Heme oxygenase (HO)-1 has emerged as a new therapeutic target for neurological diseases. We have reported that epalrestat (EPS), which is currently used for the treatment of diabetic neuropathy, increases HO-1 levels through the activation of nuclear factor erythroid 2-related factor 2 (Nrf2) in bovine aortic endothelial cells. In this study, we tested the hypothesis that EPS upregulates HO-1 via Nrf2 activation in the component cells of the nervous system, by using rat Schwann cells and human SH-SY5Y cells. Treatment of Schwann cells with EPS at near-plasma concentration led to a dramatic increase in HO-1 levels. Nrf2 knockdown by small interfering RNA (siRNA) suppressed the EPS-induced HO-1 expression. EPS did not promote the intracellular accumulation of free ferrous iron and reactive oxygen species, by increasing ferritin via Nrf2 during HO-1 induction. Moreover, EPS stimulated the expression of superoxide dismutase 1 and catalase, which also are Nrf2 target gene products. It also markedly increased HO-1 levels in SH-SY5Y cells through the activation of Nrf2. We demonstrated for the first time that EPS upregulates HO-1, superoxide dismutase, and catalase by activating Nrf2. We suggest that EPS has the potential to prevent several neurological diseases.

**Key words** epalrestat; heme oxygenase (HO)-1; superoxide dismutase (SOD); catalase

Fig. 1. Chemical Structure of Epalrestat

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proteins, including SOD, catalase, and ferritin. EPS may be associated with the increase of antioxidant protein expression. In this study, we examined the effects of EPS on the expression of HO-1, SOD, and catalase in rat Schwann cells. In addition, we investigated HO-1 expression in the human neuroblastoma cell line SH-SY5Y.

MATERIALS AND METHODS

Cell Culture and Treatment with EPS Rat Schwann cells and the human neuroblastoma cell line SH-SY5Y were purchased from Sumitomo Dainippon Pharma Co., Ltd. (Osaka, Japan). Cells were grown to 80–90% confluence in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), t-glutamine (4 mM), penicillin (100 U/mL), and streptomycin (100 µg/mL) at 37°C in a humidified atmosphere of 5% CO2 and 95% air. Then, the cells were passaged by trypsinization.

Before treating the cells with EPS (Wako Pure Chemical Industries, Ltd., Osaka, Japan), the culture medium was replaced with DMEM containing 2% FBS. EPS (10, 50, 100 µg) was subsequently added to the medium.

Knockdown of Nrf2 with Small Interfering RNA (siRNA) Oligonucleotides directed against rat Nrf2 and control siRNA (Ambion, Austin, TX, U.S.A.) were transfected into Schwann cells using Lipofectamine RNAiMAX (Invitrogen, Eugene, OR, U.S.A.), according to the manufacturer’s protocol. Briefly, both Nrf2 siRNA and control siRNA were diluted with Opti-MEM medium and then, diluted Lipofectamine RNAiMAX was added. The transfection mixture was incubated at room temperature for 20 min. When cells reached 30–50% confluence, the culture medium was replaced with DMEM (without FBS) and the transfection mixture was added to each well. The final concentration of siRNA was 50 nm.

Measurement of Protein HO-1, SOD, and catalase protein levels were analyzed by Western blotting. The cells were treated with EPS, washed with Dulbecco’s phosphate buffered saline (DPBS), and lyzed in radioimmunoprecipitation assay (RIPA) buffer (Pierce, Rockford, IL, U.S.A.) containing protease inhibitors. The lysate was centrifuged at 10,000×g for 15 min and 15 µg of protein in the supernatant was resolved by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were blotted onto a polyvinylidene difluoride (PVDF) membrane. The membrane was incubated with the following primary antibodies: anti-rabbit HO-1 polyclonal antibody (Abcam, Cambridge, U.K.), anti-rabbit SOD1 polyclonal antibody (Abcam), and anti-mouse catalase monoclonal antibody (Sigma-Aldrich, St. Louis, MO, U.S.A.). Following primary antibody incubation, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. Chemiluminescence was detected with horseradish peroxidase (HRP)-conjugated secondary antibodies. Chemiluminescence was detected with an ECL Plus Western blot detection kit (GE Healthcare, Tokyo, Japan). Protein levels were analyzed by Western blotting. The cells were treated with EPS and incubated in medium containing 5-(6)-carboxy-2’,7’-dichlorofluorescein diacetate (Sigma-Aldrich) and RhoNox-1 (Goryo Chemical, Inc., Sapporo, Japan) were used to estimate intracellular ROS and free iron levels, respectively. The cells were treated with EPS and incubated in medium containing 5-(6)-carboxy-2’,7’-dichlorofluorescein diacetate (10 µM) or RhoNox-1 (5 µM) for 20 min or 1 h, respectively. After the cells were washed with DPBS, changes in intracellular ROS and free iron levels were visualized as green or red fluorescence by confocal laser scanning microscopy.

Other Procedures Cell viability was assessed by using CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS assay) from Promega (Madison, WI, U.S.A.), as described previously. Protein concentrations were determined using the Bradford method with bovine serum albumin as the standard.

Statistical Analysis All experiments were performed independently at least three times. Data were combined and expressed as the mean±standard deviation (S.D.). Statistical significance was determined using one-way ANOVA with Bonferroni post-hoc tests. A p value of <0.05 was considered to be significant.

RESULTS

Effect of EPS on HO-1 Levels in Schwann Cells HO-1 is known as a typical antioxidant protein regulated by Nrf2 activation. In our previous work, we demonstrated that the activation of Nrf2 was observed after treatment of Schwann...
In this study, we first examined the effect of EPS on HO-1 expression in Schwann cells (Fig. 2). Schwann cells were treated with EPS at 10, 50, and 100 µM for 24h. Then, HO-1 protein levels in the EPS-treated Schwann cells were estimated by fluorescence microscopy studies (Fig. 2A) and Western blot analysis (Fig. 2B). Both fluorescence microscopy studies and Western blot analysis demonstrated a dose-dependent increase in HO-1 protein levels in the EPS-treated Schwann cells. Treatment with 10 µM EPS did not induce a significant increase in HO-1 protein levels, whereas treatment with 50 and 100 µM EPS markedly increased HO-1 protein levels. In the treatment with 50 and 100 µM EPS, the increases were 3.9- and 13.7-fold by Western blot analysis, respectively, relative to control. Figure 2C shows that EPS at 50 and 100 µM caused a dramatic increase in HO-1 mRNA levels. In Schwann cells treated with 10 µM EPS, no significant change was observed in HO-1 mRNA levels. These results indicate that EPS increases HO-1 levels in Schwann cells through transcription regulation.

Next, we examined whether Nrf2 levels could alter the increases in HO-1 levels in cells treated with EPS, by means of Nrf2 knockdown in Schwann cells. Schwann cells were transfected with control siRNA (siControl) or Nrf2 siRNA (siNrf2). Nrf2 mRNA levels in the cells transfected with Nrf2 siRNA were reduced by approximately 90% relative to those in control siRNA transfected cells (data not shown). As shown in Fig. 2D, the increase in HO-1 mRNA levels after treatment with 50 µM EPS was completely inhibited by the knockdown of Nrf2 by siRNA. These results indicate that EPS induces HO-1 upregulation via Nrf2-mediated signaling in Schwann cells.

**Effect of EPS on Intracellular Free Iron and Ferritin Levels in Schwann Cells**

HO-1 catalyzes the degradation of heme to produce ferrous iron, which easily reacts with molecular oxygen to produce superoxide that is subsequently converted into H$_2$O$_2$. It has been demonstrated that the over-expression of HO-1 promotes ROS formation and cell death.\(^6\)\(^7\)

As shown in Fig. 2, EPS at 50 and 100 µM caused a dramatic increase in HO-1 levels, implying that the excess free iron released by HO-1 may accelerate ROS production in the intracellular milieu. It was reported that the treatment of Schwann cells with EPS at 10 and 50 µM for 24h had no effect on cell viability; slightly lowered cell viability was observed at the EPS concentration of 100 µM and loss of viability was noted in only 4% of the EPS-treated cells compared to control.\(^10\)

Then, we further examined the effect of EPS on free iron levels. Figure 3B shows the intracellular free ferrous iron levels estimated by fluorescence microscopy studies using the novel turn-on fluorescent probe RhoNox-1 for the selective detection of labile ferrous iron through the formation of a red fluorescent product.\(^16\) EPS did not induce the intracellular accumulation of free ferrous iron; in fact, a dose-dependent decrease in red fluorescence was observed in Schwann cells treated with EPS.

Iron is sequestered by ferritin, an intracellular iron repository protein that is co-induced with HO-1.\(^17\)

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Fig. 2. Effect of EPS on HO-1 in Schwann Cells

Schwann cells were treated with EPS at the indicated concentrations for 24h. HO-1 protein levels were estimated by fluorescence microscopy studies (A) and by Western blot analysis (B), and HO-1 mRNA levels were quantified by using real-time RT-PCR (C). Pictures shown are representative of three independent experiments. Scale bar in A indicates 20µm. Values in B and C are the mean±S.D. of three experiments. * Significant difference from the value of control ($p<0.05$). (D) Schwann cells were transfected with control siRNA (siControl) or Nrf2 siRNA (siNrf2) and were treated or not treated with 50 µM EPS for 24h. Subsequently, HO-1 mRNA levels were measured. Values are the mean±S.D. of three experiments. * Significant difference from the value of non-treated siControl ($p<0.05$). # Significant difference from the value of siControl treated with EPS ($p<0.05$).
demonstrates that EPS at 50 and 100 µM upregulated ferritin mRNA levels. The treatment with 10 µM EPS did not cause a significant increase in ferritin mRNA levels. Nrf2 knockdown by siRNA suppressed the increase in ferritin mRNA levels after EPS treatment (Fig. 3D). It seems that EPS acts to store free iron in the non-toxic form by increasing ferritin via Nrf2 during HO-1 induction.

Fig. 4. Effect of EPS on SOD1 and Catalase Protein Levels in Schwann Cells

Schwann cells were treated with EPS at the indicated concentrations for 24 h. SOD1 and catalase protein levels were estimated by fluorescence microscopy studies (A: SOD1, B: catalase) and by Western blot analysis (C: SOD1, D: catalase). Pictures shown are representative of three independent experiments. Scale bar in A and B indicates 20 µm. Values in C and D are the mean ± S.D. of three experiments. * Significant difference from the value of control \((p < 0.05)\). 

Fig. 3. Effect of EPS on Intracellular ROS, Free Iron, and Ferritin Levels in Schwann Cells

Schwann cells were treated with EPS at the indicated concentrations for 4 h (A) or 24 h (B–D). Intracellular ROS (A) and ferrous iron (B) levels were estimated by fluorescence microscopy using 5-(6)-carboxy-2’7’-dichlorofluorescein diacetate and RhoNox-1, respectively. Each panel shows the typical fluorescence intensity from three independent experiments. Bright-field images are shown in the upper part of A. Scale bar, 20 µm. (C) Ferritin mRNA levels were quantified by using real-time RT-PCR. Values in C are the mean ± S.D. of three experiments. * Significant difference from the value of control \((p < 0.05)\). (D) Schwann cells were transfected with control siRNA (siControl) or Nrf2 siRNA (siNrf2) and were treated or not treated with 50 µM EPS for 24 h. Subsequently, ferritin mRNA levels were measured. Values are the mean ± S.D. of three experiments. * Significant difference from the value of non-treated siControl \((p < 0.05)\). 

* Significant difference from the value of control \((p < 0.05)\). 

# Significant difference from the value of siControl treated with EPS \((p < 0.05)\).
Effect of EPS on SOD and Catalase Levels in Schwann Cells

Nrf2 controls not only HO-1 gene but also the genes of many cytoprotective enzymes, including SOD and catalase. We examined whether EPS could alter SOD and catalase levels. SOD1 and catalase protein levels in Schwann cells treated with EPS were estimated by fluorescence microscopy studies and Western blot analysis (Fig. 4). As shown in Figs. 4A and B, slight increases in SOD1 and catalase protein levels were observed at EPS concentrations of 50 and 100 µM, as measured by fluorescence microscopy studies. The increases in SOD1 protein levels after treatment with 50 and 100 µM EPS were 1.8- and 2.2-fold, respectively, relative to control, as determined by Western blot analysis. At those EPS concentrations, the increases in catalase protein levels were 1.4- and 1.8-fold, respectively (Fig. 4D). EPS at 50 and 100 µM increased both SOD1 and catalase mRNA levels (Figs. 5A, B). In addition, Nrf2 knockdown by siRNA suppressed the increases in SOD1 and catalase mRNA levels after treatment with 50 µM EPS (Figs. 5C, D). The results suggest that EPS can induce SOD1 and catalase upregulation via Nrf2. However, the ability of EPS to increase SOD1 and catalase levels is suppressed compared with its ability to increase HO-1 levels.

**Effect of EPS on HO-1 Levels and Nrf2 in SH-SY5Y Cells**

It is known that HO-1 induction may play an important role in Parkinson’s disease, a degenerative disorder of the nervous system. We examined the effects of EPS on HO-1 expression using the human neuroblastoma cell line SH-SY5Y, which is a model of neuronal cells. SH-SY5Y cells were treated with EPS at 10, 50, and 100 µM for 24 h. EPS at those concentrations had no effect on cell viability (data not shown). As shown in Figs. 6A and B, treatment with EPS at 50 and 100 µM caused an increase in HO-1 levels even in SH-SY5Y cells. At those EPS concentrations, the increases were 3.2- and 17.1-fold, respectively, relative to control, as determined by Western blot analysis. A concomitant increase in HO-1 mRNA level was observed (Fig. 6C). Figure 6D demonstrates that EPS induced an increase in nuclear Nrf2 protein level. The increase was 6.6- and 11.1-fold by treatment with 50 and 100 µM EPS, respectively. EPS at 10 µM did not significantly increase the nuclear Nrf2 protein level. The results in Fig. 6D were similar to those in Figs. 6A–C; Nrf2 activation and HO-1 upregulation were observed under the same conditions for 50 and 100 µM EPS treatment. As can be seen from Fig. 6E, EPS at the concentrations used had no influence on cytosolic Nrf2 protein level. The results indicate that EPS increases HO-1 levels by stimulating the Nrf2 pathway in not only Schwann cells but also SH-SY5Y cells.

**DISCUSSION**

EPS (Ono Pharmaceuticals, Osaka, Japan) is the only aldose reductase inhibitor currently available for the treatment of
revealed that LY294002, an inhibitor of phosphatidylinositol 3-kinase, activates Nrf2. Our study of bovine aortic endothelial cells indicates that the inhibition of aldose reductase does not suggest that the kinase is associated with Nrf2 activation and contributes to the ability of EPS to increase HO-1 levels by targeting Kelch-like ECH associated protein 1 (Keap1).8,23,24) When the Keap1–Nrf2 complex is dissociated by some form of cellular stimuli, Nrf2 is translocated into the nucleus where it binds to ARE. EPS has an α,α-,α-unsaturated ketone moiety in its structure. α,β-Unsaturated ketones act as inducers of ARE genes by oxidizing sulfhydryl groups in Keap1, which leads to the dissociation and nuclear translocation of Nrf2.25) Therefore, the EPS-stimulated Nrf2 activation is most likely achieved by altering Keap1 structure. Unlike EPS, other aldose reductase inhibitors, such as sorbinil and alrestatin,26,27) failed to increase HO-1 levels (data not shown). This result indicates that sorbinil and alrestatin, which do not have an α,β-unsaturated aldehyde or ketone, have no influence on the Nrf2 pathway. Aldose reductase is known as a target gene of Nrf2.28,29) However, it seems unlikely that aldose reductase is induced by EPS because our previous study indicated that the reductase activity in Schwann cells was decreased by 30–50% after treatment with 10–100 µM EPS.10) In addition, these findings suggest that the inhibition of aldose reductase does not contribute to the ability of EPS to increase HO-1 levels by activating Nrf2. Our study of bovine aortic endothelial cells revealed that LY294002, an inhibitor of phosphatidylinositol 3-kinase,30) abolished the EPS-stimulated GSH synthesis, suggesting that the kinase is associated with Nrf2 activation induced by EPS.21) In contrast, in the case of Schwann cells, it was unlikely that the action of EPS was associated with the kinase because LY294002 had no influence on EPS-stimulated GSH synthesis (data not shown). On the other hand, Nrf2 was degraded by the ubiquitin-proteasome system and proteasome inhibitors enhanced HO-1 mRNA and protein accumulation.31) EPS had no effect on proteasome activity in Schwann cells (data not shown). In addition, EPS-induced HO-1 upregulation was observed in the presence of clasto-lactacytin-β-lactone, a proteasome-specific inhibitor32) (data not shown). We suggest that ubiquitin-proteasome is not involved in the mechanism underlying HO-1 induction by EPS. At present, it remains unclear how EPS activates the Nrf2–Keap1 pathway in Schwann cells; its clarification will require further studies.

HO-1 is induced in response to oxidative stress and protects cells from oxidative injury.20) The HO-1-mediated protection is related to the degradation of its substrate, heme.24) Non-protein-bound free heme is highly toxic and may cause oxidative stress.21) It has been shown that free heme has proinflammatory properties.20) The by-products of the HO-1 reaction also contribute to the protective response. For example, CO exerts cytoprotective effects through its potent anti-inflammatory, anti-apoptotic, and vasodilatory properties.4,18) Bile pigments biliverdin and bilirubin are pEROXy radical scavengers.5) However, some studies suggested that HO-1 induction might not always be beneficial and the release of redox-active iron from heme might enhance oxidative stress.35,36) The circulating redox-active iron is capable of promoting the generation of ROS, such as superoxide and H2O2, which is a source of hydroxyl radical. A recent study suggested that the increased expression of HO-1, which is mostly localized on the endoplasmic reticulum, resulted in significant translocation to the mitochondria and induced oxidative stress by disrupting mitochondrial function.7) Several studies also showed that HO-1 overexpression promoted mitochondrial sequestration of non-transferrin iron and induced macroautophagy that contributed to pathological iron deposition and bioenergetic failure in age-related neurodegenerative disorders.7,37,38) In the present study, EPS dramatically increased HO-1 levels. This implies that the increased HO-1 expression by EPS may lead to excessive free iron accumulation and subsequently ROS production in the intracellular milieu. In addition, because mild oxidative stress activates the Nrf2 pathway,30) it is assumed that EPS has the potential to induce oxidative stress. However, our results indicated that EPS failed to elevate ROS production (Fig. 3). EPS did not accumulate intracellular free iron; in fact, a decrease in free iron levels was observed in Schwann cells treated with EPS, probably by upregulating ferritin expression. These results suggest that EPS increases HO-1 levels without producing unintended negative effects on Schwann cells. Conveniently, the increased expression of SOD and catalase may contribute to the ability to preserve cell viability (Fig. 4). In order to identify the relationship between HO-1 induction and ROS levels, we examined whether ferritin levels could alter the increase in ROS levels in cells treated with EPS, by performing ferritin knockdown in Schwann cells. Nevertheless, EPS had a negligible effect on ROS levels in cells transfected with ferritin siRNA (data not shown). Because EPS induced a dramatic increase in GSH levels10) in the same manner as that observed in HO-1 and SOD/catalase levels, it appeared that EPS failed to elevate ROS production even in ferritin knockdown cells. It has been demonstrated that HO-1 protects cells from damage induced by oxidative stress in diverse cell types.40) In addition, HO-1 exerts cytoprotective effects by preventing apoptosis.40) These findings suggest that HO-1 up-regulation by EPS may prevent the development and progression of disorders caused by oxidative stress.

One study has described that HO-1 is a target for neuro-
protection and neuroinflammation in neurodegenerative diseases. Several phytochemicals, such as resveratrol, curcumin, flavonoids, and carnosol, can upregulate HO-1 expression via the Nrf2 pathway. Studies on the regulation and amplification of HO-1 by pharmacological approaches may lead to the discovery of novel drugs for the treatment of a variety of diseases. Meanwhile, it is necessary to examine currently available pharmacological agents for their ability to induce HO-1 with minimum adverse effects. In drug repurposing strategies, the actions of drugs in clinical use, whose safety and pharmacokinetics in humans have already been confirmed, are examined comprehensively at the molecular level and the results are used for the development of new medicines. One example is statins, which have initially been introduced to prevent arteriosclerosis due to their cholesterol-lowering effects, but have been recognized to exert anti-inflammatory effects via HO-1 induction.

Moreover, 5-aminosalicylic acid, one of the major flavonoids, can upregulate HO-1 expression and has preventive and/or therapeutic potential for the management of Parkinson’s disease. In addition, berberine, a well-known alkaloid, protects SH-SY5Y cells from cell death induced by Parkinson’s disease related neurotoxin 6-hydroxydopamine through the induction of HO-1. From the findings of the present study, we expect that the pharmacological actions of EPS would lead to break-throughs in drug discovery and development. EPS might be useful as a therapeutic agent for the treatment of Alzheimer’s disease and Parkinson’s disease.

In summary, we demonstrated that EPS, approved in Japan for use in treating subjective neuropathy symptoms, upregulates HO-1 expression in Schwann cells and SH-SY5Y cells in association with the Nrf2 pathway. The upregulation of HO-1 is considered to contribute to enhancing neuroprotection. Our findings have led us to propose that targeting the upregulation of HO-1, ferritin, and SOD/catalase by EPS is a promising therapeutic approach in nervous system diseases.

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Conflict of Interest The authors declare no conflict of interest.

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