellular reduced glutathione (GSH) levels

The levels of GSH were determined using monochlorobimane (Sigma), which forms a fluorescent conjugate with GSH, as described previously (26).

2.11. Measurement of catalase activity

Catalase activity was determined on the basis of the peroxidatic activity of catalase with methanol in the presence of hydrogen peroxide as described previously (27). The formaldehyde produced was measured spectrophotometrically using 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as a chromogen.

2.12. Statistics

The statistical significance of the difference between three or more groups of individual data was analyzed by one-way analysis of variance and *post-hoc* multiple comparisons using Turkey's test. Statistical significance was defined as $p < 0.05$. Data are expressed as mean ± SEM.

3. Results

3.1. Inhibitory effect of paraquat on 26S proteasome activity

PC12 cells transfected with pZsProSensor-1 (PC12 proteasome sensor cells) were used for quantitating cellular ubiquitin-dependent 26S proteasome activity. Treatment with paraquat (50 μM) for 24 h caused an increase in ZsGreen fluorescence, which reflected the reduced activity of the 26S proteasome (Fig. 1A). A flow cytometric analysis also showed that paraquat increased ZsGreen fluorescence in the transfected cells (Fig. 1B). The marker M1 was used to designate cells with low 26S-proteasome activity. The increases in both the proportion of cells with a low 26S-proteasome activity and the mean fluorescence intensity of ZsGreen reached a plateau 24 h after exposure to paraquat (Fig. 1C and D). The extent of inhibition of the 26S proteasome by paraquat (50 μM) corresponded to that afforded by MG132, a proteasome inhibitor, at a concentration between 100 and 150 nM (Fig. 1E). PC12 cells differentiated by NGF possess many important neurochemical and signal transduction processes similar to dopaminergic neurons. In NGF-differentiated PC12 cells, paraquat (50 μM) also time-dependently increased ZsGreen fluorescence (Fig. 1F). To examine whether reactive oxygen species or endogenous dopamine participates in paraquat-mediated 26S proteasomal inhibition, ascorbic acid or α-methyl-*p*-tyrosine, which is an inhibitor of tyrosine hydroxylase, was applied from 24 h before and simultaneously with paraquat. Ascorbic acid (1–10 μM) attenuated the paraquat-induced inhibition of 26S proteasome activity in a concentration-dependent manner (Fig. 1F). On the other hand, α-methyl-*p*-tyrosine (1 mM) slightly increased the basal and paraquat-induced level in 26S proteasome activity (Fig. 1G).

3.2. Inhibitory effect of paraquat on 20S proteasome activity

The 20S proteasome is the proteolytic core particle of the 26S proteasome. The inhibitory effect of paraquat on 26S proteasome activity may be attributed to dysfunction of 20S proteasome proteolytic core activity. However, the effect of paraquat on 20S proteasome activity in cell lysates was limited. Paraquat (50 μM) slightly decreased 20S proteasome activity 48 h after exposure to the drug (Fig. 2A). In contrast, MG132 (30–300 nM) markedly suppressed 20S proteasome activity in a concentration-dependent manner (Fig. 2B).

3.3. Influence of proteasome inhibition on the Nrf2–ARE pathway

In agreement with previous reports (13–15), treatment with MG132 (30–150 nM) or lactacystin (0.1–1 μM), which is another proteasome inhibitor, for 9 h increased the level of the Nrf2 protein (Fig. 3A and B). As a result, both MG132 and lactacystin increased ARE-dependent transcriptional activity in a time-dependent manner (Fig. 3C and D). In addition, the effects of proteasome inhibitors on paraquat-induced cytotoxicity were examined. Paraquat cytotoxicity was significantly attenuated by pre- and co-treatment with MG132 (30–100 nM) or lactacystin (0.3–1 μM; Supplementary Fig. S1). Treatment with MG132 (not less than 150 nM) for 72 h caused marked toxicity in PC12 cells (data not shown).

3.4. Activation of ARE-dependent transcription by paraquat

Next, we examined whether paraquat affected the Nrf2–ARE pathway. The amount of intracellular Nrf2 protein increased after treatment with paraquat (50 μM) in a time-dependent manner (Fig. 4A). However, no significant changes were found in the amount of Nrf2 mRNA, although Nrf2 mRNA tended to decrease just after exposure to paraquat (Fig. 4B). In response to the upregulation of the Nrf2 protein, treatment with paraquat (50 μM) increased ARE-luciferase activity (Fig. 4C). In NGF-differentiated PC12 cells, treatment with paraquat (50 μM) caused a rapid and transient increase in ARE-luciferase activity (Fig. 4D). To confirm the involvement of Nrf2 in paraquat-induced ARE activation, ARE reporter PC12 cells were transfected with an siRNA against Nrf2. In our previous reports (26), mRNA level of Nrf2 was decreased to 50% by the siRNA. Transfection of the siRNA suppressed the basal level and the paraquat-induced increase in ARE-luciferase activity (Fig. 4E). To examine whether the paraquat-induced ARE activation affected cytotoxicity, cell viability was measured in Nrf2-knockdown cells. For unknown reasons, PC12 cells acquired resistance to paraquat toxicity after exposure to Lipofectamine 2000 (Supplementary Fig. S2). Therefore, paraquat was used at a concentration of 250 μM to induce sufficient toxicity in siRNA-transfected PC12 cells. PC12 cells transfected with the Nrf2 siRNA

Fig. 1. Paraquat decreases 26S proteasome activity in living cells. (A, B) Effect of paraquat on 26S proteasome activity in fluorescence images (A) and flow cytometry (B). PC12 proteasome sensor cells were treated with paraquat (50 μM) for 24 h. Scale bar, 50 μm. A marker (M1) is placed to indicate the percentage of cells with low proteasome activity (<3%) under normal conditions. (C, D) Temporal change in 26S proteasome activity in response to paraquat. PC12 proteasome sensor cells were treated with paraquat (50 μM) for the indicated periods. C: Percentage of cells in gate M1. D: Fluorescence intensity per cell. ZsGreen fluorescence was determined by flow cytometry. (n = 3) E: Effect of MG132 on 26S proteasome activity. PC12 proteasome sensor cells were treated with MG132 (100–200 nM) or paraquat (50 μM) for 24 h. (n = 3) F: Effect of paraquat on 26S proteasome activity in NGF-differentiated PC12 cells. PC12 proteasome sensor cells were differentiated by NGF (10 ng/mL) for 4 days. Treatment with paraquat (50 μM) for 3–48 h was conducted as differentiation period was equalized. (n = 3–4) (G–H) Effects of ascorbic acid and α-methyl-*p*-tyrosine on paraquat-induced inhibition of 26S proteasome activity. PC12 proteasome sensor cells were pretreated with 1–10 μM ascorbic acid (G) or 0.1–1 mM α-methyl-*p*-tyrosine (H) for 24 h, and exposed to 50 μM paraquat in the presence of ascorbic acid or α-methyl-*p*-tyrosine for 24 h. (n = 4) $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the control. $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ compared with paraquat alone.

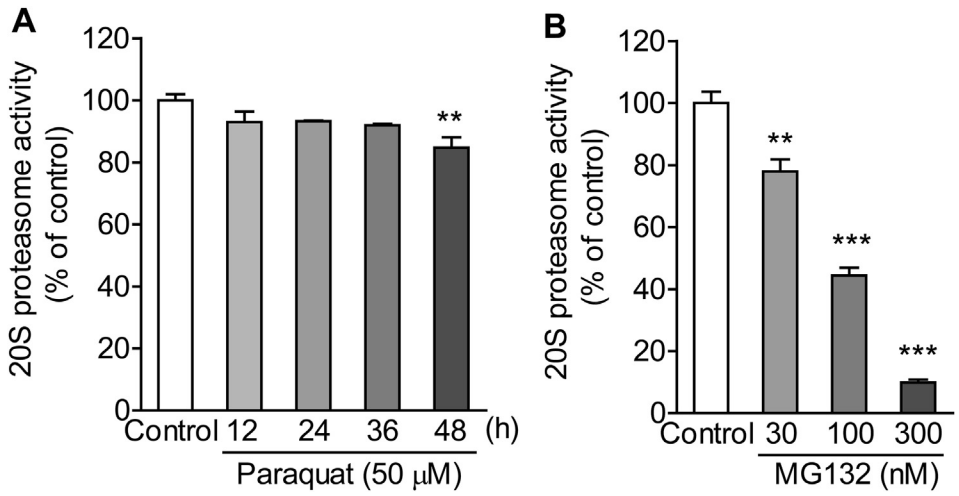


Fig. 2. Paraquat has only a limited influence on 20S proteasome activity in cell lysates. A: Effect of paraquat on 20S proteasome activity. PC12 cells were treated with paraquat (50 μM) for the indicated periods. B: Effect of MG132 on 20S proteasome activity. PC12 cells were treated with MG132 (30–300 nM) for 24 h. (n = 3) $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the control.

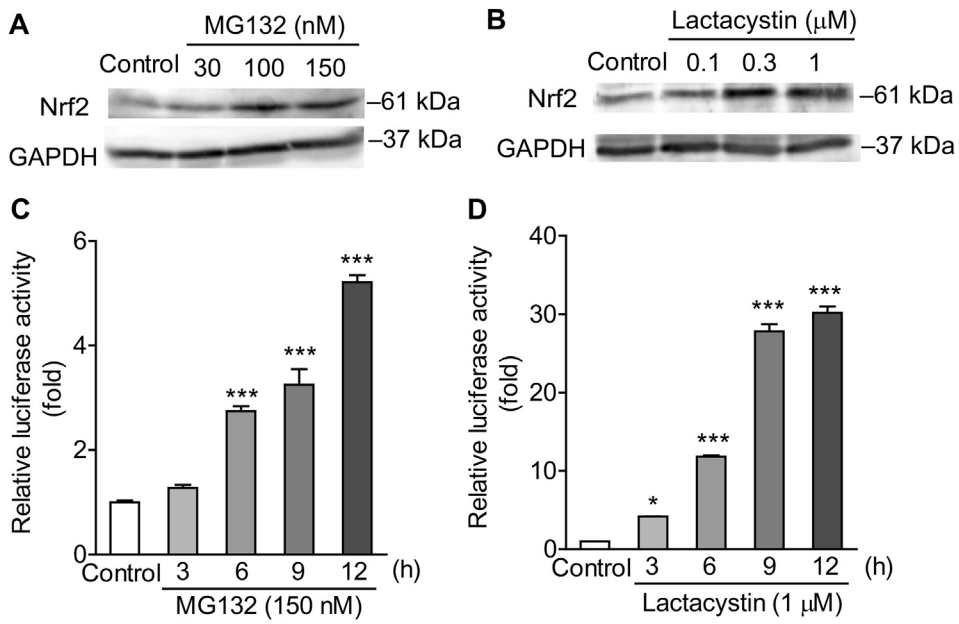


Fig. 3. Proteasome inhibitors activate the Nrf2–ARE pathway. (A, B) Effects of proteasome inhibitors on Nrf2 protein levels. PC12 cells were treated with MG132 (30–300 nM; A) or lactacystin (0.1–1 μM; B) for 9 h. (C, D) Effects of proteasome inhibitors on ARE-dependent transcriptional activity. ARE reporter PC12 cells were treated with 150 nM MG132 (C) or 1 μM lactacystin (D) for 3–12 h. (n = 3) $^{*}p < 0.05$, $^{***}p < 0.001$ compared with the control.

were more vulnerable to paraquat toxicity (250 μM) than those transfected with the control siRNA (Fig. 4F).

3.5. Involvement of the induction of γ-GCS in paraquat toxicity

The Nrf2–ARE pathway is critical in mediating the induction of antioxidant enzymes, such as γ-GCS, a rate-limiting enzyme in glutathione synthesis, catalase, and HO-1. Treatment with paraquat (50 μM) increased the γ-GCS mRNA level and GSH content in a time-dependent manner (Fig. 5A and B). To inhibit the increase in the GSH content by paraquat, BSO (1 mM), an inhibitor of γ-GCS, was co-applied with paraquat, suppressing the basal level and the paraquat-induced increase in the GSH content (Fig. 5C). Co-administration of BSO (0.03–1 mM) exacerbated paraquat-induced cytotoxicity, although BSO (1 mM) alone had no effect on cell viability (Fig. 5D).

3.6. Involvement of the induction of catalase in paraquat toxicity

Treatment with paraquat (50 μM) also increased the catalase mRNA level and activity (Fig. 6A and B). To suppress this increase in catalase activity by paraquat, 3-amino-1,2,4-triazole, an inhibitor of catalase, was co-applied with paraquat. Co-administration of 3-amino-1,2,4-triazole (1–30 mM) exacerbated the paraquat-induced cytotoxicity, although 3-amino-1,2,4-triazole (30 mM) alone had no effect on cell viability (Fig. 6C).

3.7. Noninvolvement of the induction of HO-1 in paraquat toxicity

Treatment with paraquat (50 μM) also increased HO-1 mRNA and protein levels (Fig. 7A and B). The mRNA and protein levels peaked at around 24 h, gradually decreasing. To eliminate the

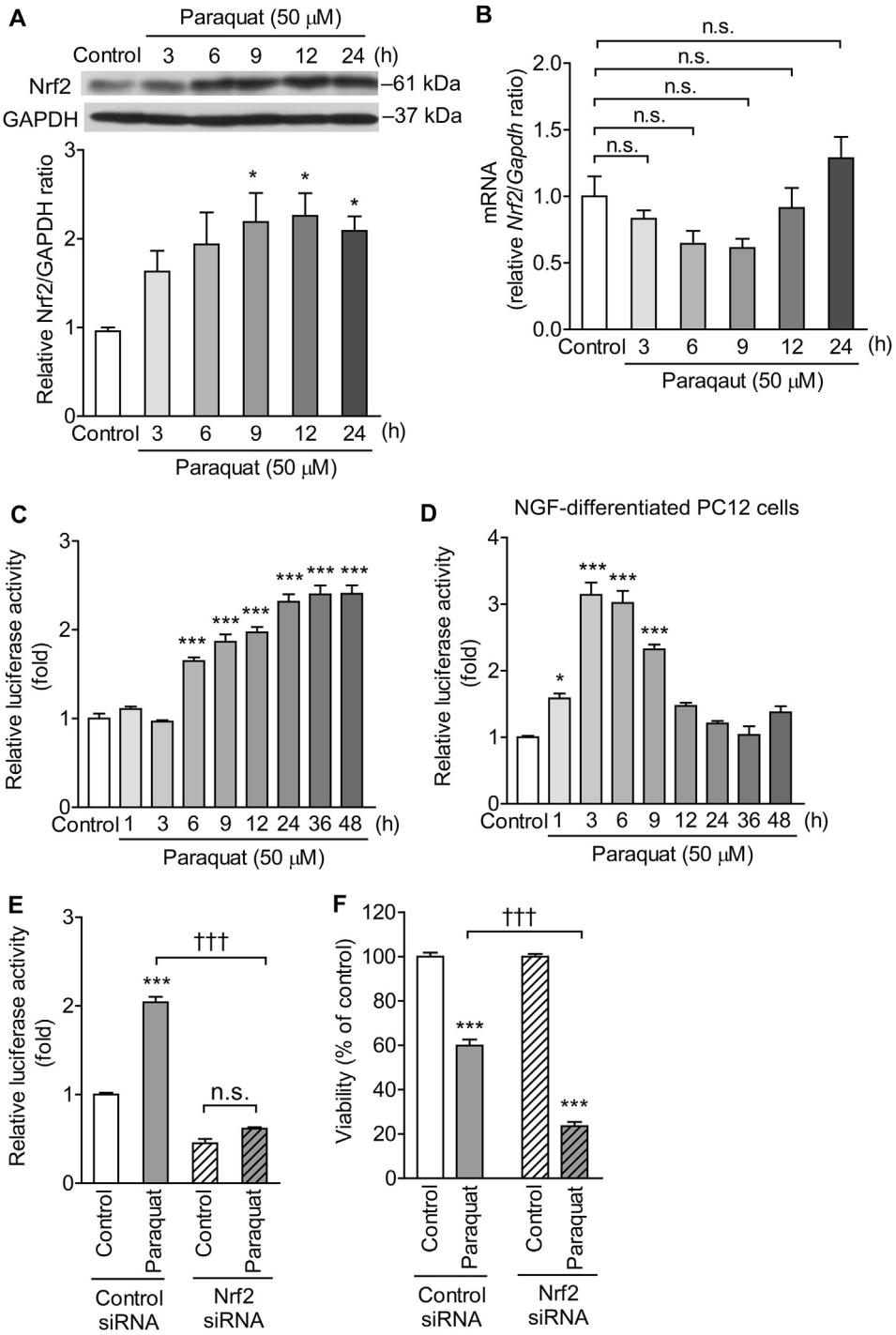


Fig. 4. Paraquat activates the Nrf2–ARE pathway. (A, B) Effect of paraquat on Nrf2 protein (A) and mRNA (B) levels. PC12 cells were treated with paraquat (50 μM) for 3–24 h. The levels of Nrf2 were normalized to those of GAPDH. (n = 4) C: Effect of paraquat on ARE-dependent transcriptional activity. ARE reporter PC12 cells were treated with paraquat (50 μM) for 1–48 h. (n = 3) D: Effect of paraquat on ARE-dependent transcriptional activity in NGF-differentiated PC12 cells. ARE reporter PC12 cells were differentiated by NGF (10 ng/mL) for 4 days. Treatment with paraquat (50 μM) for 1–48 h was conducted as differentiation period was equalized. (n = 3) E: Effect of an Nrf2 siRNA on paraquat-induced ARE activation. ARE reporter PC12 cells were transfected with an Nrf2 siRNA before treatment with paraquat (50 μM) for 24 h. (n = 3) F: Effect of an Nrf2 siRNA on paraquat toxicity. PC12 cells were transfected with an Nrf2 siRNA before treatment with paraquat (250 μM) for 48 h. (n = 4) $^{*}p < 0.05$, $^{***}p < 0.001$ compared with the control. $^{\dagger\dagger\dagger}p < 0.001$. n.s., not significant.

contribution of the upregulation of HO-1 protein by paraquat, PC12 cells were transfected with an siRNA against HO-1. Transfection of this siRNA suppressed the basal level and the paraquat-induced increase in HO-1 protein levels (Fig. 7C). However, PC12 cells transfected with the HO-1 siRNA exhibited a similar cytotoxicity to paraquat (250 μM) as did the control siRNA (Fig. 7D). Furthermore, although bilirubin, one of end products of heme metabolism by HO-1, possesses radical scavenging activity (28), co-administration of bilirubin (3–10 μM) failed to inhibit paraquat-induced cytotoxicity (Fig. 7E).

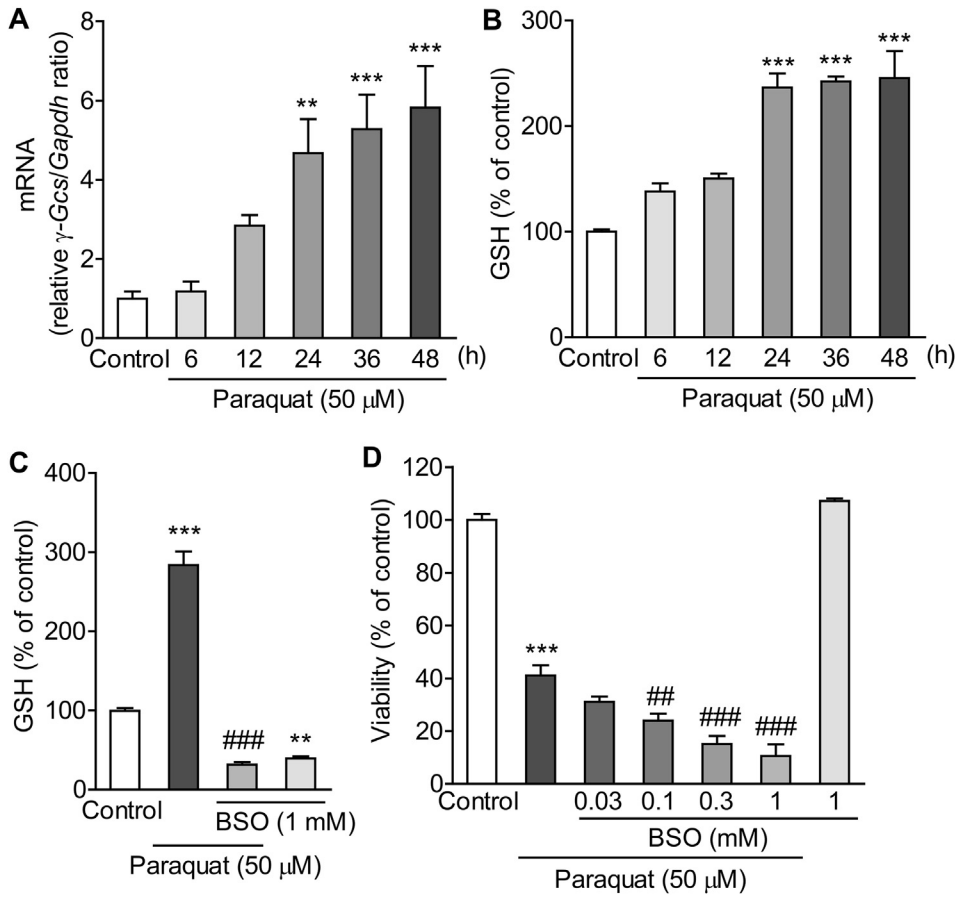


Fig. 5. Induction of γ-GCS participates in alleviation of paraquat toxicity. (A, B) Temporal changes in γ-GCS mRNA expression (A, n = 6) and intracellular GSH content (B, n = 3) in response to paraquat. PC12 cells were treated with 50 μM paraquat for 6–48 h. C: Effect of BSO on intracellular GSH content, which was upregulated by paraquat. PC12 cells were treated with 50 μM paraquat for 24 h in the presence or absence of BSO (1 mM). (n = 4) D: Effect of BSO on paraquat toxicity. PC12 cells were treated with 50 μM paraquat for 24 h in the presence or absence of BSO (0.03–1 mM). (n = 4) $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the control. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with paraquat alone.

4. Discussion

Genetic information acquired from familial PD cases implicates defective UPS in PD pathogenesis. However, because more than 90% PD cases are sporadic, the elucidation of UPS function during the cell-death process induced by environmental dopaminergic neurotoxins is important for understanding the etiology of this disease. In the present study, we demonstrated that paraquat excerted an inhibitory effect on 26S proteasome activity in living cells without a decline in 20S proteasome activity. Furthermore, we propose a role of 26S proteasome activity in the Nrf2–ARE pathway activation by paraquat.

Our data showed that paraquat inhibited 26S proteasome activity, although the precise mechanisms remain to be clarified. However, several issues were noted in this study. First, the inhibition of the 26S proteasome by paraquat was unlikely to be a consequence of a cell-death process, because the decrease in 26S proteasome activity occurred before cell death began. 26S Proteasome activity decreased from 6 h after exposure to paraquat (50 μM) in the present study, whereas cell viability reduced from 36 h onward in our previous report (24). Second, the elevation of reactive oxygen species by paraquat appears to be involved in the inhibitory effect on 26S proteasome activity. Because ascorbic acid efficiently scavenges the superoxide anion (29), the superoxide anion produced via a redox cycling mechanism of paraquat may trigger the inhibition of 26S proteasome activity. In addition, dopamine oxidation products exert strong inhibitory effects on 20S proteasome activity (30). However, the restorative effect of α-methyl-*p*-tyrosine on the inhibitory effect of paraquat on 26S proteasome activity was limited, suggesting the elevation of dopamine by paraquat does not directly cause the inhibition of 26S proteasome activity. Third, the inhibitory effect of paraquat on 26S proteasome activity is unlikely to be mediated by direct interaction with the 20S proteasome, because treatment with paraquat yielded no obvious inhibition of 20S proteasome activity in cell lysates. Reinheckel *et al.* (31) demonstrated that the 26S proteasome is more vulnerable to oxidative stress compared with the 20S proteasome. In fact, paraquat has been reported to decrease protein levels of 19S proteasome subunits, but not 20S proteasome alpha or beta subunits (32). However, in DJ-1-deficient mice, paraquat has been reported to decrease protein levels of both 19S and 20S proteasome subunits (33). Another study proposes that a decrease in proteasomal proteolytic activity by paraquat is not due to loss of the 19S or 20S components (34). Furthermore, oxidative stress could upregulate 20S proteasome subunits via the Nrf2–ARE pathway (35). Although an association between paraquat and proteasome is complicated and differs by experimental conditions, time-course study is necessary to better understand the role of proteasome activity in paraquat toxicity.

Proteasome inhibition is hypothesized to play an important role in mediating cellular toxicity in PD. Severe inhibition of proteasome activity induces dopaminergic neuronal death *in vitro* and *in vivo* (11, 12). Conversely, previous data from our and other groups demonstrated that mild inhibition of proteasome activity provided dopaminergic neuroprotection *in vitro*

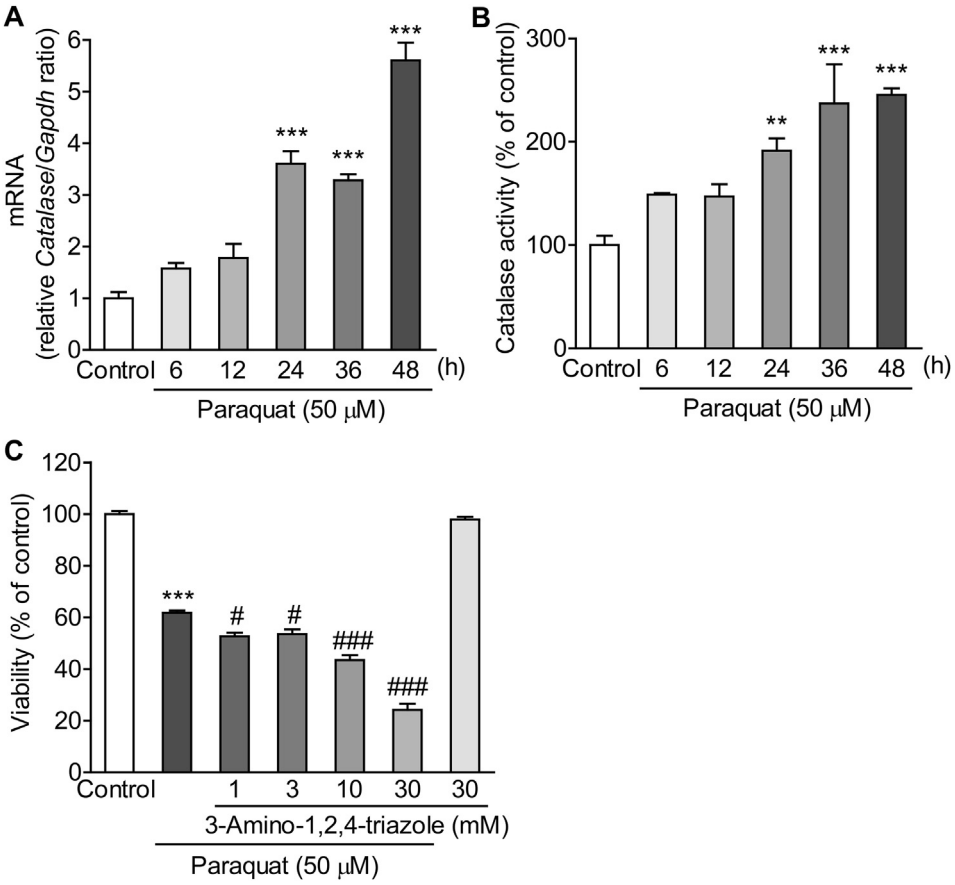


Fig. 6. Induction of catalase participates in alleviation of paraquat toxicity. (A, B) Temporal changes in catalase mRNA expression (A, n = 3) and activity (B, n = 3–4) in response to paraquat. PC12 cells were treated with 50 μM paraquat for 6–48 h. C: Effect of a catalase inhibitor on paraquat toxicity. PC12 cells were treated with 50 μM paraquat for 24 h in the presence or absence of 3-amino-1,2,4-triazole (1–30 mM). (n = 4) $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the control. $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ compared with paraquat alone.

and *in vivo* (16–18). They propose the activation of the Nrf2–ARE pathway as a probable mechanism underlying the protective effect of proteasome inhibitors (15, 18). Recently, it has been reported that knock-out of 19S proteasome regulatory subunit leads to activation of the Nrf2–ARE pathway (36). Therefore, these reports raise the possibility that a mild reduction of 26S proteasome activity during paraquat toxicity can serve as a cellular defense mechanism to protect against oxidative stress. At least in experimental models of PD, there is still a dispute over whether dysfunction of proteasome activity is responsible for cellular toxicity. Similarly, in NGF-differentiated PC12 cells, paraquat induced a decrease in 26S proteasome activity and the activation of the Nrf2–ARE pathway. Because NGF increase the expression level of the dopamine transporter in PC12 cells (37), cellular sensitivity to paraquat was likely to differ from naive cells. Further study is required to elucidate the temporal response to paraquat in NGF-differentiated PC12 cells.

Our findings demonstrated that paraquat upregulated intracellular Nrf2 protein level and activated the Nrf2–ARE pathway. Because Nrf2 is an ubiquitin–proteasome substrate (13), the inhibitory effect of paraquat on 26S proteasome activity can induce the accumulation of Nrf2. Another possibility is that activation of the Nrf2–ARE pathway by paraquat may upregulate Nrf2 protein level. However, there is a dispute over whether activation of the Nrf2–ARE pathway increases Nrf2 mRNA level (14, 38). Furthermore, we showed that paraquat did not increase Nrf2 mRNA levels. Thus, the Nrf2 protein level increased by paraquat seems to be the result from a post-transcriptional mechanism, rather than of an increase in Nrf2 transcription. Rather, Nrf2 mRNA levels tended to be transiently decreased by paraquat (Fig. 4B). Several studies have shown that microRNA-34a, one of non-coding RNA species, negatively regulates the mRNA levels of Nrf2 (39, 40). In fact, exposure to paraquat is reported to increase the expression levels of microRNA-34a (41). Nevertheless, the fact that paraquat increased Nrf2 protein levels suggested that the inhibition of degradation of Nrf2 protein predominates over the suppression of translation of Nrf2 mRNA.

More importantly, suppression of the activation of the Nrf2–ARE pathway by RNA interference potentiated paraquat toxicity. This experimental result indicates that the activation of the Nrf2–ARE pathway induced by paraquat plays a role in a cellular defense mechanism to protect against paraquat toxicity. Among phase 2 detoxifying and antioxidant enzymes which are upregulated by the Nrf2–ARE pathway, we showed paraquat increased the expression of γ-*Gcs*, *Catalase*, and *Ho-1*. Because inhibition of γ-GCS and catalase, or knockdown of HO-1 alone did not affect cell viability, PC12 cells may be maintained at low levels of oxidative stress under normal conditions. Our data indicate that the upregulation of γ-GCS and catalase contributes to the protective effect afforded by the paraquat-induced activation of the Nrf2–ARE pathway, whereas the upregulation of HO-1 by paraquat may not participate in its toxicity. Catalase and GSH seem to play a role to suppress reactive oxygen species under oxidative conditions induced by paraquat. Catalase efficiently eliminates hydrogen peroxide, which can be generated by single-electron transfer of the superoxide anion produced via a redox cycling mechanism of

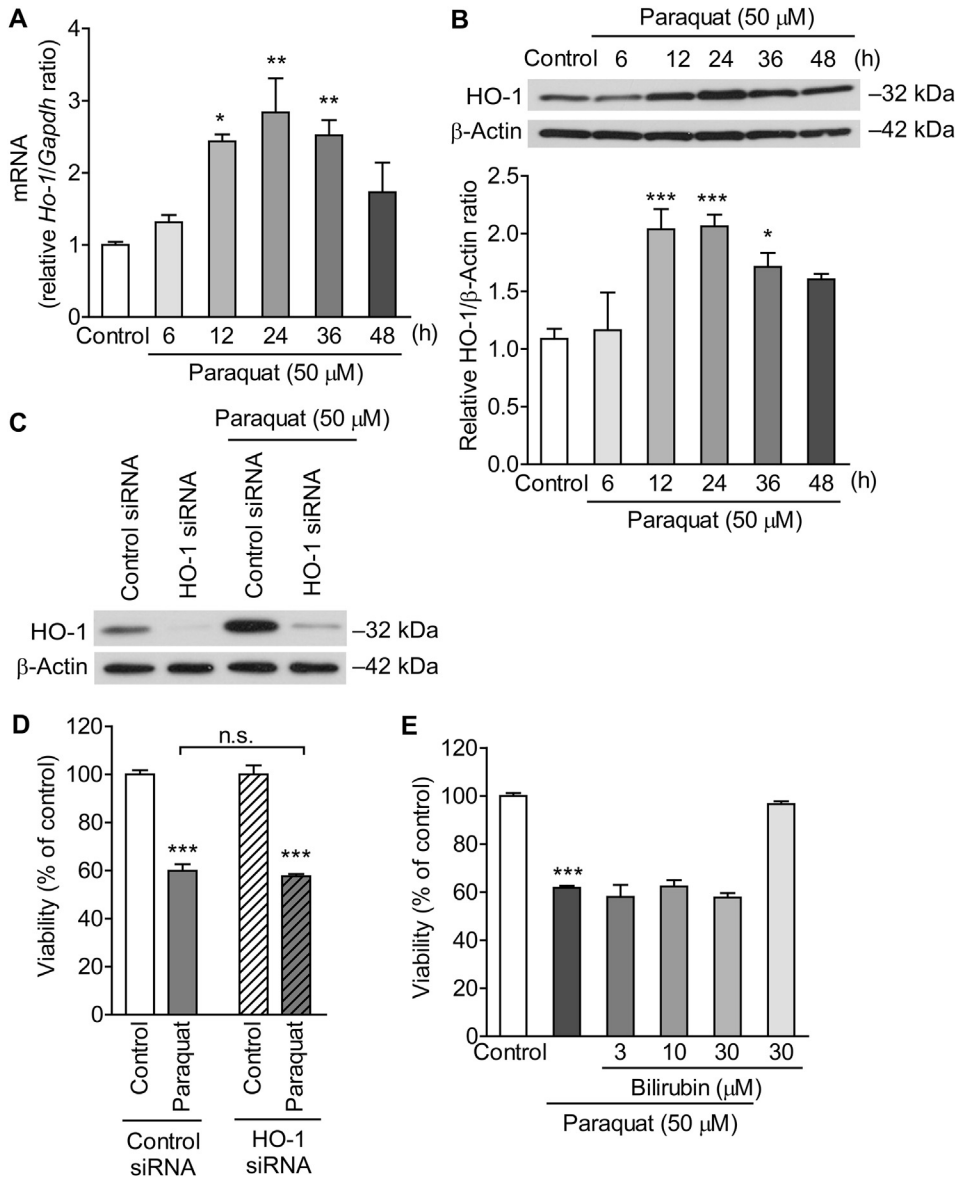


Fig. 7. Induction of HO-1 does not affect paraquat toxicity. (A, B) Temporal changes in HO-1 mRNA (A, n = 3–4) and protein (B, n = 7) expression in response to paraquat. PC12 cells were treated with 50 μM paraquat for 6–48 h. C: Effect of an HO-1 siRNA on the upregulation of HO-1 induced by paraquat. PC12 cells were transfected with an HO-1 siRNA before treatment with paraquat (50 μM) for 24 h. D: Effect of an HO-1 siRNA on paraquat toxicity. PC12 cells were transfected with an HO-1 siRNA before treatment with paraquat (250 μM) for 48 h. (n = 4) E: Effect of bilirubin on paraquat toxicity. PC12 cells were treated with 50 μM paraquat for 24 h in the presence or absence of bilirubin (3–30 μM). (n = 4) $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the control. n.s., not significant.

paraquat. GSH does not only remove cellular hydrogen peroxide as a substrate for glutathione peroxidase but also reacts directly with the superoxide anion (42). In contrast, although bilirubin is an efficient scavenger of hydroxyl radicals, the reaction of bilirubin with the superoxide anion is rather slow (28). This can explain the observation that suppression of HO-1 upregulation did not exacerbate paraquat toxicity. This finding may be relevant to our previous report, which showed that trolox (which scavenges the superoxide anion less efficiently) only had a minor effect on paraquat toxicity (24).

In summary, the present study suggests that compensatory activation of the Nrf2–ARE pathway via inhibition of 26S proteasome serves as part of a cellular defense mechanism to protect against paraquat toxicity. However, further experiments are required to verify that the 26S proteasomal inhibition is associated with the activation of the Nrf2–ARE pathway. Although it is uncertain whether the proteasomal dysfunction observed in PD is directly linked to the Nrf2–ARE pathway, the nuclear localization of Nrf2 is observed in remaining dopaminergic neurons of PD patients (6). Taken together, our findings may provide insights into the relationship between proteasome activity and the Nrf2–ARE pathway in PD pathogenesis.

Conflict of interest

The authors indicated no potential conflicts of interest.

Acknowledgments

This work was supported by JSPS KAKENHI Grant Numbers 21790070, 23790083.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jphs.2015.09.003.

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