Fundamental Toxicological Sciences (Fundam. Toxicol. Sci.)
Vol.11, No.4, 205-213, 2024

Fundamental Toxicological Sciences
URL : http://www.fundtoxicolsci.org/index_e.html

Original Article

Zrt- and Irt-like protein 8 (ZIP8) and metallothionein aid in zinc protection of cultured vascular endothelial cells against cadmium cytotoxicity

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(Received August 4, 2024; Accepted August 9, 2024)

ABSTRACT — Cadmium is a known contributing factor for cardiovascular diseases, such as atherosclerosis and hypertension. Although zinc protects against cadmium cytotoxicity in vascular endothelial cells, the detailed mechanisms, especially the differences in the roles of a metal transporter Zrt- and Irt-like protein 8 (ZIP8) and metallothionein (MT), remain unclear. ZIP8 is involved in the uptake of cadmium, whereas MT is induced by and sequesters it. Zinc protects bovine aortic endothelial cells from cadmium cytotoxicity in a culture system in a concentration-dependent manner. Zinc significantly decreased intracellular cadmium accumulation. Cadmium elevated the expression of ZIP8 mRNA, and zinc significantly suppressed this increase in a concentration-dependent manner. However, the expression of MT was not induced by zinc alone but by cadmium; the induction of MT by cadmium was suppressed by zinc at lower concentrations, but intensified by zinc at higher concentrations. However, even when MT induction was strongly suppressed by siRNA-mediated knockdown, cadmium cytotoxicity was reduced by zinc at both lower and higher concentrations. In the absence of zinc, cadmium cytotoxicity was increased by metal response element-binding transcription factor-1 (MTF-1) siRNA-mediated knockdown of MT-1/2. Consequently, it has been suggested that zinc protects the vascular endothelial cells through a concentration-dependent mechanism. Specifically, the decrease in ZIP8 expression is crucially important for the protective effect of zinc at low concentrations against cadmium cytotoxicity in vascular endothelial cells, whereas both the decrease in ZIP8 and the induction of MT contribute to the protection by zinc at high concentrations.

Key words: Cadmium, Zinc, Vascular endothelial cell, ZIP8, Metallothionein, Cytotoxicity

INTRODUCTION

Cadmium is well-known as a contributing factor for cardiovascular diseases, including atherosclerosis and hypertension (Tomera *et al.,* 1994; Navas-Acien *et al.,* 2004). These conditions arise from the dysfunction of vascular endothelial cells, which line the interior surfaces of blood vessels (Aird, 2004; Bauersachs

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and Schäfer, 2004; Davignon and Ganz, 2004; Galley and Webster, 2004). Our stuies have explored the toxicity of cadmium on vascular endothelial cell functions. We found that cadmium causes cytotoxicity and induces morphological changes, such as endothelium removal from cell monolayers (Kaji *et al.,* 1992a). Vascular endothelial cells are particularly susceptible to cadmium-induced damage (Kaji *et al.,* 1995*,* 1996). Cadmium also decreases fibrinolytic activity by inducing the expression of plasminogen activator inhibitor-1 in these cells (Yamamoto *et al.,* 1993; Hara *et al.,* 2021). Proteoglycans are macromolecules consisting of a core protein and one or more glycosaminoglycan side chains. Proteoglycans contribute to the antithrombogenic properties of the vascular endothelium by exhibiting heparin-like activity (Mertens *et al*., 1992); whereas cadmium influences endothelial proteoglycan synthesis (Ohkawara *et al*., 1997; Hara *et al*., 2023). The cytotoxicity of cadmium to vascular endothelial cells strongly depends on the intracellular accumulation of cadmium.

Zrt- and Irt-like protein (ZIP) transporters have eight transmembrane domains and a histidine-rich domain in the cytoplasmic region that recognizes zinc ions and is responsible for zinc uptake (Eide, 2004). ZIP8 transports not only zinc but also iron, manganese, and cadmium (He *et al*., 2006) from extracellular space to the cytosol of cells. This transporter is crucial for vascular endothelial cell susceptibility to cadmium *in vivo* (Dalton *et al*., 2005). We reported that cadmium induces the expression of ZIP8 in vascular endothelial cells via NF-κB signaling by the activation of the inhibitor of κBα (IκBα) and JNK signaling (Fujie *et al*., 2022a). Additionally, it was revealed that TGF-β_1 induces the expression of ZIP8 and potentiates the susceptibility of vascular endothelial cells to cadmium cytotoxicity (Ito *et al*., 2022), suggesting that the expression level of ZIP8 is a factor that determines susceptibility to cadmium in the cells. Recently, we found that lead, a toxic heavy metal, induces the expression of endothelial ZIP8 by activating the NF-κB pathway (Fujie *et al*., 2022b). These results suggest that the expression level of ZIP8 is one of a crucial factor that determines cadmium cytotoxicity in vascular endothelial cells.

Metallothionein (MT) is a cysteine-rich protein that binds and detoxifies heavy metals such as cadmium and zinc (Kägi and Valee, 1960). There are two isoforms of MT, MT-1 and MT-2, found throughout mammalian organs (Karin and Richards, 1982; Palmiter, 1994). While MT-1 and MT-2 can be induced to varying extents by heavy metals (McNeill *et al*., 2019), both isoforms are upregulated by cadmium in vascular endothelial cells (Fujie *et al*., 2020). MT induction requires the activation of metal response elements (MREs) in the promoter region of MT genes, which recruit MRE-binding transcription factor-1 (MTF-1) by zinc-binding (Dalton *et al*., 1997; Bittel *et al*., 1998; Andrews, 2000). In contrast, NF-E2 related factor 2 (Nrf2) (Itoh *et al*., 1997) is involved in endothelial MT induction by binding to the antioxidant response element (ARE) in the promoter regions of MT genes (Shinkai *et al*., 2016; Fujie *et al*., 2019a). The induction of MT-1 requires activation of both the MTF-1-MRE and Nrf2-ARE pathways, whereas only the MTF-1-MRE pathway is involved in the induction of MT-2 in vascular endothelial cells derived from the bovine aorta (Fujie *et al*., 2016a, 2016b). As MT sequesters cadmium and the concentration of toxic-type cadmium is lowered within cells, MT induction decreases the cytotoxicity of cadmium in various cell types.

Cadmium toxicity can be influenced by interactions with other heavy metals in various biological systems. In vascular endothelial cells, while zinc, a known MT inducer, does not induce MT (Kaji *et al.,* 1992b; Fujie *et al.,* 2016c), it protects cells from cadmium cytotoxicity by reducing intracellular cadmium accumulation (Kaji *et al*., 1992b). Copper also reduces cadmium cytotoxicity through similar mechanisms (Kaji *et al.,* 1992c). Since cadmium induces MT in vascular endothelial cells (Kaji *et al.,* 1992b), the effects of MT induction on cadmium-zinc interactions cannot be ignored. This study aims to investigate the roles of ZIP8 expression and MT induction in the interactions between cadmium and zinc in vascular endothelial cells.

MATERIALS AND METHODS

Materials

Bovine aortic endothelial cells were purchased from Cell Applications (San Diego, CA, USA). The following materials were purchased from the respective vendors: tissue culture plates and dishes from AGC Techno Glass (Shizuoka, Japan); Dulbecco's modified Eagle's medium (DMEM) and calcium- and magnesium-free phosphate buffered saline (CMF-PBS) from Nissui Pharmaceutical (Tokyo, Japan); fetal bovine serum (FBS) and High-Capacity cDNA Reverse Transcription kit, BCA Protein Assay Kit, Lipofectamine RNAiMAX transfection reagent, OPTI-MEM reduced serum medium, and alamarBlue cell viability reagent from Thermo Fisher Scientific (Waltham, MA, USA); QIAzol lysis reagent from Qiagen (Venlo, Netherlands); GeneAce SYBR qPCR Mixα from Nippon Gene (Tokyo, Japan); mouse monoclonal anti-MT-1/2 antibody (E9) from Dako (Glostrup, Denmark); rabbit polyclonal anti-β-actin antibody (A5060)

from Sigma-Aldrich Chemical (St. Louis, MO, USA); horseradish peroxidase conjugated anti-mouse IgG antibody (#7076) and rabbit IgG antibody (#7074) from Cell Signaling (Beverly, MA, USA); Immobilon Transfer Membranes, May-grünwald, and Giemsa stain solution from Merck KGaA (Darmstadt, Germany); cadmium chloride, zinc sulfate, Chemi-Lumi One L, and other reagents from Nacalai Tesque (Kyoto, Japan).

Cell culture and cytotoxicity evaluation

Vascular endothelial cells were cultured in DMEM supplemented with 10% FBS in 6- or 24-well culture plates at 37°C in 5% CO_2 until confluence. The medium was removed, and the cells were washed twice with serum-free DMEM. The cells were exposed to cadmium chloride (10 µM) and/or zinc sulfate (5, 10, 30, 50, or 100 µM) for 24 hr in serum-free DMEM in 24-well culture plates or 96-well culture plates. After 24-hr incubation of vascular endothelial cells with cadmium and/or zinc in 24-well culture plates, the conditioned media were discarded, and the cell layers were washed with CMF-PBS, fixed with methanol, and stained with Giemsa for morphological observations.

The cells in 96-well culture plates were treated with cadmium and/or zinc as described above, the conditioned media were discarded, and the cells were incubated with 100 µL of fresh serum-free DMEM and 10 µL of alamarBlue reagent for 2 hr at 37°C. Then, 100 µL of the control conditioned media and the conditioned media on the cell layer treated with or without metal(s) each were transferred into a 96-well black-bottom plate. The fluorescence intensity of each sample was measured at 544/590 nm by using an EnVision multilabel counter (Perkin Elmer, Waltham, MA, USA). Cytotoxicity index (%) was calculated using the following formula:

$$\text{Cytotoxicity index (\%)} = \frac{(C_C + C_M) - T_C}{(C_C + C_M)} \times 100$$

where C_C is the fluorescence intensity of the supernatant of the media on the cell layer treated without cadmium and zinc; C_M is that of the conditioned media of the cells treated without cadmium and zinc, and Tc is that of the supernatant of the media on the cell layer treated with cadmium and zinc.

Intracellular accumulation of cadmium and zinc

The intracellular accumulation of cadmium and zinc was determined as previously described (Fujie *et al.*, 2019b). The samples were used to detect intracellular cadmium (m/z = 114) and zinc (m/z = 64) by inductively coupled plasma mass spectrometry (NexION 300S, PerkinElmer, Waltham, MA, USA). Another portion of the lysate was analyzed for DNA content by the fluorometric method (Kissane and Robins, 1958) and the content of cadmium and zinc was expressed as pmol/µg DNA.

Real-time reverse transcription polymerase chain reaction (Real-time RT-PCR)

Vascular endothelial cells were treated with cadmium and/or zinc, and the level of mRNAs for ZIP proteins were analyzed by real-time RT-PCR, using GeneAce SYBR qPCR Mixα with 10 ng cDNA and the primers on a StepOnePlus real-time PCR system (ThermoFisher Scientific). The thermal cycling parameters were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. mRNA levels of ZIP1, ZIP2, ZIP3, ZIP4, ZIP6, ZIP8, ZIP10, ZIP14, DMT1, and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were quantified using the comparative Ct method. The fold change for each gene was assessed after normalizing the intensity value to that of GAPDH. The sequences of the

Table 1. Bovine gene-specific primers for quantitative real-time PCR.

Gene	Forward primer (5'→3')	Reverse primer (3'→5')
ZIP1	TTCTCTACATCACCTTCCTGG	AACCTTCCTTGCCTGTCTTG
ZIP2	GCTCTCGCTCTCCTTTCAC	ACCAGCCGCAGTCCTACA
ZIP3	GGACACACTCACCTCAACGC	CTCAAGGCTCCAAGCAGAAC
ZIP4	GACAGCCACAGTGACGACAG	CAGACATTCCGTACACAGCC
ZIP6	CCTGAAAATGATGATGATGTGG	CAAGATTGCTGGCTGCTGAG
ZIP8	GAATGAGCACTCGACAAGCC	TAGAGGAACATGCCTCCAGC
ZIP10	TTCTATCACTGTCATTAGCCTGC	GCGTCTCCACTCATTGTTCC
ZIP14	TCTCGGTAGTGCCTCTGTCC	GAATGTCTCAGTGCTGGTTGG
DMT1	CACAGGTAGCCATCAGAGCC	ACCAGGTTAGGAGTTCAGGAG
GAPDH	CAATGACCCCTTCATTGACCTTC	GGATCTCGCTCCTGGAAGATG

forward and reverse strands of the primers are listed in Table 1.

Western blot analysis

Vascular endothelial cells were treated with cadmium and zinc, and MT-1/2 protein levels were determined by western blotting as described previously (Fujie *et al.*, 2016c). The electrotransferred polyvinylidene difluoride membranes were incubated with anti-β-actin antibody (1:1,000) or anti-MT-1/2 antibody (1:200) at 4°C overnight and then with horseradish peroxidase-conjugated anti-rabbit IgG antibody or horseradish peroxidase-conjugated anti-mouse IgG antibody (1:5,000) for 1 hr at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence using Chemi-Lumi One L and scanned using LAS-3000 (Fujifilm, Tokyo, Japan).

Transient transfection

Small interfering RNA (siRNA) transfection was performed as previously described (Fujie *et al.*, 2022b). siRNAs (Bioneer, Daejeon, Korea) were transfected using the Lipofectamine RNAiMAX transfection reagent according to the manufacturer's instructions. The sequences of the sense and antisense strands of bovine MTF-1 siRNA were as follows: 5'-GCACUUUGGAGGAUGGAGAdTdT-3' (sense) and 5'-UCUUCAUCCUCCAAAGUGCCA-3' (antisense). A nonspecific sequence was used as an siRNA negative control (Qiagen).

Statistical analysis

Statistical analyses were performed using Student's *t*-test or analysis of variance with Dunnett's or Tukey-Kramer's test for multiple comparisons using Statcel3 (OMS, Tokyo, Japan). *P*-values < 0.05 were considered to indicate statistically significant differences.

RESULTS AND DISCUSSION

First, we evaluated the effect of zinc on cadmium cytotoxicity in vascular endothelial cells using morphological assessments and a cell viability assay (Fig. 1). No damage to the cell layer was observed when exposed to zinc alone at concentrations up to 100 μM for 24 hr (Fig. 1A, upper panel). In contrast, exposure to 10 μM cadmium

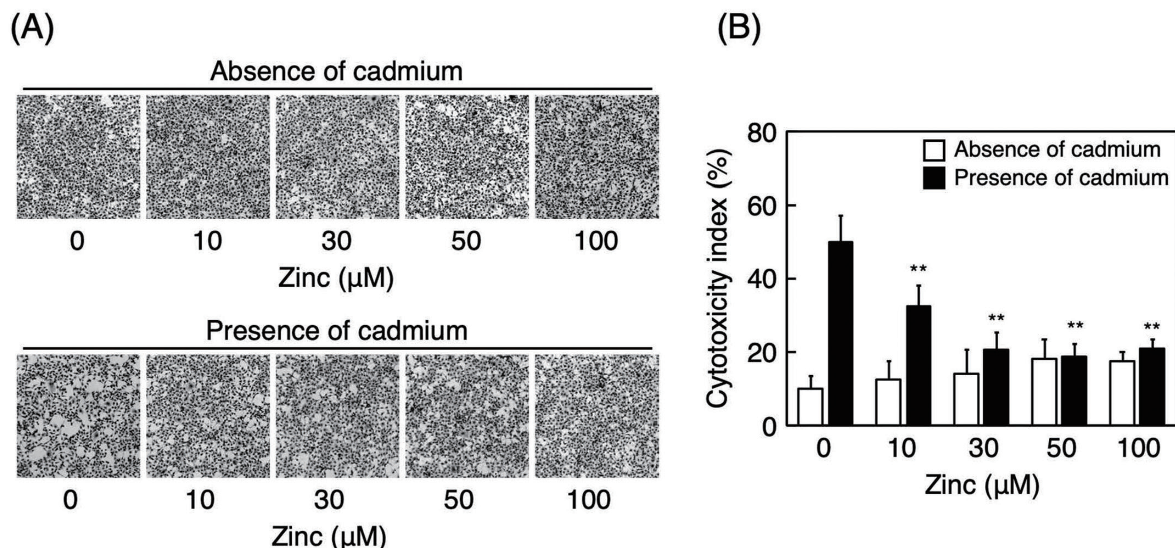

Fig. 1. Interaction of zinc with cadmium on cytotoxicity in vascular endothelial cells. **(A)** Morphological appearance of vascular endothelial cells after exposure to zinc alone (upper panel) and both cadmium and zinc (lower panel). Confluent cultures of bovine aortic endothelial cells were exposed to cadmium chloride (10 μM) in the absence or presence of zinc sulfate (10, 30, 50, and 100 μM) for 24 hr. The cell layer was stained with Giemsa. Original magnification (x40). Deendothelialized areas occurred after exposure to cadmium alone and zinc reduced the occurrence. **(B)** Interaction of zinc with cadmium on the cytotoxic index in vascular endothelial cells after exposure to cadmium, zinc, and both. Confluent cultures of bovine aortic endothelial cells were exposed to cadmium chloride (10 μM) in the absence or presence of zinc sulfate (10, 30, 50, and 100 μM) for 24 hr. The cytotoxicity was evaluated by the alamarBlue assay, and the cytotoxicity index was calculated. Values are means ± S.E. from four biological replicates. **Significantly different from the corresponding "absence of zinc" group, ** $p < 0.01$.

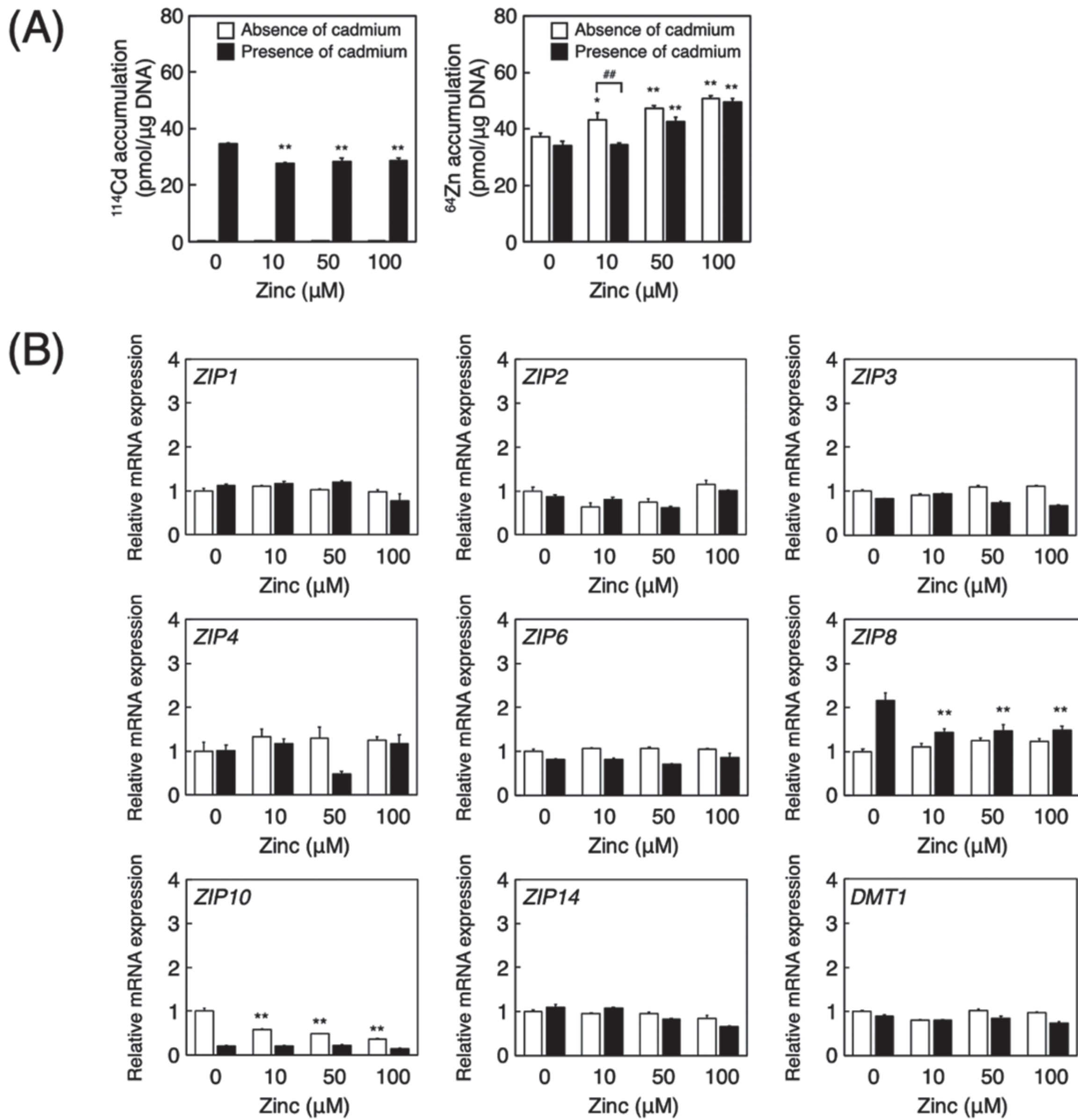

Fig. 2. Interaction of zinc with cadmium on the intracellular accumulation of cadmium and zinc and on the expression of metal transporter mRNAs in vascular endothelial cells. **(A)** Intracellular accumulation of cadmium (left panel) and zinc (right panel) in vascular endothelial cells after exposure to cadmium, zinc, or both. Confluent cultures of bovine aortic endothelial cells were exposed to cadmium chloride (10 µM) in the absence or presence of zinc sulfate (10, 50, and 100 µM) for 24 hr. Intracellular cadmium and zinc were measured by inductively coupled plasma mass spectrometry. Values are means ± S.E. from four biological replicates. Significantly different from the corresponding "absence of zinc" group, * $p < 0.05$; ** $p < 0.01$. **(B)** Expression of metal transporter mRNAs in vascular endothelial cells after exposure to cadmium, zinc, or both. Confluent cultures of bovine aortic endothelial cells were exposed to cadmium chloride (5 µM) in the absence or presence of zinc sulfate (10, 50, and 100 µM) for 24 hr. The expression of ZIP1, ZIP2, ZIP3, ZIP4, ZIP6, ZIP8, ZIP10, ZIP14, and DMT1 mRNA was determined by real-time RT-PCR. Values are means ± S.E. from technical triplicates. Significantly different from the corresponding "absence of zinc" group, **$p < 0.01$, or the corresponding "absence of cadmium" group, ##$p < 0.01$.

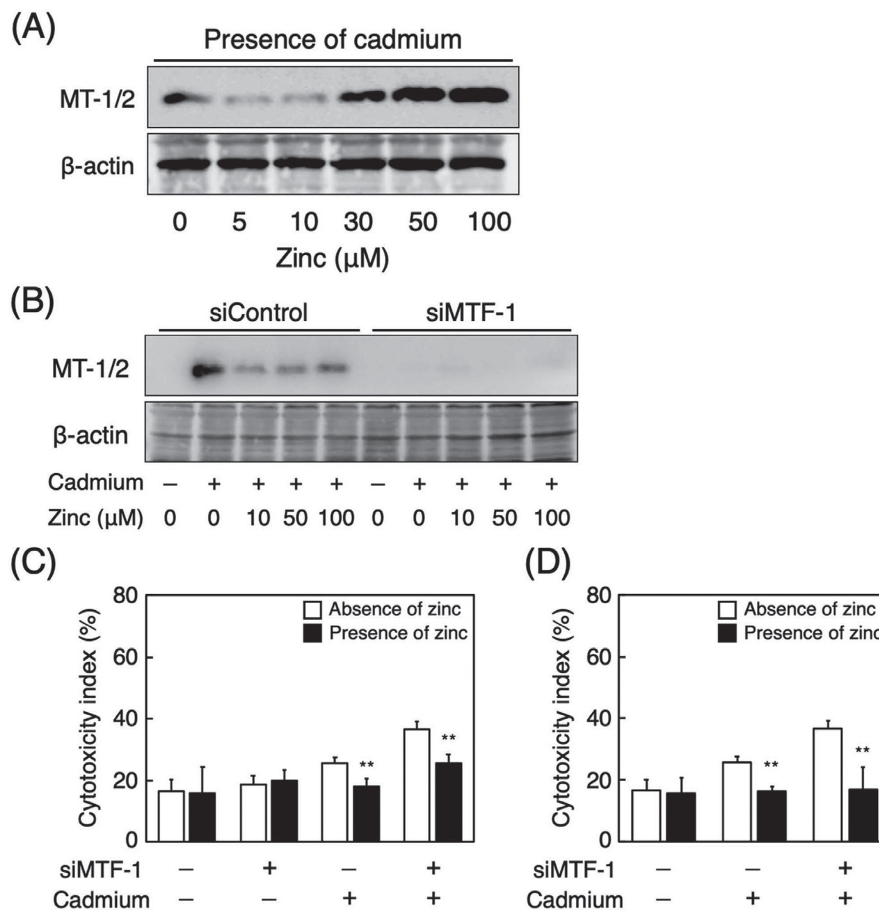

Fig. 3. Role of metallothionein in the interaction of zinc with cadmium in vascular endothelial cells. **(A)** Interaction of zinc with cadmium on the MT induction. Confluent cultures of bovine aortic endothelial cells were exposed to cadmium chloride (5 μM) in the presence of zinc sulfate (5, 10, 30, 50, and 100 μM) for 24 hr. The expression of MT-1/2 protein was determined by Western blot analysis. **(B)** Knockdown of MT by MTF-1 siRNA. Confluent cultures of bovine aortic endothelial cells were exposed to cadmium chloride (5 μM) in the absence or presence of zinc sulfate (10, 50, and 100 μM) for 24 hr. The expression of MT-1/2 protein was determined by Western blot analysis. **(C)** Interaction of zinc with cadmium on the cytotoxicity index after knockdown of MT by MTF-1 siRNA. Confluent cultures of bovine aortic endothelial cells were treated with MTF-1 siRNA and then exposed to cadmium chloride (5 μM) and/or zinc sulfate (10 μM). The cytotoxicity was evaluated by the alamarBlue assay, and the cytotoxicity index was calculated. Values are means ± S.E. from four biological replicates. **Significantly different from the corresponding "absence of zinc" group, $p < 0.01$. **(D)** Interaction of zinc with cadmium on the cytotoxicity index after knockdown of MT by MTF-1 siRNA. Confluent cultures of bovine aortic endothelial cells were treated with MTF-1 siRNA and then exposed to cadmium chloride (5 μM) and/or zinc sulfate (50 μM). The cytotoxicity was evaluated by the alamarBlue assay, and the cytotoxicity index was calculated. Values are means ± S.E. from four biological replicates. **Significantly different from the corresponding "absence of zinc" group, $p < 0.01$.

caused noticeable cell layer injury, which was mitigated by zinc (Fig. 1A, lower panel). Zinc significantly reduced the cadmium cytotoxicity (Fig. 1B), confirming its protective effect against cadmium toxicity, as previously reported (Kaji *et al.,* 1992b).

We also assessed the impact of zinc on intracellular cadmium accumulation. Zinc treatment decreased cadmium levels within the cells (Fig. 2A, left panel), while cadmium exposure reduced intracellular zinc levels at zinc concentrations of 10 μM, but was not reduced at

concentrations of 50 and 100 μM (Fig. 2A, right panel). Among the ZIP transporters and DMT1 examined—ZIP1, ZIP2, ZIP3, ZIP4, ZIP6, ZIP8, ZIP10, ZIP14, and DMT1—only ZIP8 mRNA levels were increased by cadmium exposure, and this increase was significantly reduced by zinc treatment. Cadmium also significantly decreased ZIP10 mRNA levels, while the expression of other ZIP mRNAs remained unchanged regardless of cadmium or zinc exposure. Although ZIP14 and DMT1 transport both cadmium and zinc (Tomera *et al*., 1994; Girijashanker *et al.,* 2008), their mRNA levels were unaffected by either metal, suggesting that ZIP8 is the primary transporter involved in the interaction between cadmium and zinc. In summary, cadmium accumulation in cells is driven by ZIP8 induction and results in significant cytotoxicity. Zinc counteracts this effect by suppressing ZIP8 induction, thereby reducing intracellular cadmium accumulation and its associated toxicity. This protective effect of zinc is consistent across a range of concentrations, indicating that the interaction between cadmium and zinc with ZIP8 expression occurs independently of zinc concentration.

Figure 3A and 3B illustrate the induction of MT in vascular endothelial cells following exposure to cadmium, zinc, or both. As previously reported (Fujie *et al.,* 2016c), zinc alone did not induce MT-1/2 expression in these cells. However, when both cadmium (5 μM) and zinc were present, the induction of MT-1/2 by cadmium was reduced at zinc concentrations of 5 and 10 μM, but was enhanced at concentrations of 30 μM and above (Fig. 3A). This suggests that MT induction in endothelial cells may serve as a defense mechanism against cadmium toxicity, particularly at higher zinc concentrations. In experiments where MT-1/2 induction was inhibited by knocking down MTF-1 (Fig. 3B), zinc at concentrations of 10 and 50 μM significantly mitigated cadmium cytotoxicity in vascular endothelial cells (Figs. 3C and 3D). Previous research (Kaji *et al.,* 1992b) demonstrated that MT induced by cadmium sequesters cadmium within the cells, suggesting that endothelial MT acts as a scavenger to protect against cadmium toxicity. However, the data in Fig. 3 show that zinc can protect against cadmium toxicity even without inducing MT-1/2. In other words, zinc's protective effect against cadmium is closely linked to the expression level of ZIP8, which determines intracellular cadmium accumulation. Our earlier findings (Kaji *et al*., 1992a; Kaji *et al.,* 1992b; Mishima *et al.,* 1995; Mishima *et al*., 1997) on cadmium-zinc interactions support this mechanism. It is important to note, though, that this does not imply MT is ineffective at protecting against cadmium toxicity, as cadmium toxicity was increased by the siMTF-1-mediated knockdown of MT-1/2 in the absence of zinc (Figs. 3C and 3D).

In summary, our findings indicate that: (1) Zinc protects against cadmium-induced cytotoxicity by reducing intracellular cadmium accumulation and suppressing cadmium-induced ZIP8 expression in vascular endothelial cells; (2) This protection occurs even when MT-1/2 is knocked down; and (3) Lower concentrations of zinc reduce MT induction by cadmium, whereas higher concentrations of zinc enhance MT induction. We conclude that decreased ZIP8 expression is crucial for zinc's protective effect at low concentrations, while both reduced ZIP8 expression and MT induction contribute to zinc's protective effect at high concentrations.

ACKNOWLEDGMENTS

This work was supported by JSPS KAKENHI Grant Numbers JP22K17355 (to T. H.), JP23K16312 (to T. F.), and the Study Group of the Health Effects of Heavy Metals Organized by the Ministry of the Environment, Japan. We would like to thank Editage (www.editage.com) for the English language editing.

Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES

Aird, W.C. (2004): Endothelium as an organ system. Crit. Care Med., **32** (Suppl), S271-S279.

Andrews, G.K. (2000): Regulation of metallothionein gene expression by oxidative stress and metal ions. Biochem. Pharmacol., **59**, 95-104.

Bauersachs, J. and Schäfer, A. (2004): Endothelial dysfunction in heart failure: mechanisms and therapeutic approaches. Curr. Vasc. Pharmacol., **2**, 115-124.

Bittel, D., Dalton, T., Samson, S.L., Gedamu, L. and Andrews, G.K. (1998): The DNA binding activity of metal response element-binding transcription factor-1 is activated *in vivo* and *in vitro* by zinc, but not by other transition metals. J. Biol. Chem., **273**, 7127-7133.

Dalton, T.P., Bittel, D. and Andrews, G.K. (1997): Reversible activation of mouse metal response element-binding transcription factor 1 DNA binding involves zinc interaction with the zinc finger domain. Mol. Cell. Biol., **17**, 2781-2789.

Dalton, T.P., He, L., Wang, B., Miller, M.L., Jin, L., Stringer, K.F., Chang, X., Baxter, C.S. and Nebert, D.W. (2005): Identification of mouse SLC39A8 as the transporter responsible for cadmium-induced toxicity in the testis. Proc. Natl. Acad. Sci. USA, **102**, 3401-3406.

Davignon, J. and Ganz, P. (2004): Role of endothelial dysfunction in atherosclerosis. Circulation, **109** (Suppl 1), III27-III32.

Eide, D.J. (2004): The SLC39 family of metal ion transporters. Pflugers Arch., **447**, 796-800.

Fujie, T., Ito, K., Ozaki, Y., Takahashi, S., Yamamoto, C. and Kaji,

T. (2022a): Induction of ZIP8, a ZIP transporter, via NF-κB signaling by the activation of IκBα and JNK signaling in cultured vascular endothelial cells exposed to cadmium. Toxicol. Appl. Pharmacol., **434**, 115802.

Fujie, T., Murakami, M., Yoshida, E., Yasuike, S., Kimura, T., Fujiwara, Y., Yamamoto, C. and Kaji, T. (2016b): Transcriptional induction of metallothionein by tris(pentafluorophenyl)stibane in cultured bovine aortic endothelial cells. Int. J. Mol. Sci., **17**, 1381.

Fujie, T., Muraoka, A., Ito, K., Ozaki, Y., Yamamoto, C. and Kaji, T. (2022b): Lead induces a metal transporter, ZIP8, via activation of the NF-κB signaling pathway and the induction is involved in the protection against lead cytotoxicity by intracellular lead accumulation independent mechanisms in cultured vascular endothelial cells. J. Toxicol. Sci., **47**, 493-501.

Fujie, T., Ozaki, Y., Takenaka, F., Nishio, M., Hara, T., Fujiwara, Y., Yamamoto, C. and Kaji, T. (2020): Induction of metallothionein isoforms in cultured bovine aortic endothelial cells exposed to cadmium. J. Toxicol. Sci., **45**, 801-806.

Fujie, T., Segawa, Y., Uehara, A., Nakamura, T., Kimura, T., Yoshida, E., Yamamoto, C., Uchiyama, M., Naka, H. and Kaji, T. (2016c): Zinc diethyldithiocarbamate as an inducer of metallothionein in cultured vascular endothelial cells. J. Toxicol. Sci., **41**, 217-224.

Fujie, T., Segawa, Y., Yoshida, E., Kimura, T., Fujiwara, Y., Yamamoto, C., Satoh, M., Naka, H. and Kaji, T. (2016a): Induction of metallothionein isoforms by copper diethyldithiocarbamate in cultured vascular endothelial cells. J. Toxicol. Sci., **41**, 225-232.

Fujie, T., Takenaka, F., Yoshida, E., Yasuike, S., Fujiwara, Y., Shinkai, Y., Kumagai, Y., Yamamoto, C. and Kaji, T. (2019a): Possible mechanisms underlying transcriptional induction of metallothionein isoforms by tris(pentafluorophenyl)stibane, tris(pentafluorophenyl)arsane, and tris(pentafluorophenyl)phosphane in cultured bovine aortic endothelial cells. J. Toxicol. Sci., **44**, 327-333.

Fujie, T., Yamamoto, T., Yamamoto, C. and Kaji, T. (2019b): Bis(1,4-dihydro-2-methyl-1-phenyl-4-thioxo-3-pyridiolato) zinc(II) exhibits strong cytotoxicity and a high intracellular accumulation in cultured vascular endothelial cells. J. Toxicol. Sci., **44**, 113-120.

Galley, H.F. and Webster, N.R. (2004): Physiology of the endothelium. Br. J. Anaesth., **93**, 105-113.

Girijashanker, K., He, L., Soleimani, M., Reed, J.M., Li, H., Liu, Z., Wang, B., Dalton, T.P. and Nebert, D.W. (2008): Slc39a14 gene encodes ZIP14, a metal/bicarbonate symporter: similarities to the ZIP8 transporter. Mol. Pharmacol., **73**, 1413-1423.

Hara, T., Matsuura, S., Aikawa, K., Shirai, M., Yoshida, M., Kaji, T. and Yamamoto, C. (2023): Cadmium induces chondroitin sulfate synthase 1 via protein kinase Cα and elongates chondroitin/dermatan sulfate chains in cultured vascular endothelial cells. J. Toxicol. Sci., **48**, 457-467.

Hara, T., Sakuma, M., Fujie, T., Kaji, T. and Yamamoto, C. (2021): Cadmium induces plasminogen activator inhibitor-1 via Smad2/3 signaling pathway in human endothelial EA.hy926 cells. J. Toxicol. Sci., **46**, 249-253.

He, L., Girijashanker, K., Dalton, T.P., Reed, J., Li, H., Soleimani, M. and Nebert, D.W. (2006): ZIP8, member of the solute-carrier-39 (SLC39) metal-transporter family: characterization of transporter properties. Mol. Pharmacol., **70**, 171-180.

Itoh, K., Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, Y., Oyake, T., Hayashi, N., Satoh, K., Hatayama, I., Yamamoto, M. and Nabeshima, Y. (1997): An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem. Biophys. Res. Commun., **236**, 313-322.

Ito, K., Fujie, T., Shimomura, M., Nakano, T., Yamamoto, C. and Kaji, T. (2022): TGF-β_1 potentiates the cytotoxicity of cadmium by induction of a metal transporter, ZIP8, mediated by the ALK5-Smad2/3 and ALK5-Smad3-p38 MAPK signal pathways in cultured vascular endothelial cells. Int. J. Mol. Sci., **23**, 448.

Kägi, J.H. and Valee, B.L. (1960): Metallothionein: a cadmium- and zinc-containing protein from equine renal cortex. J. Biol. Chem., **235**, 3460-3465.

Kaji, T., Fujiwara, Y., Koyanagi, E., Yamamoto, C., Mishima, A., Sakamoto, M. and Kozuka, H. (1992c): Protective effect of copper against cadmium cytotoxicity on cultured vascular endothelial cells. Toxicol. Lett., **63**, 13-20.

Kaji, T., Mishima, A., Koyanagi, E., Yamamoto, C., Sakamoto, M. and Kozuka, H. (1992b): Possible mechanism for zinc protection against cadmium cytotoxicity in cultured vascular endothelial cells. Toxicology, **76**, 257-270.

Kaji, T., Mishima, A., Yamamoto, C., Sakamoto, M. and Koizumi, F. (1992a): Effect of cadmium on the monolayer maintenance of vascular endothelial cells in culture. Toxicology, **71**, 267-276.

Kaji, T., Suzuki, M., Yamamoto, C., Imaki, Y., Miyajima, S., Fujiwara, Y., Sakamoto, M. and Kozuka, H. (1996): Sensitive response of cultured vascular smooth-muscle cells to cadmium cytotoxicity: comparison with cultured vascular endothelial cells and kidney epithelial LLC-PK1 cells. Toxicol. Lett., **89**, 131-137.

Kaji, T., Yamamoto, C., Miyajima, S., Suzuki, M., Fujiwara, Y., Sakamoto, M. and Koizumi, F. (1995): Vascular smooth muscle cells in culture are highly sensitive to cadmium cytotoxicity without species-related differences: comparison with Chang liver cells. Biol. Pharm. Bull., **18**, 1392-1395.

Karin, M. and Richards, R.I. (1982): Human metallothionein genes--primary structure of the metallothionein-II gene and a related processed gene. Nature, **299**, 797-802.

Kissane, J.M. and Robins, E. (1958): The fluorometric measurement of deoxyribonucleic acid in animal tissues with special reference to the central nervous system. J. Biol. Chem., **233**, 184-188.

McNeill, R.V., Mason, A.S., Hodson, M.E., Catto, J.W. and Southgate, J. (2019): Specificity of the metallothionein-1 response by cadmium-exposed normal human urothelial cells. Int. J. Mol. Sci., **20**, 1344.

Mertens, G., Cassiman, J.J., Van den Berghe, H., Vermylen, J. and David, G. (1992): Cell surface heparan sulfate proteoglycans from human vascular endothelial cells. Core protein characterization and antithrombin III binding properties. J. Biol. Chem., **267**, 20435-20443.

Mishima, A., Kaji, T., Yamamoto, C., Sakamoto, M. and Kozuka, H. (1995): Zinc-induced tolerance to cadmium cytotoxicity without metallothionein induction in cultured bovine aortic endothelial cells. Toxicol. Lett., **75**, 85-92.

Mishima, A., Yamamoto, C., Fujiwara, Y. and Kaji, T. (1997): Tolerance to cadmium cytotoxicity is induced by zinc through non-metallothionein mechanisms as well as metallothionein induction in cultured cells. Toxicology, **118**, 85-92.

Navas-Acien, A., Selvin, E., Sharrett, A.R., Calderon-Aranda, E., Silbergeld, E. and Guallar, E. (2004): Lead, cadmium, smoking, and increased risk of peripheral arterial disease. Circulation, **109**, 3196-3201.

Ohkawara, S., Yamamoto, C., Fujiwara, Y., Sakamoto, M. and Kaji, T. (1997): Cadmium induces the production of high molecular

weight heparan sulfate proteoglycan molecules in cultured vascular endothelial cells. Environ. Toxicol. Pharmacol., **3**, 187-194.

Palmiter, R.D. (1994): Regulation of metallothionein genes by heavy metals appears to be mediated by a zinc-sensitive inhibitor that interacts with a constitutively active transcription factor, MTF-1. Proc. Natl. Acad. Sci. USA, **91**, 1219-1223.

Shinkai, Y., Kimura, T., Itagaki, A., Yamamoto, C., Taguchi, K., Yamamoto, M., Kumagai, Y. and Kaji, T. (2016): Partial contribution of the Keap1-Nrf2 system to cadmium-mediated metallothionein expression in vascular endothelial cells. Toxicol. Appl. Pharmacol., **295**, 37-46.

Tomera, J.F., Lilford, K., Kukulka, S.P., Friend, K.D. and Harakal, C. (1994): Divalent cations in hypertension with implications to heart disease: calcium, cadmium interactions. Methods Find. Exp. Clin. Pharmacol., **16**, 97-107.

Yamamoto, C., Kaji, T., Sakamoto, M. and Kozuka, H. (1993): Cadmium stimulation of plasminogen activator inhibitor-1 release from human vascular endothelial cells in culture. Toxicology, **83**, 215-223.