Zinc diethyldithiocarbamate as an inducer of metallothionein in cultured vascular endothelial cells

Tomoya Fujie1, Yukino Segawa1, Akane Uehara1, Takehiro Nakamura1, Tomoki Kimura2, Eiko Yoshida1, Chika Yamamoto3, Masanobu Uchiyama4, Hiroshi Naka5 and Toshiyuki Kaji1

1Department of Environmental Health, Faculty of Pharmaceutical Sciences, Tokyo University of Science, 2641 Yamazaki, Noda 278-8510, Japan
2Department of Life Science, Faculty of Science and Engineering, Setsunan University, 17-8 Ikedanakamachi, Neyagawa 573-0101, Japan
3Department of Environmental Health, Faculty of Pharmaceutical Sciences, Toho University, 2-2-1 Miyama, Funabashi 274-8510, Japan
4Graduate School of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo-to 113-0033, Japan
5Graduate School of Science and Research Center for Materials Science, Nagoya University, Chikusa, Nagoya 464-8602, Japan

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ABSTRACT — Vascular endothelial cells are in direct contact with blood. Inorganic zinc is thought to be incapable of inducing metallothionein, which protects cells from heavy metal toxicity and oxidative stress, in vascular endothelial cells. Here, we aimed to further characterize the induction of metallothionein in vascular endothelial cells. Our results confirmed that inorganic zinc could not induce metallothionein in vascular endothelial cells. Moreover, ZnSO4 could not activate both the metal response element (MRE) transcription factor 1 (MTF-1)/MRE and Nrf2/antioxidant response element (ARE) pathways and was incapable of inducing metallothionein. In addition, bis(l-cysteinato)zincate(II), a zinc complex that activates the MTF-1/MRE pathway, increased MRE promoter activity but failed to induce metallothionein, suggesting that vascular endothelial metallothionein was not induced only by activation of the MTF-1/MRE pathway. Further analysis of a library of zinc complexes showed that zinc(II) bis(diethyldithiocarbamate) activated the MTF-1/MRE pathway but not the Nrf2/ARE pathway, increased MT-1A, MT-1E, and MT-2A mRNA levels, and induced metallothionein proteins. These data indicated that zinc complexes may be excellent tools to analyze metallothionein induction in vascular endothelial cells.

Key words: Antioxidant response element, Metallothionein, Metal response element, Vascular endothelial cell, Zinc, Zinc complex

INTRODUCTION

Metallothionein (MT) is a low-molecular-weight, cysteine-rich, metal-containing, inducible protein (Margoshes and Vallee, 1957). MT functions to protect cells from the cytotoxicity of toxic heavy metals, such as cadmium, by sequestration (Kägi and Schaffer, 1988). There are four isoforms of MT, i.e., MT-1, MT-2, MT-3, and MT-4 (Stennard et al., 1994; Yeiser et al., 1999; Quaife et al., 1994), with MT-1 having seven subisoforms in human tissue (Stennard et al., 1994). Of the MT isoforms, MT-1 and MT-2 are induced by cadmium and protect against metal cytotoxicity. Expression of the MT gene is mediated by metal response element (MRE)-binding transcription factor-1 (MTF-1), which binds a consensus sequence (the MRE) in the upstream region of the MT gene (Redtko et al., 1993; Zhang et al., 2001). Zinc is the only metal that can bind MTF-1 and activate the MTF-1/ MRE pathway (Bittel et al., 1998). In addition, the MT gene contains a consensus sequence, called the antioxidant response element (ARE), in the promoter region (Ohtsui et al., 2008), which is activated by the transcriptional factor nuclear factor-erythroid 2-related factor 2 (Nrf2) (Itoh et al., 1997). The ARE is involved in the transcriptional activation of MT genes by hydrogen peroxide (Dalton et al., 1994), suggesting that the Nrf2/ARE
pathway may regulate MT gene expression.

Vascular endothelial cells cover the luminal surface of blood vessels and contribute to the antithrombogenic properties of the vascular endothelium by synthesizing and secreting anticoagulant and fibrinolytic substances, such as anticoagulant heparan sulfate proteoglycans (Mertens et al., 1992), thrombomodulin (Esmon et al., 1982), and tissue plasminogen activator (Levin and Loskutoff, 1982), thereby prevent atherosclerosis (Harker et al., 1981). Because cadmium exposure is a risk factor for atherosclerosis (Houtman, 1993; Fagerberg et al., 2012), we previously studied the cytotoxicity of cadmium in vascular endothelial cells using a cell culture system. Our analyses showed that vascular endothelial cells are sensitive to cadmium cytotoxicity (Kaji et al., 1996); cadmium influences the synthesis of heparan sulfate proteoglycans (Ohkawara et al., 1997) and lowers fibrinolytic activity by promoting the secretion of plasminogen activator inhibitor-1 (Yamamoto et al., 1993; Yamamoto and Kaji, 2002). Endothelial cells can be protected from cadmium cytotoxicity by zinc (Kaji et al., 1992; Mishima et al., 1997). In general, zinc-induced MT provides protection against cadmium; however, in vascular endothelial cells, zinc does not induce MT protein expression, despite reducing the accumulation of cadmium within the cells (Kaji et al., 1992). Therefore, regulation of MT induction in vascular endothelial cells may be distinct from that in other cell types.

Organic-inorganic hybrid molecules, which are composed of an organic structure and metal(s), have been used as reagents in chemical synthetic reactions and can exhibit unique biological activities (Fujiiwa et al., 2005; Kimura et al., 2012; Murakami et al., 2015; Kohri et al., 2015). Therefore, the bio-elements strategy, i.e., bio-organic metallics, appears to be an effective method for analysis of biological systems. Previously, we found that the zinc complex bis(l-cysteinato)zincate(II) [Zn(cys)] induces the transcriptional activation of MT in fibroblastic cells by transferring zinc to MTF-1 (Kimura et al., 2012). However, because the mechanisms underlying vascular endothelial MT induction may be unique, it is unclear whether Zn(cys), induces MT in vascular endothelial cells. Induction by inorganic zinc at a transcriptional level has also not been confirmed, and inorganic zinc cannot be applied as an effective tool for identification of the mechanisms underlying endothelial MT induction.

Here, we examined MT induction by inorganic zinc and sought to identify an organic-inorganic hybrid molecule that could induce MT in bovine aortic endothelial cells from a library of zinc complexes. Our results showed that the zinc complex zinc(II) bis(diethylidithiocarbamate) [Zn(edtc)] elevated MT mRNAs in bovine cells and increased MT protein expression by activating MRE promoter activity.

**MATERIALS AND METHODS**

**Materials**

Zn(cys)2 was synthesized as described previously (Viladkar et al., 1993). Zn(edtc)2 was purchased from Tokyo Chemical Industry (Tokyo, Japan). Bovine aortic endothelial cells were purchased from Cell Applications (San Diego, CA, USA). The following materials were purchased from the respective vendors: Dulbecco’s modified Eagle’s medium (DMEM) and calcium- and magnesium-free phosphate-buffered saline from Nissui Pharmaceutical (Tokyo, Japan); fetal bovine serum (FBS) from HyClone Laboratories (Waltham, MA, USA); bicinchoninic acid protein assay reagent kits from Thermo Fisher Scientific (Waltham, MA, USA); polyvinylidene difluoride membranes (0.2 μm) from Millipore (Bedford, MA, USA); horseradish peroxidase-conjugated anti-rabbit IgG antibodies (#7074) and horseradish peroxidase conjugated anti-mouse IgG antibodies (#7076) from Cell Signaling (Beverly, MA, USA); Gene Ace SYBR qPCR Mix from Nippon Gene (Tokyo, Japan); the Dual-Luciferase Reporter Assay System and pRL-SV40 from Promega (Madison, WI, USA); and Chemi-Lumi One L and other reagents from Nacalai Tesque (Kyoto, Japan).

**Cell culture**

Vascular endothelial cells were cultured at 37°C in 5% CO2 in DMEM supplemented with 10% FBS until confluent. The medium was removed, and the cells were washed twice with serum-free DMEM. The cells were treated with or without ZnSO4 (5, 10, 30, 50, or 100 μM), Zn(cys)2 (10, 20, 30, 50 or 100 μM), or Zn(edtc)2 (1, 2, 3, or 5 μM) at 37°C for 3, 6, 12, 18, 24, or 48 hr in serum-free DMEM.

**Western blot analysis**

Confluent cultures of bovine aortic endothelial cells treated with ZnSO4, Zn(cys)2, or Zn(edtc)2 were lysed in sodium dodecyl sulfate sample buffer (50 mM Tris-HCl buffer solution containing 2% sodium dodecyl sulfate and

T. Fujie et al.
10% glycerol, pH 6.8) and incubated at 95°C for 5 min. Protein concentrations were determined using a bicinchoninic acid protein assay reagent kit. 2-Mercaptoethanol and bromophenol blue (1.67% each) were added to samples (10 μg protein) and incubated at 95°C for 3 min for detection of Nrf2; for detection of MT-1/2, 20 μg of the cellular protein was added to 10 mM EDTA, 50 mM dithiothreitol, 5% 2-mercaptoethanol, and 1.67% glycerol. The samples were then heated at 95°C for 3 min, incubated with 200 mM iodoacetamide for 30 min at room temperature in the dark, and mixed with 0.75 M Tris-HCl (pH 8.8). The cellular proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% or 15% polyacrylamide gels for Nrf2 or MT-1/2, respectively, and electrotransferred to polyvinylidene difluoride membranes (0.2 μm) at 2 mA/cm² for 1 hr. The membranes were blocked with 5% skim milk in 20 mM Tris-HCl buffer solution containing 15 mM NaCl and 0.1% Tween 20 (pH 7.5) and then incubated with anti-Nrf2 (1:200), anti-MT-1/2 (1:200), or anti-β-actin antibodies (1:1,000) at 4°C overnight. After washing with 20 mM Tris-HCl buffer solution containing 15 mM NaCl and 0.1% Tween 20 (pH 7.5), the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibodies (1:5,000) for 1 hr at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence using Chemi-Lumi One L and scanned with an LAS3000 (Fujifilm, Tokyo, Japan).

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

Total RNA was extracted from bovine aortic endothelial cells treated with ZnSO₄, Zn(cys)₂, or Zn(edtc)₂. cDNA was synthesized using a High-Capacity cDNA Reverse Transcription kit. Real-time PCR was performed using Gene Ace SYBR qPCR Mix α with 10 ng cDNA and 100 nM primers on a StepOnePlus RT-PCR system (Applied Biosystems). The thermal cycling parameters were as follows: 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min. Expression levels of MT-1A, MT-1E, MT-2A, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs were quantified by the comparative Ct method. Fold changes in expression were assessed after normalization of the intensity value to that of GAPDH. The following primer pairs were used: bovine MT-1A, 5'-CACCTGCAAGGCTTGCAAGA-3' (forward) and 5'-CGAGGGCCCCCTTTCGACATA-3' (reverse); bovine MT-1E, 5'-C ACAATTGCTCTGCCCCCACA-3' (forward) and 5'-CACACTTGCCAAGGCCACCA-3' (reverse); bovine MT-2A, 5'-GGCTCTCTGCAAATGCAAAGAT-3' (forward) and 5'-CCGAAGCCCCCTTTGACAGAC-3' (reverse); bovine GAPDH, 5'-AACACCCTCAAGATTGTCAGCAGA-3' (forward) and 5'-ACAGTCTTCTGGGGTCGAGTA-3' (reverse).

**Luciferase assay**

The firefly reporter plasmids pGL4.12-MREG and pGL4.12-AREG × 3 were cloned as previously described (Kimura et al., 2002, 2009) and used for MRE- or ARE-driven reporter assays. Cells were transfected with reporter plasmids using Lipofectamine LTX reagent with PLUS reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Briefly, confluent cultures of bovine aortic endothelial cells were prepared in 24-well plates. Firefly reporter plasmid (0.01 μg/mL), control Renilla reporter plasmid (0.01 μg/mL), and transfection reagent (0.05 μL/mL) were mixed with Opti-MEM and incubated for 5 min at room temperature. The mixture was added to the culture medium, and the cells were incubated at 37°C in Opti-MEM. After 1 hr, the medium was changed to 10% DMEM supplemented with 10% FBS. After 24 hr, the cells were incubated with ZnSO₄ (10, 50, or 100 μM), Zn(cys) (10, 50, or 100 μM), or Zn(edtc) (1, 5, or 10 μM) for 3 or 6 hr and lysed. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System and a GloMax 20/20n luminometer (Promega). MRE- and ARE-driven activities were normalized according to the luminescence of pRL-SV40.

**Statistical analysis**

The data are presented as the mean ± standard error (SE) for each experimental group. Statistical analysis were performed using one-way analysis of variance followed by Bonferroni’s-type multiple t-test. Differences with P values of less than 0.05 were considered significant.

**RESULTS AND DISCUSSION**

Zinc is known to protect against cadmium toxicity in various biological systems (Webb, 1972; Early and Schnell, 1978), and the protective effects are generally due to induction of MT by zinc, which in turn sequesters cadmium from interactions with critical target molecules. Figure 1 shows the expression of MT in vascular endothelial cells treated with ZnSO₄. After a 24-hr treatment with inorganic zinc, MT protein expression was not observed (Fig. 1A), consistent with our previous study (Kaji et al., 1992). Furthermore, ZnSO₄ did not alter the expression of MT-1A, MT-1E, or MT-2A mRNAs, MT subforms expressed in bovine tissue (Fig. 1B). The promoter activities of the MRE (Fig. 1C) and ARE (Fig. 1E) were
unaffected by ZnSO₄; ZnSO₄ also failed to activate Nrf2 (Fig. 1D), suggesting that the zinc ion could not activate the MTF-1/MRE and Nrf2/ARE pathways, which regulate MT induction. Previously, we investigated the interaction between zinc and cadmium in vascular endothelial cells and found that zinc accumulates within the cells but does not induce MT at the protein level; however, zinc protects cells from cadmium cytotoxicity by decreasing the intracellular accumulation of cadmium (Kaji et al., 1992). In the present study, we confirmed that ZnSO₄ could not induce MT protein or mRNA. In addition, both the MRE and Nrf2/ARE pathways were not activated, suggesting that the zinc ion did not function in any step of MT induction in vascular endothelial cells. Although the mechanisms through which zinc may not participate in the regulation of endothelial MT induction are unclear, it is likely that these mechanisms contribute to maintenance of the constitutive expression of MT in vascular endothelial cells, the only cell type that is in direct contact with the blood, even when the blood zinc concentration is altered by foods and other factors.

Zn(cys)₂ is a zinc complex that causes transcriptional induction of MT in mouse embryo fibroblasts (Kimura et al., 2012). The complex activates MTF-1 without elevating the expression of mRNA for heme oxygenase-1, a cell stress-related protein whose expression is mediated by Nrf2 (Kaspar and Jaiswal, 2010). Zn(cys)₂ serves as a donor of the zinc ion to MTF-1 and specifically activates MTF-1. Therefore, we next examined whether Zn(cys)₂ could induce MT in vascular endothelial cells. Our results showed that Zn(cys)₂ (Fig. 2A) did not increase the expression of MT proteins when used at a concentration of 100 μM or less for up to 48 hr (Fig. 2B). Transcriptional induction of MT subisoforms was also unchanged by treatment with Zn(cys)₂ at 100 μM or less for 12 hr (Fig. 2C), and exposure to 100 μM Zn(cys)₂ for up to 24 hr did not upregulate MT-1A, MT-1E, or MT-2A mRNA levels (data not shown). However, although Zn(cys)₂ did

Fig. 1. Effects of ZnSO₄ on the induction of MT in vascular endothelial cells. [A] Expression of MT proteins after treatment with ZnSO₄ for 24 hr. [B] Expression MT-1A, MT-1E, and MT-2A mRNAs after treatment with ZnSO₄ for 12 hr. [C] Promoter activity of the MRE after treatment with ZnSO₄ for 12 hr. [D] Activation of Nrf2 after treatment with ZnSO₄ for 6 hr. [E] Promoter activity of the ARE after treatment with ZnSO₄ for 12 hr.
not activate Nrf2 (Fig. 2E) and ARE promoter activity (Fig. 2F), MRE promoter activity was activated by the zinc complex (Fig. 2D). These results indicated that Zn(cys)₂ served as a donor of the zinc ion specifically for MTF-1 but that MT isoforms could not be induced only by activation of the MTF-1/MRE pathway in vascular endothelial cells. We postulate that there is an epigenetic regulation in endothelial MT gene expression, including demethylation of DNA (Lieberman et al., 1983), acetylation of histone (Okumura et al., 2011), and acetylation of Nrf2 (Sun et al., 2009). It is reported that acetylation of Nrf2 can change AREs to which Nrf2 molecules are recruited (Sun et al., 2009; Mercado et al., 2011). It is possible that this change may be important for endothelial MT induction. In addition, it is unclear why Zn(cys)₂ but not ZnSO₄ could activate MTF-1. We postulate that vascular endothelial cells may have a relatively large zinc pool, which may make it difficult for the zinc ion to come in contact with MTF-1. Moreover, the zinc ion in the Zn(cys)₂ molecule would be protected from translocation to the zinc pool.

Because both Zn(cys)₂ and ZnSO₄ failed to induce endothelial MT, we sought to identify MT inducers from a library of 33 zinc complexes. Interestingly, we found that Zn(edtc)₂ (Fig. 3A) increased the expression of MT proteins in a concentration-dependent manner when used at a concentration of 10 μM or less for 6 hr (Fig. 3B, left panel); this increase was observed after 6 hr or more when cells were treated with 10 μM Zn(edtc)₂ (Fig. 3B, right panel). The expression of MT-1A, MT-1E, and MT-2A mRNAs was also elevated by exposure to Zn(edtc)₂, at 10 μM or less for 6 hr (Fig. 3C). Moreover, induction of all three isoforms was noted after 3 hr, with the most dramatic elevation observed after 6 hr (data not shown). When used at a concentration of 10 μM or less, Zn(edtc)₂ elevated the promoter activity of the MRE (Fig. 3D) but
failed to activate Nrf2 (Fig. 3 F) after 3 hr. Additionally, Nrf2 was not activated by the zinc complex at 10 μM or less after up to 12 hr, indicating that Zn(edtc)₂ induced MT expression by activation of the MTF-1/MRE pathway in vascular endothelial cells. Although both Zn(edtc)₂ and Zn(cys)₂ activated the MTF-1/MRE pathway but not the Nrf2/ARE pathway, only Zn(edtc)₂ induced endothelial MT. As stated above, endothelial MT induction appeared to be regulated epigenetically. In addition, release of the zinc ion from the zinc pool within vascular endothelial cells may be required for activation of MTF-1. We hypothesize that Zn(cys)₂ may be a specific activator of MTF-1, whereas Zn(edtc)₂ not only activates MTF-1 by providing the zinc ion and/or activating zinc transporters that transport the zinc ion from the zinc pool to cytosol (Fukada, and Kambe, 2011) but also acts on the epigenetic system for endothelial MT induction. However, further studies are required to elucidate these mechanisms.

In summary, in the current study, we demonstrated that inorganic zinc (ZnSO₄) could not activate both the MTF-1/MRE and Nrf2/ARE pathways and was incapable of inducing MT in vascular endothelial cells. Moreover, Zn(cys)₂, a donor of the zinc ion to MTF-1, was also incapable of inducing MT, despite activation of the MTF-1/MRE pathway. Finally, we showed that another zinc complex, Zn(edtc)₂, induced the expression of MT subisoforms through activation of the MTF-1/MRE pathway but not the Nrf2/ARE pathway. The characteristics of Zn(edtc)₂, as an endothelial MT inducer appear to be useful for analyzing mechanisms other than the signaling pathway, including the epigenetic regulation of endothelial MT induction and the corresponding zinc transport system. Recently, we found that copper diethyldithiocarbamate, which has the same ligand as Zn(edtc)₂ but with
copper but not zinc, also induces endothelial MT protein and mRNA expression (unpublished data). In this case, both the MTF-1/MRE and Nrf2/ARE pathways are activated. The mechanisms underlying endothelial MT induction using these metal complexes are under investigation in our laboratory. Our current findings suggested that organic-inorganic hybrid molecules may be used as tools for analysis of the mechanisms underlying biological systems. Since organic-inorganic hybrid molecules can exhibit unique biological activities (Murakami et al., 2015; Kohri et al., 2015), further studies are needed to clarify the unique regulation of MT induction in vascular endothelial cells using organic-inorganic hybrid molecules.

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

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