Epalrestat suppresses cadmium-induced cytotoxicity through Nrf2 in endothelial cells

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Abstract. Cadmium (Cd) is an industrial and environmental pollutant that targets the vascular endothelium. The vascular system is critically affected by Cd toxicity. Recent studies have indicated an association between Cd and vascular diseases, although the mechanisms of Cd implications in vascular diseases are not clear. The purpose of the present study was to determine whether epalrestat (EPS), which is used for the treatment of diabetic neuropathy, protects against Cd-induced cytotoxicity in bovine aortic endothelial cells (BAECs). In the present study, the effects of EPS at near-plasma concentration were examined on Cd-induced cytotoxicity in BAECs. Cd-induced cytotoxicity was suppressed by pretreatment with EPS. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a key transcription factor that serves a role in regulating the expression of glutamate cysteine ligase, the rate-limiting enzyme in glutathione (GSH) synthesis. In a previous study, EPS was demonstrated to increase GSH levels in BAECs in association with the Nrf2 pathway. In the present study, EPS increased GSH levels in BAECs exposed to Cd. The protective ability of EPS against the Cd-induced cytotoxicity disappeared following Nrf2 small interfering RNA transfection. In addition, EPS affected the intracellular levels of Cd, Cd transporter ZIP8 and metallothionein. To the best of our knowledge, the current study demonstrated, for the first time, that EPS suppresses Cd-induced cytotoxicity in BAECs. The upregulation of GSH may be associated with the suppression of Cd-induced cytotoxicity by EPS. From these findings, it may be proposed that the regulation of GSH, ZIP8 and metallothionein by EPS is a promising therapeutic approach to prevent Cd-induced toxicity.

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Introduction

Cadmium (Cd) is a toxic heavy metal that is commonly found at industrial worksites or in the environment (1). The vascular system is a critical target of Cd toxicity and the action of Cd on the vascular system may play important roles in mediating the pathophysiological effects of Cd in specific target organs (1). Vascular endothelial cells are exposed to Cd circulating in bloodstream and, if Cd is present at sufficiently high concentrations, the endothelial cells are injured. Endothelial dysfunction and damage may be attributable to toxicity in parenchymal cells of various target organs, such as kidney and liver (2). The most commonly used therapeutic strategy against Cd toxicity is chelation therapy to promote metal excretion. The chelating agent EDTA is most widely used clinically. However, Cd chelators themselves present a number of safety and efficacy concerns (1). In fact, CaNa2EDTA can cause renal toxicity (in the proximal tubule in particular). Because of lack of specificity, such essential metals as zinc, iron, and manganese are excreted and depleted following CaNa2EDTA therapy (3). Therefore, the development of safe and efficient strategies against Cd toxicity is required.

As a thiol-binding metal, free Cd primarily targets highly abundant cellular glutathione (GSH), a reactive oxygen species (ROS) scavenger (4). Depletion of the GSH pool leads to poor scavenging of Cd, which results in the disturbance of cellular redox balance leading to oxidative stress. Cd interferes with not only antioxidant defense systems but also the mitochondrial electron transport chain. Although the complete pathology evoked by Cd toxicity is unknown, the ability of Cd to elicit an oxidative stress response seems apparent. Aiba *et al* (5) revealed a novel pathway for the GSH-mediated reduction of Cd toxicity in mammalian cells, namely, elevated GSH levels can downregulate Cd uptake through the downregulation of Cd transporter Zrt-, Irt-like protein 8 (ZIP8). Therefore, elevation of cellular GSH levels is an important method for modulating Cd toxicity.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a key transcription factor that plays a central role in regulating

the expression of glutamate cysteine ligase (GCL). GCL is an enzyme that catalyzes the first and rate-limiting step in de novo GSH synthesis. Sulforaphane, a compound found in broccoli sprouts, is a potent Nrf2 activator (6). Recent studies have shown that sulforaphane protects cells and tissues against Cd toxicity (7,8). *In vitro* and *in vivo* studies have demonstrated that Nrf2 activation protects against Cd toxicity by increasing GSH levels (9). Interestingly, in vascular endothelial cells after Cd exposure, Nrf2 partially contributes to the expression of metallothionein (MT), the most potent protective measure against Cd-induced toxicity (2).

Epalrestat (5-[(1Z,2E)-2-methyl-3-phenyl propenylidene]-4-oxo-2-thioxo-3-thiazolidine acetic acid; EPS; Ono Pharmaceuticals), which received approval for use in Japan in 1992, is currently being used for the treatment of diabetic neuropathy. EPS is an inhibitor of aldose reductase, a rate-limiting enzyme in the polyol pathway. Under hyperglycemic conditions, EPS reduces intracellular sorbitol accumulation, which is implicated in the pathogenesis of diabetic complications (10). EPS is easily absorbed by neural tissue and inhibits aldose reductase with minimum adverse effects (11). Recently, we found that EPS increased GSH levels in rat Schwann cells by upregulating GCL via Nrf2 activation (12). In addition, EPS increased GSH levels in bovine aortic endothelial cells (BAECs) (13). The purpose of the present study was to determine whether: i) EPS protects against Cd-induced cytotoxicity in BAECs; ii) EPS affects GSH levels in cells exposed to Cd; and iii) EPS has an effect on the intracellular levels of Cd and MT.

Materials and methods

Cell culture and treatment with EPS and Cd. BAECs were purchased from Dainippon Sumitomo Pharma Co., Ltd. BAECs were grown to 80-90% confluence in DMEM containing 10% fetal bovine serum (FBS), L-glutamine (4 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37˚C in a humidified atmosphere of 5% CO_2 and 95% air. Then, the cells were passaged by trypsinization.

Before treating the cells with EPS (Wako Pure Chemical Industries, Ltd.), the culture medium was replaced with DMEM containing 2% FBS. EPS (10, 50 and 100 μM) was subsequently added to the medium. After the treatment with EPS for 16 h, cells were exposed to Cd chloride (25 and 50 μM).

Cell viability. Viability of BAECs was assessed by measuring lactate dehydrogenase (LDH) release. After treatment of BAECs in 12-well plates with EPS and Cd, aliquots of the medium were taken to measure the activity of LDH released from cells. The remaining intracellular LDH was released by adding 0.1% Triton X-100 in phosphate-buffered saline (PBS) at pH 7.4. LDH activity was measured spectrophotometrically on the basis of the increase in absorbance at 340 nm with 60 mM lithium lactate in 0.3 M diethanolamine buffer (pH 9.0), after the reaction was initiated by adding 3 mM (final concentration) NAD^+. Released LDH activity was expressed as percentage of total LDH activity (activities of LDH in the medium and in the remaining cells).

Treatment with N-acetylcysteine and buthionine sulfoximine. BAECs were exposed to Cd at 25 μM for 24 h in the presence or absence of N-acetylcysteine (NAC) (Sigma-Aldrich; Merck KGaA) or GSH (free) (Wako Pure Chemical Industries, Ltd.) at 1 mM. BAECs were pretreated with 100 μM buthionine sulfoximine (BSO) (Sigma-Aldrich; Merck KGaA) for 16 h. Subsequently, the untreated or BSO-treated cells were exposed to Cd at 25 μM for 24 h. The effects of NAC, GSH, and BSO on cell viability was estimated by measuring LDH release as described above.

Knockdown of Nrf2 in BAECs with small interfering RNA (siRNA). Oligonucleotides directed against bovine Nrf2 (Sigma-Aldrich; Merck KGaA) and control siRNA (Ambion) were transfected into BAECs using Lipofectamine RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc.) 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 BAECs 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 20 nM.

Intracellular Cd concentrations. Intracellular Cd concentrations were measured by graphite furnace atomic absorption spectrometry (GF-AAS; AA-7000 Atomic Absorption Spectrophotometer), according to the method described by Luczak *et al* (14). After treatment of BAECs in 6-cm dishes with EPS and Cd, Cd-containing media were removed. Attached cells were washed twice with warm DPBS and the cells were harvested with a cell scraper in DPBS, collected by centrifugation, and washed twice with ice-cold DPBS. The cells were then suspended in 400 μl of ice-cold deionized water and this was followed by the addition of 400 μl of 10% nitric acid. After the samples were heated at 50˚C for 60 min, Cd-containing extracts were collected by centrifugation. The supernatants were diluted with water to give 2% nitric acid prior to Cd measurements by GF-AAS.

For protein measurements, which were necessary for the normalization of Cd concentrations, Cd-extracted cell pellets were washed with ice-cold 5% nitric acid, centrifuged, and solubilized in 100 μl of 0.5 M NaOH by incubation at 37˚C for 30 min. The dissolved pellets were used for protein measurements.

ZIP8 and MT mRNA levels. RT-qPCR analysis was carried out to measure mRNA levels. Total RNA from the treated cells was extracted with RNAspin Mini (GE Healthcare) according to the manufacturer's protocol. mRNAs were reverse-transcribed into cDNA with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). RT-qPCR was performed with a 7500 Fast Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). Primers for bovine ZIP8 (Bt04283914_m1), bovine MT (Bt03279283_m1), and GAPDH (Bt03210913_g1) were purchased from Applied Biosystems;Thermo Fisher Scientific, Inc. Data were analyzed using the $2^{-\Delta\Delta Cq}$ method (15) and

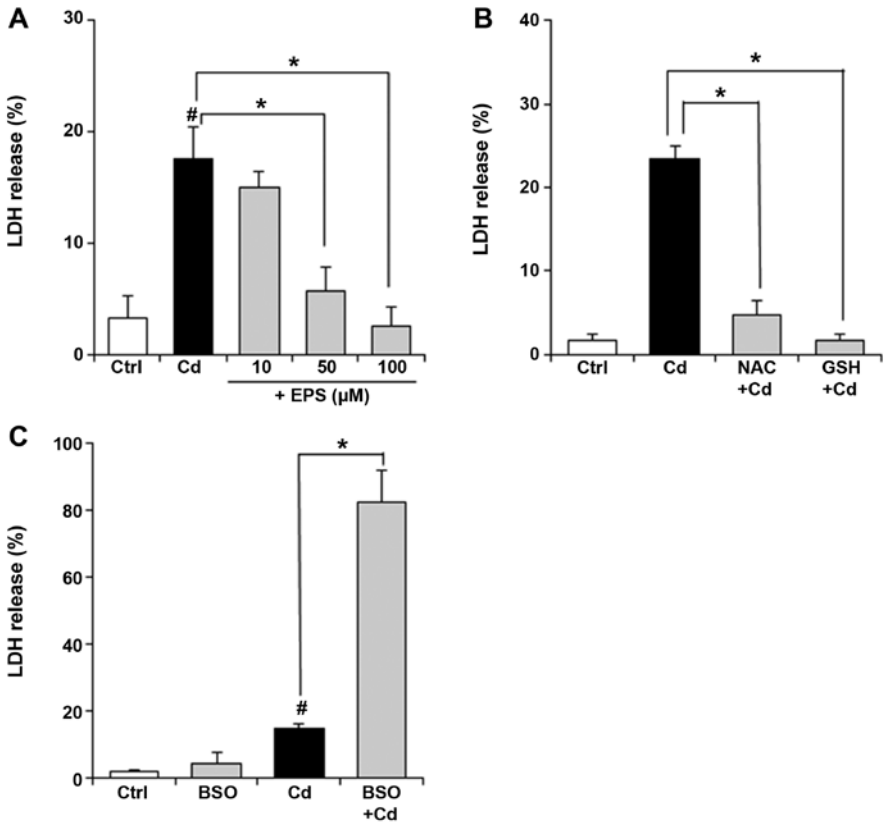

Figure 1. Effect of EPS on viability of BAECs exposed to Cd. (A) BAECs were pretreated with EPS (10, 50 or 100 μM) for 16 h. Subsequently, the untreated or EPS-treated cells were exposed to Cd at 25 μM for 24 h. Cell viability was estimated by measuring LDH release. Values are means ± SD of three experiments. (B) BAECs were exposed to Cd at 25 μM for 24 h in the presence or absence of NAC or GSH at 1 mM. Cell viability was estimated by measuring LDH release. Values are means ± SD of three experiments. (C) BAECs were pretreated with 100 μM BSO for 16 h. Subsequently, the untreated or BSO-treated cells were exposed to Cd at 25 μM for 24 h. Cell viability was estimated by measuring LDH release. Values are means ± SD of three experiments. *P<0.01. #P<0.01 vs. control. EPS, epalrestat; BAECs, bovine aortic endothelial cells; Cd, csadmium; LDH, lactate dehydrogenase; SD, standard deviation; NAC, N-acetylcysteine; GSH, glutathione; BSO, buthionine sulfoximine.

normalized to the internal reference gene GAPDH. Relative mRNA levels were compared and expressed as percentage of control levels.

MT protein levels. MT protein levels were analyzed by western blotting. A total of 20 μg of protein per well was resolved using sodium dodecyl sulfate polyacrylamide gel electrophoresis and electro-transferred to a PVDF membrane. After blocking and washing, the membrane was incubated with the following primary antibody: anti-mouse MT polyclonal antibody (Dako) or anti-mouse β-actin (Sigma-Aldrich; Merck KGaA). Following primary antibody incubation, the membrane was incubated with horseradish-peroxidase-conjugated secondary antibodies. Chemiluminescence was detected with an ECL Plus western blot detection kit (GE Healthcare). Protein expression in each sample was determined by normalizing target band intensity to β-actin band intensity. Band intensities were quantified using ImageJ software.

Measurement of GSH. Intracellular GSH levels were measured by spectrophotometric methods, as previously described (16). Untreated or 50 μM EPS-pretreated cells for 16 h in 12-well plates were measured after exposure to 50 μM Cd for 4 h. Each sample for GSH measurement was mixed with 0.6 mM 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), 0.2 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), and 5 mM ethylenediaminetetraacetic acid (EDTA) in 0.1 M sodium phosphate buffer (pH 7.5). The reaction was initiated by adding glutathione reductase.

Other procedures. Protein concentrations were determined using the Bradford method with bovine serum albumin (BSA) as the standard.

Statistical analysis. All experiments were performed independently at least three times. Data were combined and expressed as means ± SD. Statistical significance was determined using the Student's t-test, one-way ANOVA or two-way analysis of variance (ANOVA) with Tukey's post-hoc test. A P-value of <0.01 was considered to be significant.

Results

Effect of EPS on Cd toxicity in BAECs. The vascular system is a critical target of Cd toxicity (1). Endothelial dysfunction and damage may be attributed to toxicity in parenchymal cells of such target organs as kidney (1). In our previous work, we demonstrated that EPS increased GSH levels in BAECs by upregulating GCL via Nrf2 activation (13). In the present study, we first examined the effect of EPS on

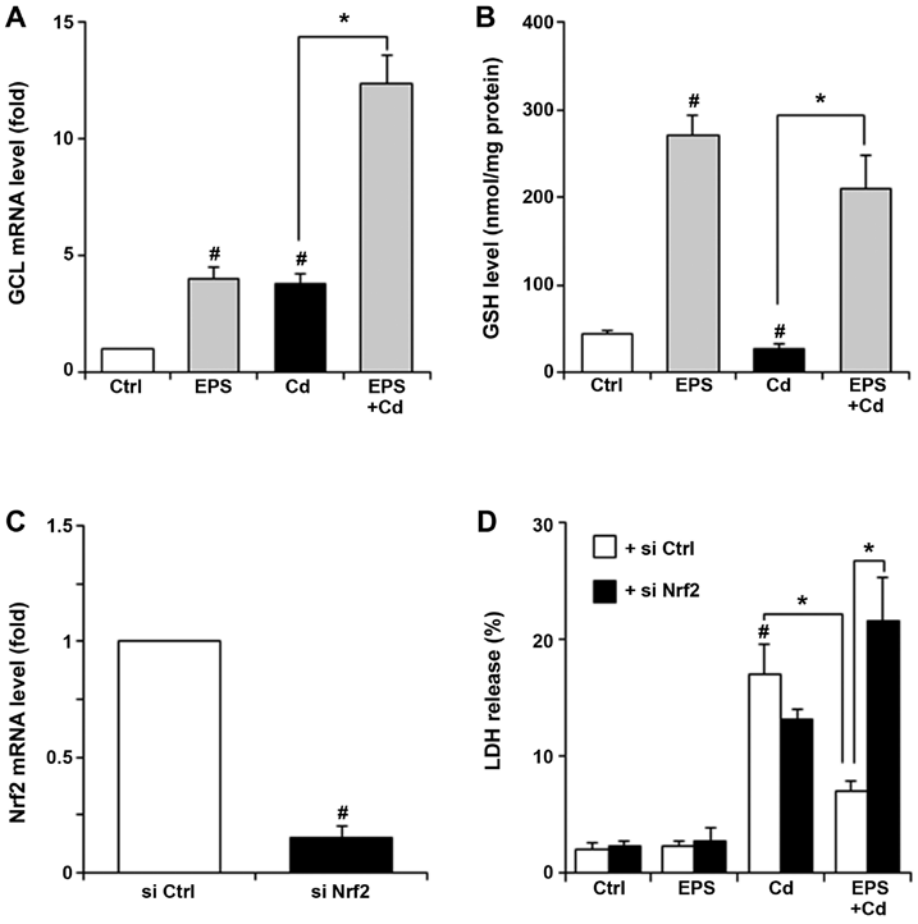

Figure 2. Effect of EPS on changes in GSH levels in BAECs. (A) GCL mRNA and (B) GSH levels in untreated or 50 μM EPS-pretreated cells were measured after exposure to 50 μM Cd for 4 h. Values are means ± SD of three experiments. (C) Control siRNA (si ctrl) or Nrf2 siRNA (si Nrf2) were diluted with Opti-MEM medium and then, diluted Lipofectamine RNAiMAX was added. The transfection mixture was incubated at room temperature for 20 min. The final concentration of siRNA was 20 nM. Values are means ± SD of three experiments. (D) BAECs were transfected with control siRNA (si ctrl) or Nrf2 siRNA (si Nrf2) and were pretreated or not pretreated with EPS (50 μM) for 16 h. Subsequently, the cells were exposed to Cd at 25 μM for 24 h. Values are means ± SD of three experiments. *P<0.01. $^{\#}$P<0.01 vs. control. EPS, epalrestat; GSH, glutathione; BAECs, bovine aortic endothelial cells; GCL, glutamate cysteine ligase; Cd, cadmium; SD, standard deviation; si, small interfering.

Cd-induced cytotoxicity, using BAECs as an *in vitro* model of the vascular endothelium. Fig. 1A shows the protective ability of EPS against Cd-induced cytotoxicity in BAECs, which was estimated by measuring LDH release, a frequently used endpoint for cytotoxicity studies. Cd-induced release of LDH was almost completely suppressed by pretreatment with EPS at 50 and 100 μM. EPS at 10 μM failed to suppress the LDH release. Our previous study demonstrated that 50 and 100 μM EPS, but not 10 μM EPS, increased GSH levels in BAECs (13). As shown in Fig. 1B, the addition of NAC or GSH (free) reduced Cd-induced LDH release from BAECs, consistent with published results (17). Intracellular GSH depletion by BSO aggravated Cd-induced toxicity in BAECs (Fig. 1C). These results indicate that GSH plays a protective role against Cd-induced cytotoxicity.

GCL is an enzyme that catalyzes the first and rate-limiting step in *de novo* GSH synthesis (18). We measured GCL mRNA and GSH levels in BAECs (Fig. 2A and B). EPS increased GCL mRNA levels as well as intracellular GSH levels. Increases in GCL mRNA levels were also observed in both untreated and EPS-treated cells after exposure to Cd. Cd exposure significantly decreased GSH levels. These effects of Cd on GCL and GSH levels are consistent with published results (19), probably reflecting GSH consumption as a result of Cd exposure. When EPS-pretreated BAECs were exposed to Cd, GCL mRNA levels markedly increased by 3-fold relative to that of EPS-pretreated cells without Cd exposure. In the EPS-pretreated cells, high GSH level was observed after Cd exposure, even though Cd exposure may result in the consumption of excess GSH resulting from the upregulation of GCL. We examined whether Nrf2 levels could alter the cell viability treated with Cd and/or EPS, by means of Nrf2 knockdown in BAECs. BAECs were transfected with control siRNA or Nrf2 siRNA. Nrf2 mRNA expression levels in cells transfected with Nrf2 siRNA were reduced by 80% relative to those in control siRNA transfected cells (Fig. 2C). Fig. 2D demonstrates that the protective ability of EPS against the Cd-induced LDH release completely disappeared following Nrf2 siRNA transfection.

Effect of EPS on Cd uptake in BAECs. We next measured intracellular Cd levels in BAECs. After untreated and EPS-pretreated BAECs were exposed to 50 μM Cd for 4 h, intracellular Cd levels were determined by measuring Cd

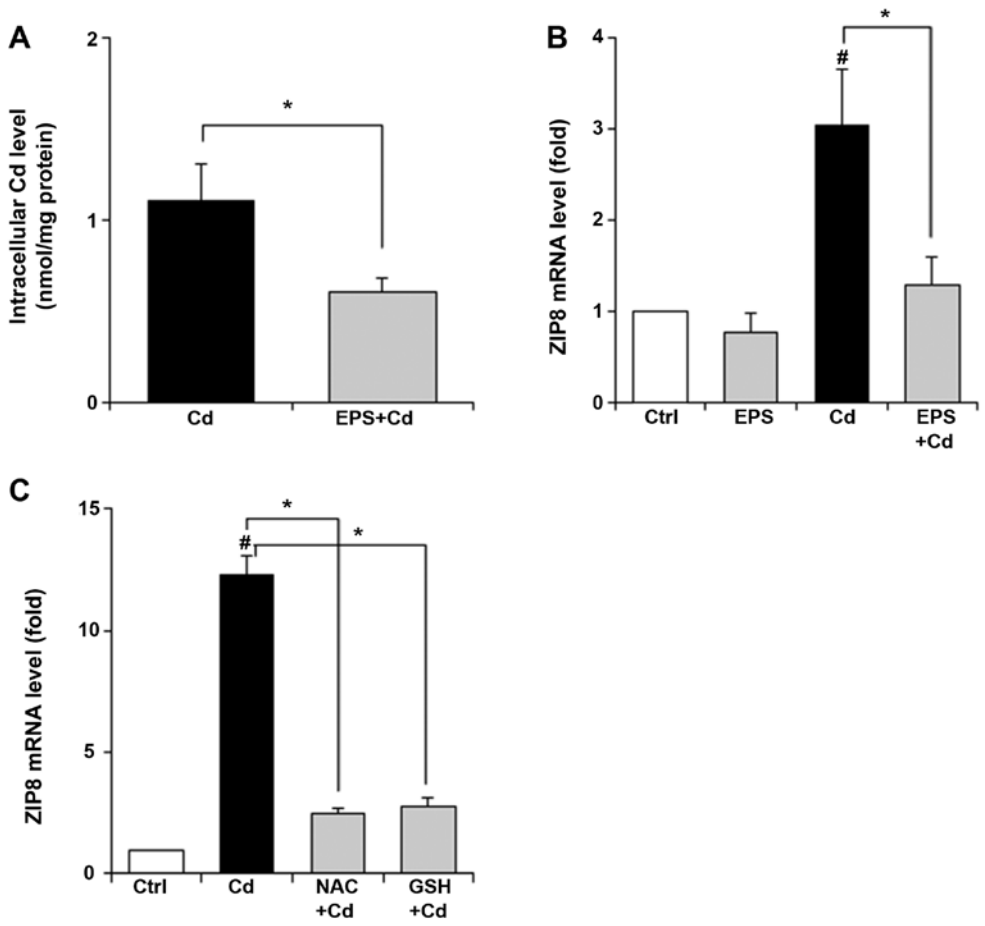

Figure 3. Effect of EPS on intracellular Cd level and ZIP8 mRNA levels in BAECs. (A) BAECs were pretreated with EPS (50 μM) for 16 h. After the cells were exposed to Cd at 50 μM for 4 h, intracellular Cd accumulation was determined by measuring Cd content in the pellet suspended in HNO_3 solution by GF-AAS. (B) BAECs were pretreated with EPS (50 μM) for 16 h. After the cells were exposed to Cd at 25 μM for 24 h, ZIP8 mRNA levels were measured. (C) BAECs were pretreated with NAC (1 mM) and GSH (free, 1 mM) for 16 h. After the cells were exposed to Cd at 25 μM for 24 h, ZIP8 mRNA levels were measured. Values are means ± SD of three experiments. *P<0.01. $^{\#}$P<0.01 vs. control. EPS, epalrestat; Cd, cadmium; ZIP8, Zrt-, Irt-like protein 8; BAECs, bovine aortic endothelial cells; GF-AAS, graphite furnace atomic absorption spectrometry; NAC, N-acetylcysteine; GSH, glutathione.

content in the pellet suspended in HNO3 solution using GF-AAS. As shown in Fig. 3A, pretreatment with EPS at 50 μM reduced intracellular Cd levels in BAECs. Then, we examined that the effect of EPS on ZIP8 levels in BAECs. When BAECs were exposed to 25 μM Cd for 24 h, increases in ZIP8 mRNA levels were confirmed (Fig. 3B). EPS pretreatment inhibited the increases in ZIP8 mRNA levels induced by Cd exposure. In other experiments in which NAC or GSH (free) was added, a similar trend was observed; both NAC and GSH inhibited the Cd-induced increases in ZIP8 mRNA levels (Fig. 3C).

Effect of EPS on MT levels in BAECs. Finally, we examined whether EPS affected MT in BAECs. Exposure to Cd (25 μM) for 4 h resulted in elevated MT mRNA and protein levels (Fig. 4A and B). EPS pretreatment led to further increases in the MT levels. In control cells, EPS alone had no effect on the MT levels.

Discussion

Cd is an industrial and environmental pollutant that targets the vascular endothelium (1). Recent advances in Cd toxicity research have suggested an association between Cd and vascular diseases (20). Although the molecular targets of Cd toxicity are poorly understood, there are studies indicating that Cd causes endothelial dysfunction and exhibits cytotoxicity, indicating that functional damage to the endothelium may be attributable to toxicity in parenchymal cells of various target organs, such as kidney and liver (2). However, the mechanisms of Cd implications in vascular diseases have yet to be explained.

EPS is the only aldose reductase inhibitor currently available for the treatment of diabetic neuropathy. EPS is easily absorbed by neural tissue and inhibits aldose reductase with minimum adverse effects (11). The usual dosage of EPS for oral use is 50 mg three times a day. The plasma EPS concentration of 3.9 μg/ml (12 μM) was observed 1 h after a single oral dose of 50 mg (21). In this study, we examined the effects of EPS at near-plasma concentration on Cd-induced cytotoxicity in BAECs. Our new findings are that: i) EPS protects against Cd-induced cytotoxicity in BAECs; ii) EPS increases GSH levels in cells exposed to Cd; and iii) EPS has an effect on the intracellular levels of Cd, ZIP8, and MT.

Some antioxidants decrease the cytotoxic effects of Cd (7,22,23). Sulforaphane, which is a natural and highly

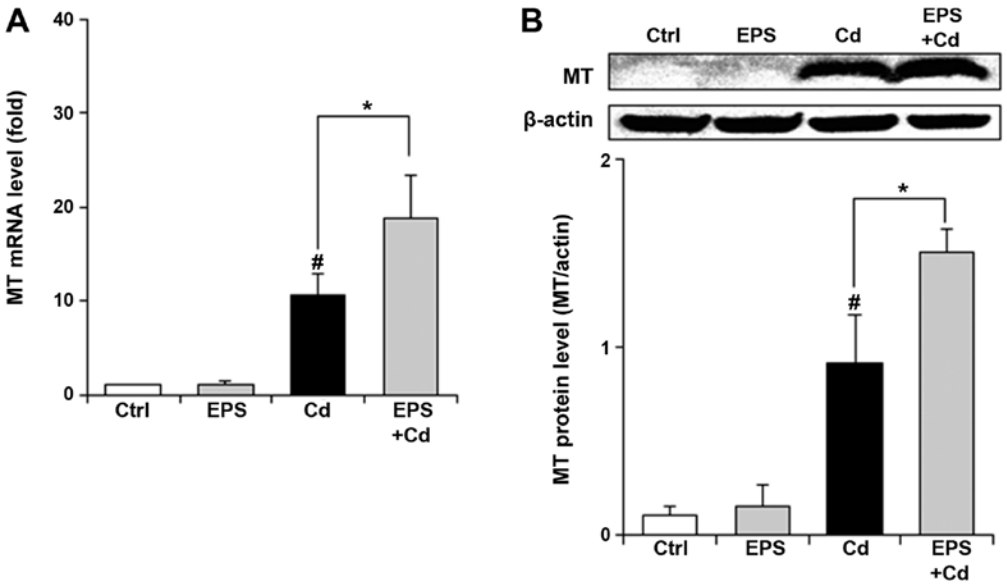

Figure 4. Effect of EPS on MT levels in BAECs. BAECs were pretreated with EPS (50 μM) for 16 h. After the cells were exposed to Cd at 25 μM for 4 h, (A) MT mRNA and (B) protein levels were measured. Values are means ± standard deviations of three experiments. *P<0.01. $^{\#}$P<0.01 vs. control. EPS, epalrestat; MT, metallothionein; BAECs, bovine aortic endothelial cells; Cd, cadmium.

effective antioxidant, reduced Cd-induced cell death in lymphocytes and monocytes (8). Sulforaphane is a potent Nrf2 activator that promotes the restoration of cellular GSH levels (24). Sulforaphane prevents testicular damage by Cd in association with the Nrf2 pathway (8). Quercetin attenuates Cd-induced oxidative damage and apoptosis in ovarian granulosa cells (25). Both sulforaphane and quercetin are Nrf2 activators (26). Sulforaphane and quercetin modulate GSH homeostasis via Nrf2. In kidney cells, the activation of Nrf2 is an adaptive intracellular response to Cd-induced oxidative stress, and that Nrf2 is protective against Cd-induced apoptosis (27). Nrf2 has important roles in suppression the carcinogenicity of Cd in terms of protection from oxidative stress-induced DNA damage (28). Therefore, the therapeutic Nrf2 activator dose may be a new strategy against Cd-induced toxicity. In our previous reports, we showed that EPS increases GSH levels in rat Schwann cells and BAECs in association with the Nrf2 pathway (12,13). EPS could be expected to protect against Cd-induced toxicity in the same manner as sulforaphane and quercetin. In fact, our present study indicates that EPS protects against Cd-induced cytotoxicity in BAECs (Fig. 1A). Cd exposure to untreated cells resulted in decreased GSH levels. GSH plays a protective role against Cd-induced cytotoxicity (Fig. 1B and C). It is possible that the EPS-induced protection against Cd-induced cytotoxicity is mainly due to the increase in GSH levels. The mechanism seems to involve the upregulation of GSH via Nrf2. When EPS-pretreated cells were exposed to Cd, the increase in GSH levels prevented the loss of viable cells induced by the exposure to Cd. EPS increased GCL mRNA levels and an increase in GCL mRNA levels was also observed in both untreated and EPS-treated cells after exposure to Cd in BAECs (Fig. 2A). However, in the EPS-pretreated BAECs, there were no significant differences in GSH levels between EPS treatment alone and EPS treatment followed by Cd exposure (Fig. 2B), implying that Cd exposure may result in the consumption of GSH. Cd is tightly bound to GSH for chelation (29). In addition, the experiments using Nrf2 knockdown cells (Fig. 2D) indicated that Nrf2 may be involved in the protective ability of EPS against Cd-induced cytotoxicity. Together, these results suggest that EPS protects against Cd-induced cytotoxicity in BAECs by increasing GSH levels. Possibly, Nrf2 may be involved in the protection against Cd-induced cytotoxicity.

Accumulating evidence indicates that oxidative stress could be partially responsible for some cases of Cd-induced cytotoxicity (30). We examined whether ROS was generated by Cd in BAECs using the ROS probe and found that ROS production was not induced by Cd in our present study (data not shown). Cd is not able to produce radicals via Fenton-type chemistry. Nonetheless, it induces oxidative stress through a multifaceted mechanism, including the reduction of antioxidative defense and ROS production as a result of mitochondrial damage (9). The use of antioxidants, the induction of antioxidant enzymes, and the complexation of Cd with GSH and MT are the most potent protective measures against Cd-induced oxidative stress.

Cd-induced cytotoxicity is dependent on the amount of intracellular Cd (9). Fig. 3A shows that the pretreatment with EPS reduced the intracellular accumulation of Cd in BAECs. This reduction of the intracellular Cd accumulation may be involved in the suppression of Cd-induced cytotoxicity by EPS. ZIP8 plays an important role in Cd uptake in mammalian cells (31). One report has indicated that the absence of ZIP8 expression in vascular endothelial cells is associated with resistance to Cd-induced testicular toxicity (32). That finding suggests that ZIP8 expression in endothelial cells is crucial for the Cd-induced cytotoxicity. Our results in Fig. 3B indicate that the effect of EPS on ZIP8 mRNA levels may contribute to the suppression of Cd-induced cytotoxicity, albeit in a limited data. Further studies might be needed to clarify the effect of EPS on ZIP8 expression levels. Fig. 3C shows that NAC or GSH inhibited Cd-induced ZIP8 mRNA levels. One study has revealed that elevated GSH levels can downregulate Cd uptake

by downregulating the Cd transporter ZIP8 (5). EPS inhibited Cd-induced ZIP8 mRNA levels (Fig. 3B). Therefore, we attribute this inhibitory effect to the increase in GSH levels by EPS.

MT as well as GSH is the most potent protective measure against Cd-induced oxidative stress (9). Exposure to such heavy metals as Cd triggers the induction of MT, which confers cells with resistance to heavy-metal-induced toxicities (33). Exposure to Cd elevated MT mRNA and protein levels and EPS pretreatment further increased the levels of MT (Fig. 4A and B), indicating that EPS contributed to the expression of MT in BAECs treated with Cd. Metal-responsive transcription factor 1 (MTF-1) is considered to be a major activator for MT gene expression (34,35). On the other hand, Nrf2 partially contributes to MT expression in vascular endothelial cells after Cd exposure (2). Our results indicate that the effect of EPS on MT levels may contribute to mediate Nrf2 involved in the protective ability of EPS partially regulates to MT levels in BAECs treated with Cd. Further studies might be needed to clarify the effect of EPS on MT levels.

In summary, we demonstrated for the first time that EPS suppresses Cd-induced cytotoxicity in BAECs. The upregulation of GSH may be involved in the suppression of Cd-induced cytotoxicity by EPS. Our findings have led us to propose that targeting the regulation of GSH, ZIP8 and MT by EPS is a promising therapeutic approach in Cd poisoning.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author's contributions

RT and KS conceived and designed the experiments and wrote the manuscript. YM and KY performed the experiments and analyzed and interpreted the data. YY and SO performed the experiments. YT analyzed and interpreted the data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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