Hydrogen Peroxide-Reducing Factor Released by PC12D Cells Increases Cell Tolerance against Oxidative Stress

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PC12D cells, a subline of rat adrenal pheochromocytoma PC12 cells, extend neurites rapidly in response to differentiation stimuli and are used to investigate the molecular mechanisms of neurite extension. In the present study, we found significant tolerance of PC12D cells against Parkinson’s disease-related stimuli such as dopamine and 6-hydroxydopamine; this tolerance was significantly decreased by a change in the medium. Conditioned medium from PC12D cells induced tolerance against oxidative stress, which suggests that cytoprotective factor may be released by PC12D cells into the culture medium. Conditioned medium-induced tolerance was not found for PC12 cells or human neuroblastoma SH-SY5Y cells. A cytoprotective factor generated by PC12D cells exhibited hydrogen peroxide-reducing activity. Chemical characterization showed that this cytoprotective factor is water soluble and has a molecular weight about 1000Da, and that its activity is inhibited by sodium cyanide. Release of this cytoprotective factor was increased by differentiation stimuli and oxidative stress. Taken together, these results suggest that release of a hydrogen peroxide-reducing factor by PC12D cells increases cell tolerance against oxidative stress. This study provides new insights into the antioxidative properties of factors in extracellular fluid.

Key words PC12D cell; extracellular fluid; hydrogen peroxide; cytoprotective factor; low molecular compound

Reactive oxygen species (ROS) with varying degrees of reactivity are present in vivo, and their diverse roles in physiological and pathophysiological events have received much attention.1,2) Free radicals such as the hydroxyl radical and peroxyl radical are highly reactive and oxidize biological materials rapidly, whereas hydroperoxides such as hydrogen peroxide (H2O2) exhibit lower reactivity and are involved not only in oxidative stress-induced damage but also in redox regulation and signal transduction in the cell.3) ROS are reduced preferentially by the antioxidative system, which removes free radicals through the actions of radical scavenging antioxidants, and hydroperoxides are reduced and detoxified by peroxidases and catalase.3–5) Heme, an iron protoporphyrin, can also mediate the reduction of H2O2 directly.6)

Numerous studies have implicated ROS and oxidative stress in several neurodegenerative diseases including Parkinson’s disease (PD).7,8) PD is a progressive, age-related, neurodegenerative condition characterized by movement disorders such as bradykinesia, rigidity, and tremor. These symptoms are caused by the degradation of dopamine (DA)-producing neurons in the substantia nigra pars compacta of the midbrain.9) Increasing evidence suggests that oxidative stress is an important mediator of the pathogenesis of PD. It is thought that nigral dopaminergic neurons are rich in ROS because the enzymatic and nonenzymatic metabolism of DA leads to the generation of ROS.10) Experimental addition of DA induces neuronal cell death, which was inhibited by antioxidants, and ROS are involved in cell death induced by DA.11) DA oxidation can occur spontaneously in the presence of iron and leads to the generation of ROS and DA oxidation products such as 6-hydroxydopamine (6-OHDA), which is a selective catecholaminergic neurotoxin that is used for in vitro and in vivo models of PD.10,12) 6-OHDA can induce a type of toxicity that mimics the neuropathological and biochemical characteristics of PD, while the concentrations of DA and 6-OHDA are different between cellular experiments and clinical/pathophysiological status of PD.13) It has been reported that 6-OHDA is oxidized rapidly by molecular oxygen to generate the superoxide anion, H2O2, and 2-hydroxy-5-(2-aminoethyl)-1,4-benzoquinone (p-quinone) as follows:

6-OHDA + O2 → p-quinone + H2O2

ROS and p-quinone mediate 6-OHDA-induced cell death and these compounds exhibit characteristic cytotoxicity and raise cellular response.14–16) The toxicity of 6-OHDA is attenuated by thiol antioxidants such as glutathione (GSH) and n-acetyl cysteine (NAC).14–16)

PC12 cells, which are rat adrenal pheochromocytoma cells, are used as a model of neuronal cells that can undergo 6-OHDA-induced apoptotic cell death.14,15) PC12 cells are used as a model of neuronal differentiation because they respond to nerve growth factor (NGF) by exhibiting neurite growth and increased catecholamine synthesis, activation of adenylate cyclase, and generation of intracellular cAMP.17) Sano and colleagues were found, by chance, a subline of PC12 cells (PC12D cells) that extend neurites very rapidly in response to NGF and permeable analogues of cAMP such as dibutyryl cAMP.18) NGF-dependent neurite growth in PC12D cells does not require RNA and protein synthesis.19) Since PC12
cells respond to NGF only, the signal transduction pathways for NGF-dependent rapid neurite growth in PC12D cells have been analyzed and compared with those by dibutyryl cAMP. However, the resistance of PC12D cells to oxidative stress has not been investigated.

In the present study, we found a potent tolerance of PC12D cells against oxidative stress compared with other neuronal cells including PC12 cells. We demonstrate for the first time that PC12D cells can release a H$_2$O$_2$-reducing factor into extracellular fluid.

MATERIALS AND METHODS

**Materials** Dulbecco’s modified Eagle’s medium (DMEM), DMEM/nutrient mixture Ham’s F-12/1:1 (DMEM/F-12), horse serum (HS), and N2 Supplement (####17502048) were obtained from Invitrogen (Carlsbad, CA, U.S.A.). 6-OHDA (purity: ≥97%, H4381), GSH (G4251), catalase (C40), and scopoletin (S2500) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). PD-10 (####17085101), HiLoad 16/600 Superdex 30-pg (S2500) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). PBS was used for elution at 1 mL/min, and the active fractions were collected at 3 mL/fraction.

**Preparation of Fresh and Conditioned Medium** To prepare the conditioned medium, PC12D cells were grown in a 10 cm dish to 70% confluency and were then washed twice with phosphate-buffered saline (PBS) and cultured in 5 mL of DMEM containing 5% N2 Supplement and antibiotics for 16 h. After the culture period, the medium was collected and centrifuged at 450×g for 5 min. The supernatant was used as the conditioned medium and medium samples were stored at −80°C. The fresh control medium was prepared by using same procedure without cells, namely DMEM containing 5% N2 Supplement and antibiotics was added to a 10 cm dish without cells, incubate for 16 h, and then the supernatant was used as control. In the case of fresh control serum medium and conditioned serum medium, DMEM containing 10% heat-inactivated HS, 5% heat-inactivated FBS and antibiotics was used as same as described above.

**Neutral Red Staining** To examine changes in nuclear morphology, treated cells were stained with 0.01% neutral red solution at 37°C for 45 min. Nuclear condensation within the cells was observed through an Olympus IX-71 epifluorescence microscope (Olympus, Tokyo, Japan). The experimental results were evaluated as visual and qualitative things.

**Measurement of p-Quinone Generation** The generation of p-quinone by autoxidation of 6-OHDA was measured spectrophotometrically. The assay was conducted in a cell-free system under conditions corresponding to the cellular 6-OHDA treatment. Conditioned medium or fresh control medium (phenol red free) with or without GSH (2 mM) was maintained thermostatically at 37°C during the experiment. The experiment was initiated by the addition of 6-OHDA to give a final concentration of 100 μM. Maximum absorption of p-quinone (490 nm) was monitored every 10 s.

**Measurement of H$_2$O$_2$ Generation** H$_2$O$_2$ concentration was measured using a scopoletin assay. Briefly, 200 μL of 100 μM scopoletin, and 200 μL of 32.5 U/mL horseradish peroxidase were added to samples, and the volume was adjusted to 1 mL with PBS. After 5 min at room temperature, fluorescence was measured at an excitation wavelength of 366 nm and emission wavelength of 460 nm. H$_2$O$_2$ concentration in each sample was determined by using 30% H$_2$O$_2$ (Wako) as the standard. H$_2$O$_2$ concentration in the standard was confirmed by using extinction coefficient 61 μmol/L cm$^{-1}$ at 230 nm. Reduced H$_2$O$_2$ was calculated from the equation: reduced H$_2$O$_2$(determined H$_2$O$_2$ in fresh control medium)−(determined H$_2$O$_2$ in conditioned medium).

**Column Chromatography** An AKTAprime plus system (GE Healthcare) was used for fractionation of the cytoprotective factor. Medium samples were prepared as described above, and DMEM (phenol red free) without N2 supplement and antibiotics was used to collect the cytoprotective factor. Microscopy confirmed that there were no changes in cell morphology. The conditioned medium was concentrated by 20-fold using a SpeedVac concentrator SAVANT SPD1010 (Thermo Scientific), and the concentrated samples were applied to a HiLoad 16/600 Superdex 30-pg column (GE Healthcare). PBS was used for elution at 1 mL/min, and the fractionated samples were collected at 3 mL/fraction. The H$_2$O$_2$-reducing activity of each fraction was determined, and the active fractions 32 to 38 were stored at −20°C and used for further experiments.

Active fractions separated using the Superdex 30-pg column were applied to a Hitrap SP FF cation-exchange chro-
matography column (GE Healthcare) equilibrated with PBS. After washing with PBS, phosphate buffer (pH 7.4) containing 1 M NaCl was applied. The H₂O₂-reducing activity of each fraction was determined, and active fractions 1 and 2 were applied to a HiTrap Q FF anion-exchange chromatography column (GE Healthcare) equilibrated with PBS. After washing with PBS, phosphate buffer (pH 7.4) containing 1 M NaCl was applied. Active fraction 2 was used for the inhibitor experiments.

Protein Assay The protein concentration was measured using a BCA protein assay kit (#23225, Pierce Biotechnology, Inc., Rockford, IL, U.S.A.) with bovine serum albumin as a standard.

Statistical Analysis Data are reported as mean±standard deviation (S.D.) of at least three independent experiments. Differences between determinations were analyzed using either Student’s t-test or ANOVA with Tukey’s test for multiple comparisons. p<0.05 was considered to be significant.

RESULTS

Cytoprotective Factor in the Cultured Medium of PC12D Cells The tolerance of PC12D cells against PD-related stimuli such as DA and 6-OHDA was investigated. The change in cells viability was dependent on the stressor concentration, and the median lethal concentration was differed by the medium change (Fig. 1A). Effective concentration 50 (EC₅₀) of DA and 6-OHDA in each condition was estimated as follows: Control, 460 µM DA; Medium change, 220 µM DA; Control, 270 µM 6-OHDA; and Medium change, 130 µM 6-OHDA. Thus, the difference of EC₅₀ between each condition was calculated as 240 µM DA and 140 µM 6-OHDA, respectively (Fig. 1A). These results suggest the cytoprotective factor in the cultured medium of PC12D cells. By contrast, the medium change caused a smaller change in the viability of PC12 cells (Fig. 1B) and human neuroblastoma SH-SY5Y cells (Fig. 1C). The difference of EC₅₀ in each condition was calculated as: 60 µM DA and 23 µM 6-OHDA in PC12 cells (Fig. 1B); and 380 µM DA (calculated from non-linearity point) and 17 µM 6-OHDA in SH-SY5Y cells (Fig. 1C).

To identify the cytoprotective factor in conditioned medium from PC12D cells, the effects of the addition of this medium were investigated. After culture of PC12D cells for 16–18 h, the medium was replaced by either fresh control serum medium or conditioned serum medium, and the cells were treated with different concentrations of DA or 6-OHDA. These solutions were prepared as described in the Materials and Methods. As shown in Fig. 2A, the decrease in PC12D cell viability was attenuated significantly by the replacement with conditioned serum medium. EC₅₀ of DA and 6-OHDA was estimated as follows: Fresh control, 400 µM DA; Conditioned, >600 µM DA; Fresh control, 160 µM 6-OHDA; and Conditioned, 500 µM 6-OHDA. Thus, the difference of EC₅₀ between each condition was calculated as >200 µM DA and 340 µM 6-OHDA, respectively (Fig. 2A). Conditioned serum medium protected cells from DA- or 6-OHDA-induced cell death in a concentration-dependent manner (Fig. 2B). The addition of conditioned serum medium also attenuated the increase in the fluorescence intensity of nuclear condensation, an indicator of apoptotic cell death, induced by DA or 6-OHDA.
6-OHDA (Fig. 2C). These results suggest the presence of cytoprotective factor in the conditioned serum medium released by PC12D cells. The cytoprotective effects were observed in the conditioned medium obtained from PC12D cells grown in serum-free N2 medium (Supplementary Fig. S1); therefore, cultured N2 medium was used for further experiments.

To examine further the cytoprotective factor produced by PC12D cells, the following three conditions were examined (Supplementary Fig. S2). PC12D cells were pretreated with conditioned medium for 2 h and the cells were treated with 6-OHDA in the presence (condition I) or absence (condition II) of conditioned medium. PC12D cells were treated with 6-OHDA in conditioned medium without pretreatment (condition III). The cytoprotective activity of conditioned medium disappeared in condition II (Supplementary Fig. S3), which suggests that the presence of both the cytoprotective factor and 6-OHDA is important. GSH is known to protect from cell death induced by 6-OHDA via direct antioxidative action in the extracellular fluid; GSH exhibited cytoprotective activity in conditions I and III but not in condition II (Supplementary Fig. S3).

H₂O₂-Reducing Activity of the Cytoprotective Factor Released by PC12D Cells

6-OHDA is readily oxidized in the presence of oxygen to yield H₂O₂ and p-quinone, which plays an important role in cytotoxicity induced by 6-OHDA. To determine the role of the cytoprotective factor in conditioned medium released by PC12D cells, the effects of conditioned medium on the formation of H₂O₂ and p-quinone from 6-OHDA were evaluated. The spectrophotometric assay showed an increase in the absorption at 490 nm, which is attributed to the production of p-quinone, and this reaction was not influenced by the presence of conditioned medium (Fig. 3A). By contrast, this increase was not observed in the presence of GSH, as reported previously (Fig. 3A). This result suggests that the cytoprotective factor does not influence the autoxidation step of 6-OHDA. Next, the scopoletin assay was used to quantify the H₂O₂ content in the presence of conditioned medium. As shown in Fig. 3B, H₂O₂ content in medium added H₂O₂ or 6-OHDA decreased significantly in the presence of conditioned medium from PC12D cells, which suggests that the cytoprotective factor generated by PC12D cells exhibits H₂O₂-reducing activity. Consistent with this observation, H₂O₂-induced cell death was inhibited significantly by the addition of conditioned medium (Fig. 3C). EC₅₀ of H₂O₂ was estimated as follows: Fresh control, 58 μM H₂O₂; and Conditioned, 270 μM H₂O₂. Thus, the difference of ED₅₀ between each condition was calculated as 212 μM H₂O₂ (Fig. 3C). The H₂O₂-reducing enzyme, catalase, inhibited cell death induced by 6-OHDA (Fig. 3D). We examined whether the H₂O₂-reducing activity of the conditioned medium was dependent on the substrate concentration. H₂O₂ content decreased linearly to 800 μM and reached a plateau at a concentration of ca. 1000 μM (Fig. 3E).

Chemical Characterization of the Cytoprotective Factor Released by PC12D Cells

To estimate the molecular weight of the cytoprotective factor released by PC12D cells,
Conditioned medium was first separated using a Sephadex G-25 PD-10 column, which can separate molecules of 5000 Da (fraction number 5) from those of 1000 Da (fraction number 9). After fractionation of the conditioned medium, the H2O2-reducing activity and cytoprotective activity were determined. As shown in Fig. 4A, the peak of both activities was observed in fraction number 7, which suggests that the molecular weight of the cytoprotective factor is between 5000 and 1000 Da. These fractions were collected and concentrated, and then further separated using Superdex 30-pg gel column chromatography, which has an optimal molecular weight range <10000 Da. The peak of H2O2-reducing activity was observed between fraction numbers 34 and 37, and was estimated at ca. 10000 Da against standard molecules (Fig. 4B). The active fractions were pooled and applied to ion-exchange column chromatography. The cytoprotective factor in PBS solution did not bind to the HiTrap SP FF cation-exchange column, and its activity was detected in the flow-through fraction (Fig. 4C). This fraction was applied to a HiTrap Q FF anion-exchange column. Again, the H2O2-reducing activity was detected in the flow-through fraction (Supplementary Fig. S4). This fraction containing the partially purified cytoprotective factor was used for the following experiments.

The chemical properties of the cytoprotective factor were evaluated further. Neither heat treatment for 60 min at 100°C nor protease treatment for 60 min at 37°C altered the cytoprotective activity against DA and 6-OHDA (Fig. 5A). Acidification by the addition of trifluoroacetic acid (TFA) inhibited the H2O2-reducing activity of the cytoprotective factor (Fig. 5B). EDTA cannot coordinate with stably bound iron, such as the iron in heme.20) We next examined the effects of NaN3 and NaCN on the H2O2-reducing activity of the cytoprotective factor. We found that NaN3 significantly inhibited the H2O2-reducing activity of the cytoprotective factor. EDTA cannot coordinate with stably bound iron, such as the iron in heme.20) We next examined the effects of NaN3 and NaCN on the H2O2-reducing activity of the cytoprotective factor. We found that NaN3 significantly inhibited the H2O2-reducing activity of the cytoprotective factor. 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the cytoprotective factor as a typical heme were examined using optical analysis and inhibitors. However, the characteristic absorbance of heme was not observed in the cytoprotective factor isolated from conditioned medium (Supplementary Fig. S7). The heme inhibitor, imidazole, inhibited the H$_2$O$_2$-reducing activity of heme in a concentration-dependent manner, but the H$_2$O$_2$-reducing activity of the cytoprotective factor was not inhibited by imidazole (Supplementary Fig. S8).

Increased Release of the Cytoprotective Factor in Response to Differentiation Stimuli and Oxidative Stress

The quantity of cytoprotective factor released by PC12D cells was investigated in response to well-known differentiation stimuli such as NGF and retinoic acid. PC12D cells were differentiated by exposure to NGF or retinoic acid for 72 h. After culture in each condition, the cells were washed and cultured with fresh serum-free N2 medium for 16 h. The conditioned medium was collected, and its H$_2$O$_2$-reducing activity was determined. Cells grown in each condition were also col-

Fig. 4. Column Chromatography to Fractionate the Cytoprotective Factor in Conditioned Medium Released by PC12D Cells

(A) Control fresh medium or conditioned medium was separated on a Sephadex G-25 PD-10 chromatography column, and the H$_2$O$_2$-reducing activity (upper panel, n=4) and cytoprotective activity against 6-OHDA (200 µM, lower panel, n=3) were determined. **p<0.01, *p<0.05 compared with fresh control medium (Student’s t-test). (B) The cytoprotective factor was separated on a Superdex 30-pg gel chromatography column, and the H$_2$O$_2$-reducing activity of each fraction was determined (n=4). (C) The cytoprotective factor in PBS solution was applied to a HiTrap SP FF cation-exchange chromatography column, and the H$_2$O$_2$-reducing activity (left, n=4) and cytoprotective activity against 6-OHDA (200µM, right, n=3) were determined. The peak and fractions containing the cytoprotective factor are indicated by a black arrow and black bar, respectively. *p<0.05 compared with fresh control medium (Student’s t-test).

Fig. 5. Chemical Properties of the Cytoprotective Factor in Conditioned Medium Released by PC12D Cells

(A) Effects of heat and protease treatment on the cytoprotective factor. The cytoprotective factor was incubated for 60 min at 100°C (heat) or incubated with proteases (0.1% trypsin) for 60 min at 37°C, proteases were removed by filter treatment, and the cytoprotective effect against DA or 6-OHDA was evaluated (n=3). **p<0.01 compared with control solution (ANOVA and Tukey’s test). (B) Effects of acidification on the H$_2$O$_2$-reducing activity of the cytoprotective factor. The indicated amount of TFA was added to conditioned medium, 200 µM H$_2$O$_2$ was added, and the solution was incubated at 37°C for 15 min. The H$_2$O$_2$ content was measured using the scopoletin assay (n=4). **p<0.01, *p<0.05 compared with 0% TFA (ANOVA and Tukey’s test).
lected, and the total protein content of whole-cell lysates was measured to estimate the cell number. Reduced H$_2$O$_2$ content under constant protein content is shown in Fig. 7A. The release of cytoprotective factor tended to increase under the low-serum-medium condition, and the maximum H$_2$O$_2$-reducing activity was observed after stimulation with retinoic acid in the low-serum medium (Fig. 7A). The significant increase in H$_2$O$_2$-reducing activity was also observed after NGF stimulus (Fig. 7A).

The effects of oxidative stress on the release of the cytoprotective factor were investigated. PC12D cells were treated with different concentrations of H$_2$O$_2$ for 24h, and the cells were cultured further with fresh medium for 16h. In this culture condition, obvious cell death was not observed (Fig. 3C). The H$_2$O$_2$-reducing activity and protein content were measured. As shown in Fig. 7B, an increase in H$_2$O$_2$-reducing activity was observed in PC12D cells treated with 100 µM H$_2$O$_2$.

DISCUSSION

PC12D cells are used to investigate the molecular mechanisms of neurite extension. The proteasome-mediated regulation of tyrosine hydroxylase was discovered recently in PC12D cells, although the resistance of PC12D cells to oxidative stress has not been investigated thoroughly. In the present study, we found that PC12D cells released cytoprotective factor, which increased their resistance to oxidative stress by removing extracellular H$_2$O$_2$. Results in the present study indicate that the H$_2$O$_2$-reducing factor is a water-soluble, low-molecular-weight compound with stably bound metal ions that is important for the redox activity of this factor. Chemically, porphyrins such as heme are stable to heat but not to strong acid because strong acidic conditions accelerate the release of the metal from the porphyrin center. Because the affinity of the cyanide ion is stronger than that of azide ion, the cyanide ion exhibits strong inhibitory activity against heme. These properties suggest that the cytoprotective factor is a heme; however, optical analysis and the effects of imidazole suggest that the cytoprotective factor is not a typical heme. We further evaluated this hypothesis by using MS analysis; however, we could not identify promising candidates, namely organic materials containing metal, by several MS analysis such as electrospray ionization (ESI)-MS, matrix assisted laser desorption/ionization-time of flight (MALDI-TOF)-MS, and LC-MS (SHIMADZU, Kyoto, Japan). The identification of the precise chemical structure is a limit of the present study. To achieve this, it is considered that large amounts of samples and highly sensitive MS are required.

Fig. 6. Effects of Metal Chelators on the Cytoprotective Factor in Conditioned Medium Released by PC12D Cells

(A–C) Effects of the metal chelators EDTA (A), NaCN (B), and NaN$_3$ (C) on the H$_2$O$_2$-reducing activity of the cytoprotective factor. The indicated amount of metal chelators was added to the cytoprotective factor solution, and the H$_2$O$_2$-reducing activity was determined. For experiments using EDTA, 0.15 mM EDTA was added. The mean±S.D. values are shown (n=4). **p<0.01, *p<0.05 compared with each control solution (A) or 0 mM NaCN (B) (ANOVA and Tukey’s test). N.S.: not significant. Significant difference of determined H$_2$O$_2$ in conditioned medium from 0 to 6 mM NaN$_3$ was not observed (C).

Fig. 7. Increased Release of the Cytoprotective Factor in Response to Differentiation Stimuli and Oxidative Stress

(A, B) After attachment for 16–18h, cells were differentiated by exposure to 20 µM retinoic acid or 10 ng/mL NGF in control serum medium or low-serum medium for 72h (A). To induce oxidative stress, cells were treated with the indicated amount of H$_2$O$_2$ for 24h (B). After these stimuli, cells were washed, conditioned medium was collected, and the H$_2$O$_2$-reducing activity was measured. Reduced H$_2$O$_2$ content is shown in the y-axis (n=4). **p<0.01 compared with control medium (A) or 0 µM H$_2$O$_2$ (B) (ANOVA and Tukey’s test).
Several kinds of porphyrin with different structures and functions have been discovered.\textsuperscript{20,25,26} Several other porphyrin-related molecules are known, such as chlorophyll, which contains magnesium, and cyanocobalamin, which contains cobalt.\textsuperscript{26} Further, chemically synthesized porphyrins and expanded porphyrin have been reported to possess diverse molecular functions.\textsuperscript{27} Chemical modification greatly influences the reactivity of porphyrin.\textsuperscript{20,27} Thus, it is hypothesized that the cytoprotective factor released by PC12D cells is chemically modified to increase its H$_2$O$_2$-reducing activity. It has been reported that chemical modification of porphyrin greatly increases potential peroxynitrite decomposition catalysts.\textsuperscript{27} Therefore, it might be an interesting subject to determine the reactivity of cytoprotective factor against several reactive species.

The secretion of porphyrin, mostly protoporphyrin IX without metal ion, is produced in the Harderian gland, which is an exocrine gland that produces secretions to lubricate and protect the eye.\textsuperscript{28} ABCG2, a member of ATP-binding cassette (ABC) transporter gene family, binds and transports porphyrin in the Harderian gland,\textsuperscript{29} although the biological function of porphyrin secretion has not been fully elucidated. Understanding of the biological role and molecular mechanisms of heme and/or porphyrin secretion is limited, and further studies are needed to determine whether the H$_2$O$_2$-reducing factor released into the culture medium in this study has biological functions.

PC12D cells exhibited higher tolerance against oxidative stress than did PC12 cells and SH-SY5Y cells. However, the medium change caused this difference to disappear almost completely, which suggests that the factor released by PC12D cells may involve in their tolerance to oxidative stress. Release of the H$_2$O$_2$-reducing factor by PC12D cells was accelerated by treatment with differentiation stimuli such as retinoic acid or NFG. It would be interesting to determine whether the synthesis and release of this H$_2$O$_2$-reducing factor, which lead to removal of H$_2$O$_2$, are related to neurite extension. We also found that oxidative stress increased the release of the cytoprotective factor by PC12D cells. The physiologic significance of these responses is unclear. However, the redox environment differs between the intra- and extracellular fluids; that is, the former is rich in reductants such as GSH, while the latter is poor in reductants.\textsuperscript{30,31} DA oxidation might easily occur in the extracellular fluid and would generate ROS. We suggest that the release of the H$_2$O$_2$-reducing factor might be one way of protecting the cells against oxidative stress in the extracellular fluid.

In conclusion, we found that PC12D cells release an antioxidative compound that behaves as a H$_2$O$_2$-reducing factor. The current findings suggest that a newly identified antioxidative factor is released by cultured cells into extracellular fluid and may protect the cells against oxidative stress. This study might provide the new strategy for antioxidative therapy of neurodegenerative diseases including PD.

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**Conflict of Interest** The authors declare no conflict of interest.

**Supplementary Materials** The online version of this article contains supplementary materials.

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