Study on the mechanisms for vascular endothelial metallothionein induction using organic-inorganic hybrid molecules

（有機–無機ハイブリッド分子を活用した血管内皮細胞メタロチオネインの誘導メカニズムに関する研究）

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CONTENTS

Abbreviations

ARE	Antioxidant response element
As35	tris(pentafluorophenyl)arsane
BPM	Biotin-PEAC$_5$-maleimide
Cu01	Bis(hexafluoroactylacetonato)copper(II)
Cu02	*N,N′*-Bis(2-methoxycarbonyl-3-oxobutylidene)ethylenediaminatocopper(II)
Cu03	Bis(salicylidene)ethylenediaminatocopper(II)
Cu04	Copper(II) diacetate
Cu07	Bis(1,3-propanediamine)copper(II) dichloride
Cu09	Copper(II) bis(2-hydroxyethyl)dithiocarbamate
Cu10	Copper(II) bis(diethyldithiocarbamate)
Cu15	*N,N′*-Bis(3,5-di-*tert*-butyl-2-oxidobenzyl)ethylenediaminatocopper(II)
Cu17	Copper(II) bis(dimethyldithiocarbamate)
Cu18	Copper(II) bis(dibutyldithiocarbamate)
Cu19	Copper(II) bis(dibenzyldithiocarbamate)
DAPBi	2-(*N,N*-dimethylaminomethyl)phenylbis(4-methylphenyl)bismuthane
DAPSb	2-(*N,N*-dimethylaminomethyl)phenylbis(4-methylphenyl)stibane
DMEM	Dulbecco's modified Eagle's medium
Fe01	Tris(acetylacetonato)iron(III)
Fe02	Iron(II) diacetate
Fe03	Iron(II) phthalocyanine
Fe04	Sodium iron(III) ethylenediaminetetraacetate
Fe05	Iron(III) tris(diethyldithiocarbamate)
Keap1	Kelch-like ECH-associated protein 1
MG132	Z-Leu-Leu-Leu-CHO

MRE	Metal response element
MT	Metallothionein
MTF-1	Metal response element-binding transcription factor-1
Na01	Sodium diethyldithiocarbamate trihydrate
Ni06	Nickel(II) bis(diethyldithiocarbamate)
Nrf2	NF-E2-related factor 2
P35	tris(pentafluorophenyl)phosphane
PMTABi	*Bi*-phenyl-*N*-methyl-5,6,7,12-tetrahydrodibenz[*c*,*f*][1,5]azabismocine
PMTAS	*Sb*-phenyl-*N*-methyl-5,6,7,12-tetrahydrodibenz[*c*,*f*][1,5]azastibocine
Sb25	triphenylstibane
Sb33	Tris(4-fluorophenyl)stibane
Sb35	tris(pentafluorophenyl)stibane
Sb49	tris(3,4,5-trifluorophenyl)stibane
$Zn(cys)_2$	bis(L-cysteinato)zincate(II)
$Zn(edtc)_2$ (Zn01)	Zinc(II) bis(diethyldithiocarbamate)

General Introduction

Organic-inorganic hybrid molecules —organometallic compounds and metal coordination compounds— are composed of an organic structure and metal(s) in a common feature. Since the molecules used as synthetic reagents by pioneers such as Grignard and Wittig (Grignard, 1900; Wittig and Schöllkopf, 1954), organic element chemistry has developed rapidly. However, little has archived in the contribution of organic-inorganic hybrid molecules to the research of life sciences. Bio-organometallics is a new biology in which organic-inorganic hybrid molecules are used to analyze biological systems. This should be called the bio-element strategy.

Organic-inorganic hybrid molecules are expected to exhibit unique biological activities, different from those of the organic structure and metal(s) alone, because of their unique three-dimensional structures and electronic states. Intramolecular metal may intensify the biological activities of the organic structure; conversely, the organic structure may intensify the biological activities of intramolecular metal. It is possible that intramolecular interaction between the organic structure and metal(s) produces novel biological activities. It was found that an organobismuth compound —tris[2-(*N,N*-dimethylaminomethyl)-phenyl]bismuthane— exhibits cytotoxicity specific to vascular endothelial cells (Fujiwara et al., 2005). Bis(L-cysteinato)zincate provides the zinc ion selectively to metal response element binding transcription factor-1 (MTF-1) containing six C_2H_2 zinc finger motifs (Kimura T et al., 2012). Bio-organometallics appears to be effective strategy for analysis of unknown biological systems.

Blood vessels are composed of vascular endothelial cells that cover the luminal surface of vascular endothelium and other cell types such as vascular smooth muscle cells and pericytes. Since blood vessels ubiquitously exist in every organ. It is impossible for hazardous chemicals to enter parenchymal cells in the target organs without contact with vascular endothelial cells. Functional damage of endothelial cells will influence to the organ toxicity of

the chemicals. In fact, neurotoxicity of methylmercury in the cerebral cortex can be understood as a result of vascular toxicity of methylmercury (Hirooka and Kaji, 2012). On the other hand, vascular endothelial cells regulate the blood coagulation-fibrinolytic system (Levin and Loskutoff, 1982; Mourik et al., 1984) and vascular tone (Furchgott and Zawadzki, 1980; Yanagisawa et al., 1988). Therefore, clarification of the defense mechanisms against toxic heavy metals (Revis et al., 1981; Houston, 2011) and oxidative stress in vascular endothelial cells is important for understanding the metal toxicity and vascular disorders such as atherosclerosis.

Metallothionein (MT) is a protein that was found as a cadmium-binding protein (Margoshes and Vallee, 1957). MT is defined as low-molecular-weight and cysteine-rich protein with a high affinity for heavy metal ions (Kägi, 1991). MT is a family classified as four isoforms—MT-1, MT-2, MT-3, and MT-4—. The genes of the isoforms express on the same chromosome (Stennard et al., 1994; Yeiser et al., 1999; Quaife et al., 1994). MT-1 and MT-2 are expressed ubiquitous tissues and protect against toxic heavy metals by being induced by zinc and cadmium and sequestering the metals (Kägi, 1991; Sato and Bremner, 1993). MT-3 and MT-4 exist in specific tissue: MT-3 is in the neural tissue (Yeiser et al., 1999) and MT-4 is in stratified squamous epithelia (Quaife et al., 1994). Induction of MT essentially requires the activation of metal response element (MRE) by binding a transcription factor MTF-1 that is activated by zinc ion (Stuart et al., 1985; Culotta and Hamer, 1989). However, there appears to be complex mechanisms underlying MT induction especially in vascular endothelial cells.

NF-E2 related factor 2 (Nrf2) is a transcription factor that regulates phase 2 xenobiotic metabolizing and antioxidant enzymes by being bound to antioxidant response element (ARE) (Itoh et al., 1997). Although ARE(s) exist in the promoter region of MT genes (Palmiter, 1987), little was known about the roles of the Nrf2–ARE pathway in the induction of MT in vascular endothelial cells.

The purpose of the present study is to clarify the involvement of the MTF-1—MRE and Nrf2—ARE pathways in the induction of MT in vascular endothelial cells. Since it was found that inorganic zinc is not able to induce MT in vascular endothelial cells, we sought organic-inorganic hybrid molecule that induce MT in the cells and found out several molecules. Using these hybrid molecules, we investigated the roles of the MTF-1–MRE and Nrf2–ARE pathways in endothelial MT induction using these hybrid molecules.

Materials and Methods

Materials

Bis(L-cysteinato)zincate(II) ($Zn(cys)_2$), *N,N′*-Bis(2-methoxycarbonyl-3-oxobutylidene) ethylenediaminatocopper(II) (Cu02), bis(salicylidene)ethylenediaminatocopper(II) (Cu03), and *N,N′*-bis(3,5-di-*tert*-butyl-2-oxidobenzyl)ethylenediaminatocopper(II) (Cu15) were synthesized by employing literature procedures (Viladkar et al., 1993; Rybka et al., 2006; Ribeiro et al., 2004; Saint-Aman et al., 1998). Bis(hexafluoroactylacetonato)copper(II) (Cu01), bis(1,3-propanediamine)copper(II) dichloride (Cu07), copper(II) bis(2-hydroxy-ethyl)dithiocarbamate (Cu09), copper(II) bis(diethyldithiocarbamate) (Cu10), iron(II) phthalocyanine (Fe03), sodium iron(III) ethylenediaminetetraacetate (Fe04), iron(III) tris(diethyldithiocarbamate) (Fe05), nickel(II) bis(diethyldithiocarbamate) (Ni06), and zinc(II) bis(diethyldithiocarbamate) ($Zn(edtc)_2$ or Zn01) iron(II) diacetate (Fe02), zinc(II) bis(dibutyldithiocarbamate), zinc(II) bis(dibenzyldithiocarbamate) and 3,5-diaminobenzoic acid hydrochloride were purchased from Tokyo Chemical Industry (Tokyo, Japan). Bovine aortic endothelial cells were purchased from Cell Applications (San Diego, CA, USA). Smooth muscle cells derived from bovine aorta, Fibroblastic IMR-90 cells from human fetal lung and epithelial LLC-PK_1 cells from porcine kidney were obtained from DS Pharma Biomedical (Osaka, Japan). 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); May-Grunwald and Giemsa stain solution (Merck KGaA, Darmstadt, Germany); sodium diethyldithiocarbamate trihydrate (Na01), copper(II) bis(dimethyldithiocarbamate) (Cu17), polyvinylidene difluoride membranes (0.2 μm) and mouse monoclonal anti-β-actin monoclonal antibodies from Wako

Pure Chemical Industries (Osaka, Japan); mouse monoclonal anti-MT-1/2 antibodies (E9) from Dako (Glostrup, Denmark); rabbit polyclonal anti-human Nrf2 antibodies (H-300) and rabbit polyclonal anti CTR1 antibody (FL-190) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); horseradish peroxidase-conjugated anti-rabbit IgG antibodies (#7074) and horseradish peroxidase conjugated anti-mouse IgG antibodies (#7076) from Cell Signaling (Beverly, MA, USA); High-Capacity cDNA Reverse Transcription kits from Applied Biosystems (Foster, CA, USA); Gene Ace SYBR qPCR Mixα from Nippon Gene (Tokyo, Japan); Cytotox 96 Non-Radioactive Cytotoxicity Assay, a lactate dehydrogenase kit, 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).

Synthesis

2-(*N,N*-dimethylaminomethyl)phenylbis(4-methylphenyl)bismuthane (DAPBi), 2-(*N,N*-dimethylaminomethyl)phenylbis(4-methylphenyl)stibane (DAPSb), *Bi*-phenyl-*N*-methyl-5,6,7,12-tetrahydrodibenz[*c,f*][1,5]azabismocine (PMTABi), and *Sb*-phenyl-*N*-methyl-5,6,7,12-tetrahydrodibenz[*c,f*][1,5]azastibocine (PMTAS) (Fig. 2-1-1) were synthesized as reported previously (Kawahara et al., 2011; Tokunaga et al., 2000; Ohkawa et al., 1989; Kakusawa et al., 2006). Tris(4-fluorophenyl)stibane (Sb33), tris(3,4,5-trifluorophenyl)stibane (Sb49), tris(pentafluorophenyl)stibane (Sb35), tris(pentafluorophenyl)arsane (As35), and tris(pentafluorophenyl)phosphane (P35) (Fig. 2-2-1) were synthesized according to previously reported procedures (Fild et al., 1964; Kant et al., 2008; Schäfer et al., 2011; Jiang et al., 2013).

N,N′-Bis(2-methoxycarbonyl-3-oxobutylidene)ethylenediaminatocopper(II) (Cu02), bis(salicylidene)ethylenediaminatocopper(II) (Cu03), and *N,N′*-bis(3,5-di-*tert*-butyl-2-oxidobenzyl)ethylenediaminatocopper(II) (Cu15) were synthesized by employing literature procedures [14-16]. Copper(II) bis(dibutyldithiocarbamate) (Cu18) was synthesized by

mixing copper(II) diacetate (3.63 g, 20 mmol) and zinc(II) bis(dibutyldithiocarbamate) (9.48 g, 20 mmol) in a 1:1 molar ratio in a biphasic mixture of dichloromethane (1 L), water (100 mL), and 25% aqueous ammonia (200 mL) at room temperature for 1 h under aerated conditions. The organic layer was separated, washed with water, concentrated under reduced pressure, and dried in vacuum to yield the desired product as a black solid (9.32 g, 99%). Elemental analysis calculated for [$C_{18}H_{36}CuN_2S_4$]: C, 45.78; H, 7.68; N, 5.93 and found: C, 46.04; H, 7.80; N, 5.65. Copper(II) bis(dibenzyldithiocarbamate) (Cu19) was prepared analogously using zinc(II) bis(dibenzyldithiocarbamate) (92% yield). Elemental analysis calculated for [$C_{30}H_{28}CuN_2S_4$]: C, 59.23; H, 4.64; N, 4.60 and found: C, 59.64; H, 4.68; N, 4.24. The elemental analyses were recorded on a Yanaco CHN recorder MT-6 at the Chemical Instrumental Center, Research Center for Materials Science, Nagoya University.

Cell culture and treatment

Vascular endothelial cells, vascular smooth muscle cells, IMR-90 cells, and LLC-PK_1 cells were cultured in 6-well culture plates at 37 °C in a humid atmosphere of 5% CO_2 in DMEM supplemented with 10% fetal bovine serum until confluent. The medium was discarded, and the cells were washed twice with DMEM supplemented with 10% fetal bovine serum. The cells were then treated with DAPBi, DAPSb, PMTABi, or PMTAS (0.5, 1, 2, or 5 μM) and incubated for 24 h in fresh DMEM supplemented with 10% fetal bovine serum.

Confluent cultures of bovine aortic endothelial cells were washed twice with serum-free DMEM. The cells were treated with or without $ZnSO_4$, $CuSO_4$ [Cu(II)], $CuSO_4$ with 1 mM ascorbate [Cu(I)](Lee et al., 2002), sulforaphane, or indicated organic-inorganic hybrid molecules at 37°C for 3, 6, 12, 18, 24, or 48 h in serum-free DMEM.

Since organic-inorganic hybrid molecules used in this study were insoluble in water, they were solved in dimethylsulfoxide and added to the culture medium. The concentration of dimethylsulfoxide was less than 0.1%.

Cytotoxicity assay

The bovine aortic endothelial cells were cultured in DMEM supplemented with 10% FBS in 24-well culture plates at 37°C in a humidified atmosphere of 5% CO_2 until they attained confluence. The medium was discarded and the cell layer was washed twice with serum-free DMEM and then incubated at 37°C for 24 h in 0.25 mL of serum-free DMEM in the presence of increasing concentrations of DAPBi, DAPSb, PMTABi, PMTAS, Sb25, Sb33, Sb49, Sb35, As35, or P35. After incubation, the conditioned medium was harvested, and an aliquot was used for the determination of LDH activity as an indicator of cytotoxicity. The cell layer was washed twice with ice-cold Ca^{2+}- and Mg^{2+}-free PBS, fixed with methanol, and stained with Giemsa for morphological observation.

Intracellular accumulation of metals

Confluent cultures of vascular endothelial cells were incubated in 6-well plates at 37°C for 3 or 24 h in serum-free DMEM in the presence of indicated compounds.DAPBi, DAPSb, PMTABi, or PMTAS, Sb25, Sb33, Sb49, Sb35, As35, or P35, Na01, Zn01, Fe05, Cu09, or Cu10 (10 μM each), $CuSO_4$ [Cu(II)], $CuSO_4$ with 1 mM ascorbate [Cu(I)](Lee et al., 2002), or Cu10 (10 μM each). In another experiment, subconfluent cultures of bovine aortic endothelial cells were transfected with control or CTR1 small interfering RNA (siRNA) as described below and incubated at 37°C for 3 h in the presence of Cu10, Cu17, Cu18, and Cu19 (10 μM each). After incubation, the medium was discarded and the cells were washed twice with ice-cold calcium- and magnesium-free phosphate buffered saline. The cell lysates were prepared by addition of 100 μL 50 mM Tris-HCl containing 2% SDS and 10% glycerol (pH 6.8). The cell lysate was incubated at 95℃ for 3 min and a portion was treated with nitric acid-H_2O_2 at 130℃ for 1 day to degrade proteins and dissolved with 4 mL of 0.1 M nitric acid; the diluted samples were used for determination of zinc, copper, and iron content by

inductively coupled plasma mass spectrometry (NexION300S, PerkinElmer, MA, USA). Another portion of the cell lysate was analyzed for DNA content by fluorometric method (Kissane and Robins, 1958) to normalize the content of the metals per μg DNA.

Transfection

Bovine aortic endothelial cells were cultured and small interfering RNAs (siRNAs)(Bioneer, Daejeon, Korea) were transfected using RNAiMAX reagent (Invitrogen, Crlsbad, CA, USA) according to the manufacturer's instructions. Briefly, the cells were cultured in DMEM supplemented with 10% fetal bovine serum in 35-mm dishes until 70-80% confluent. Separately, siRNA duplex (35 pmol/mL) and transfection reagent (2 μL/mL) were mixed with Opti-MEM (Thermo Fisher Scientific) and incubated for 20 min at room temperature. The mixture was added to the culture medium and the cells were incubated at 37°C. After 24 h, the cells were incubated at 37°C for 24 h and then treated with indicated compounds. The sequences of the sense and antisense strands of siRNAs were as follows: bovine Nrf2 siRNA, 5′-CCAUUGAUCUCUCUGAUCUdTdT-3′ (sense) and 5′-AGAUCAGAGAGAUCAAUGGGC-3′ (antisense); bovine MTF-1 siRNA-1, 5′-GCACUUUGGAGGAUGAAGAdTdT-3′ (sense) and 5′-UCUUCAUCCUCCAAAGUGCCA-3′ (antisense); bovine MTF-1 siRNA-2, 5′-GAGAACACUUGCCUUUUCUdTdT-3′ (sense) and 5′-AGAAAAGGCAAGUGUUCUCCG-3′ (antisense); bovine CTR1 siRNA, 5′-AUAAGGAUGGUUCCAUUUGdTdT-3′ (sense) and 5′-CAAAUGGAACCAUCCUUAU-3′ (antisense). A nonspecific sequence was used as the siRNA negative control (Qiagen, Valencia, CA, USA).

Luciferase assay

Firefly reporter plasmid pGL4.12-MRE_{d4} and pGL4.12-ARE_4×3 were cloned as previously

described (Kimura et al., 2002; Kimura et al., 2009) and used for the MRE or ARE-driven reporter assay. The reporter plasmids were transfected 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 culture 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 h, the medium was replaced by 10% Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. After 24 h, the cells were incubated with $ZnSO_4$ (10, 50, or 100 μM), $Zn(cys)_2$ (10, 50, or 100 μM), or $Zn(edtc)_2$ (1, 5, or 10 μM) for 3 or 6 h, Sb35 (10, 50, 100, 150, or 200 μM), or Cu10 (1, 2, 3, 5, or 10 μM) for 3, 6, 8, or 12 h, lysed, and the luciferase activity was measured using the Dual-Luciferase Reporter Assay System and GloMax 20/20n luminometer (Promega). MRE- and ARE-driven activities were normalized to the luminescence of pRL-SV40.

Western blot analysis

Confluent cultures of bovine aortic endothelial cells treated with indicated compounds, were lysed in sodium dodecyl sulfate sample buffer (50 mM Tris-HCl buffer solution containing 2% sodium dodecyl sulfate and 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 iodoacetoamide 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/cm^2 for 1 h. 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 primary antibodies (1:200) 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 secondary antibodies (1:5,000) for 1 h 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 indicated compound. cDNA was synthesized using a High-Capacity cDNA Reverse Transcription kit. Real-time PCR was performed using Gene Ace SYBR qPCR Mixα 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 s 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 *MTF-1*, 5′-TAATGGTGATGCGGAGTC-3′ (forward) and 5′-TGCTGAGGCTGGTAGTAGA-3′ (reverse); bovine *MT-1A*, 5′-CACCTGCAAGGCCTGCAGA-3′ (forward) and 5′-CGAGGCCCCTTTGCAGACA-3′ (reverse); bovine *MT-1E*, 5′-CCAATTGCTCCTGCCCCACTA-3′ (forward) and 5′-CACACTTGGCACAGCCCACA-3′ (reverse); bovine *MT-2A*,

5′-GGCTCCTGCAAATGCAAAGAT-3′ (forward) and 5′-CCGAAGCCCCTTTGCAGAC-3′ (reverse); bovine *GAPDH*, 5′-AACACCCTCAAGATTGTCAGCAA-3′ (forward) and 5′-ACAGTCTTCTGGGTGGCAGTGA-3′ (reverse).

Keap1 and biotin-PEAC$_5$-maleimide-labeling assay *in vitro*

The mouse recombinant Keap1 construct was prepared as described previously (Miura et al., 2011; Toyama et al., 2013). The recombinant Keap1 protein was expressed as a C-terminal His-tagged fusion protein in BL21(DE3)pLysS *E. coli* cells and purified using a ProBond nickel-resin. The BPM-labeling assay was performed according to the method described by Toyama et al (2013). Briefly, mouse recombinant Keap1 protein (2 μg) was incubated with Cu10 (1, 10, or 100 μM) at 37°C for 30 min in 100 mM Tris-HCl buffer solution (pH 7.5). After incubation, 25 μM biotin-PEAC$_5$-maleimide was added to the samples and the samples were incubated at 37°C for 30 min. The samples were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel in 50 mM Tris-HCl containing 2% SDS, 8% glycerol, and 0.005% bromophenol blue (pH 6.8) without 2-mercaptoethanol and were incubated at 37°C for 30 min. They were then subjected to immnoblotting as described above.

Statistical analysis

The data was analyzed for statistical significance by Student's *t*-test when possible. *P* values less than 0.01 were considered statistically significant.

Chapter 1. Zinc diethyldithiocarbamate as an inducer of metallothionein in cultured vascular endothelial cells

1. 1. 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, $ZnSO_4$ 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.

1. 2. 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, 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 (Redtke 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 (Ohtsuji 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 et al., 1982), thereby prevent atherosclerosis (Harkar 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 synthesis and 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 (Fujiwara et al., 2005; Kimura et al., 2012; Murakami et al., 2015; Kohri et al., 2015). Therefore, the bio-elements strategy, i.e., bio-organometallics, appears to be an effective method for analysis of biological systems. Previously, we found that the zinc complex bis(L-cysteinato)zincate(II) [$Zn(cys)_2$] 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 $Zn(cys)_2$ 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(diethyldithiocarbamate) [$Zn(edtc)_2$] elevated *MT* mRNAs in bovine cells and increased MT protein expression by activating MRE promoter activity.

1. 3. 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 $ZnSO_4$. After a 24-h treatment with inorganic zinc, MT protein expression was not observed (Fig. 1-1A), consistent with our previous study (Kaji et al., 1992). Furthermore, $ZnSO_4$ did not alter the expression of *MT-1A*, *MT-1E*, or *MT-2A* mRNAs, MT subisoforms expressed in bovine tissue (Fig. 1-1B). The promoter activities of the MRE (Fig. 1-1C) and ARE (Fig. 1-1E) were unaffected by $ZnSO_4$; $ZnSO_4$ also failed to activate Nrf2 (Fig. 1-1D), 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 $ZnSO_4$ 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. 1-2A) did not increase the expression of MT proteins when used at a concentration of 100 μM or less for up to 48 h (Fig. 1-2B). Transcriptional induction of MT subisoforms was also unchanged by treatment with $Zn(cys)_2$ at 100 μM or less for 12 h (Fig. 1-2C), and exposure to 100 μM $Zn(cys)_2$ for up to 24 h did not upregulate *MT-1A*, *MT-1E*, or *MT-2A* mRNA levels (data not shown). However, although $Zn(cys)_2$ did not activate Nrf2 (Fig. 1-2E) and ARE promoter activity (Fig. 1-2F), MRE promoter activity was activated by the zinc complex (Fig. 1-2D). 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 $ZnSO_4$ 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 $ZnSO_4$ 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. 1-3A) increased the expression of MT proteins in a concentration-dependent manner when used at a concentration of 10 μM or less for 12 h (Fig. 1-3B, left panel); this increase was observed after 6 h or more when cells were treated with 10 μM $Zn(edtc)_2$ (Fig. 1-3B, 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 h (Fig. 1-3C). Moreover, induction of all three isoforms was noted after 3 h, with the most dramatic elevation observed after 6 h (data not shown). When used at a concentration of 10 μM or less, $Zn(edtc)_2$ elevated the promoter activity of the MRE (Fig. 1-3D) but failed to activate Nrf2 (Fig. 1-3 F) after 3 h. Additionally, Nrf2 was not activated by the zinc complex at 10 μM or less after up to 12 h, 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 ($ZnSO_4$) 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 (Fujie et al., 2016c).

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.

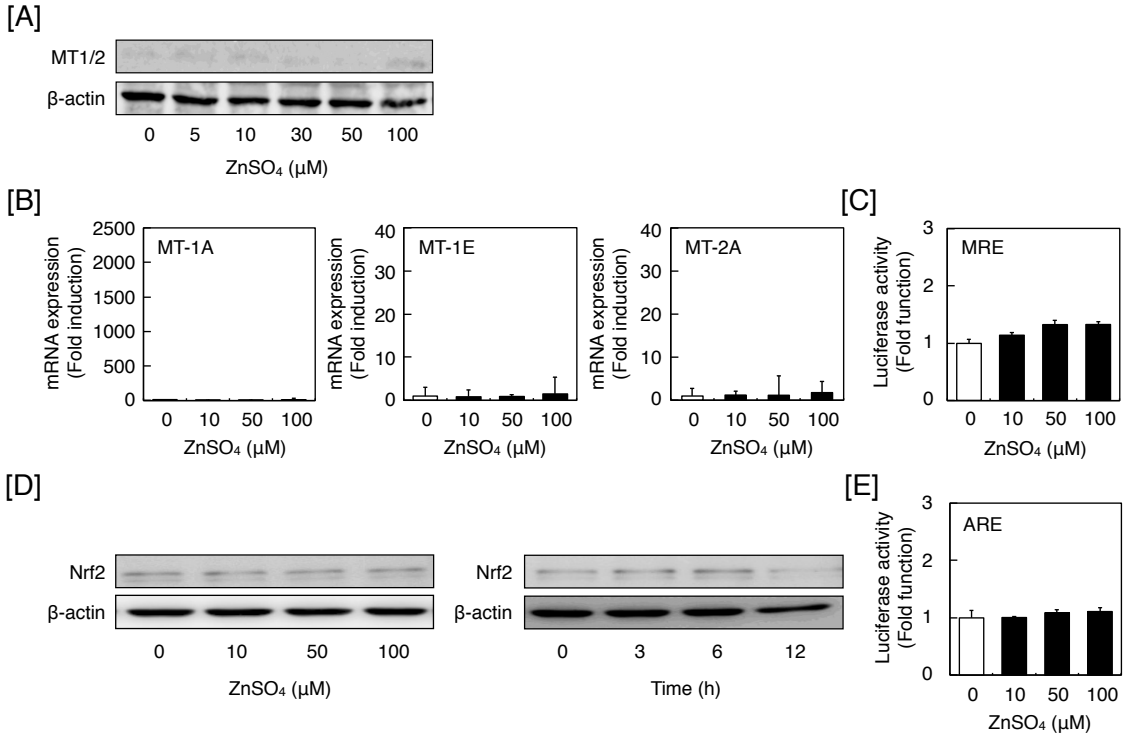

Fig. 1-1. Effects of $ZnSO_4$ on the induction of MT in vascular endothelial cells. [A] Expression of MT proteins after treatment with $ZnSO_4$ for 24 h. [B] Expression *MT-1A*, *MT-1E*, and *MT-2A* mRNAs after treatment with $ZnSO_4$ for 12 h. [C] Promoter activity of the MRE after treatment with $ZnSO_4$ for 12 h. [D] Activation of Nrf2 after treatment with $ZnSO_4$ for 6 h. [E] Promoter activity of the ARE after treatment with $ZnSO_4$ for 12 h.

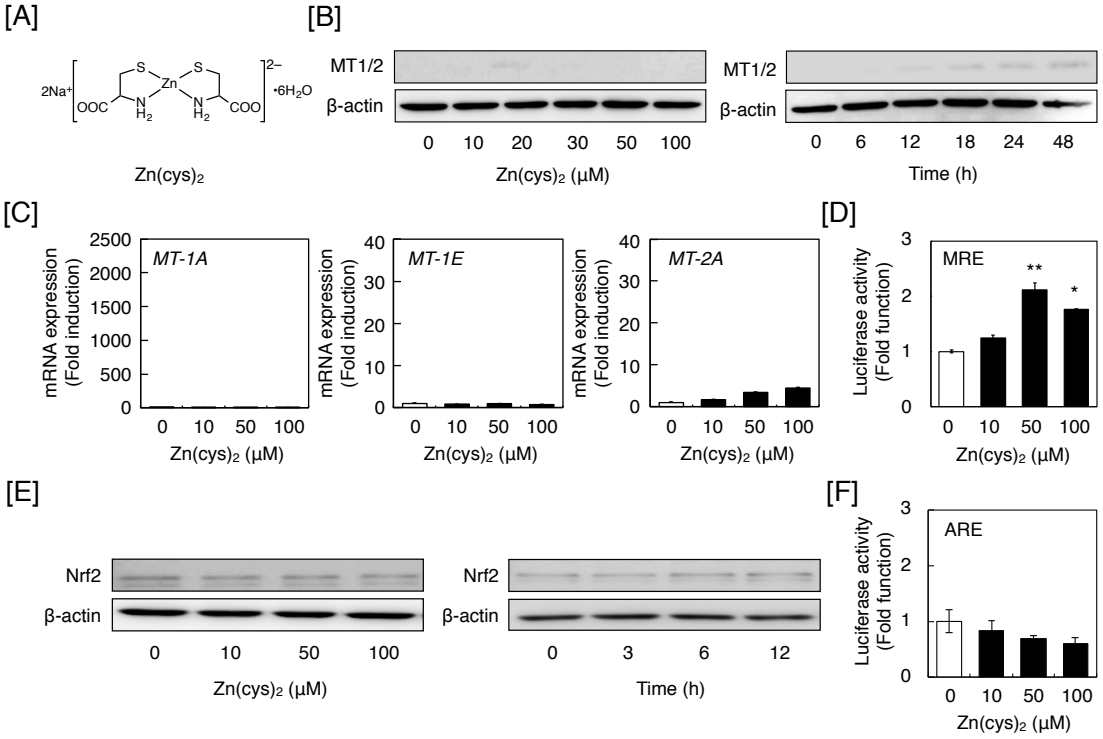

Fig. 1-2. Effects of $Zn(cys)_2$ on the induction of MT in vascular endothelial cells. [A] Structure of $Zn(cys)_2$. [B] Expression of MT proteins after treatment with $Zn(cys)_2$ at 10, 20, 30, 50, or 100 μM for 24 h (left panel) or at 100 μM for 6, 12, 18, 24, or 48 h (right panel). [C] Expression of *MT-1A*, *MT-1E*, and *MT-2A* mRNAs after treatment with $Zn(cys)_2$ for 12 h. [D] Promoter activity of MRE after treatment with $Zn(cys)_2$ for 6 h. [E] Activation of Nrf2 after treatment with $Zn(cys)_2$ at 10, 50, or 100 μM for 6 h (left panel) or at 100 μM for 3, 6, or 12 h (right panel). [F] Promoter activity of the ARE after treatment with $Zn(cys)_2$ for 6 h.

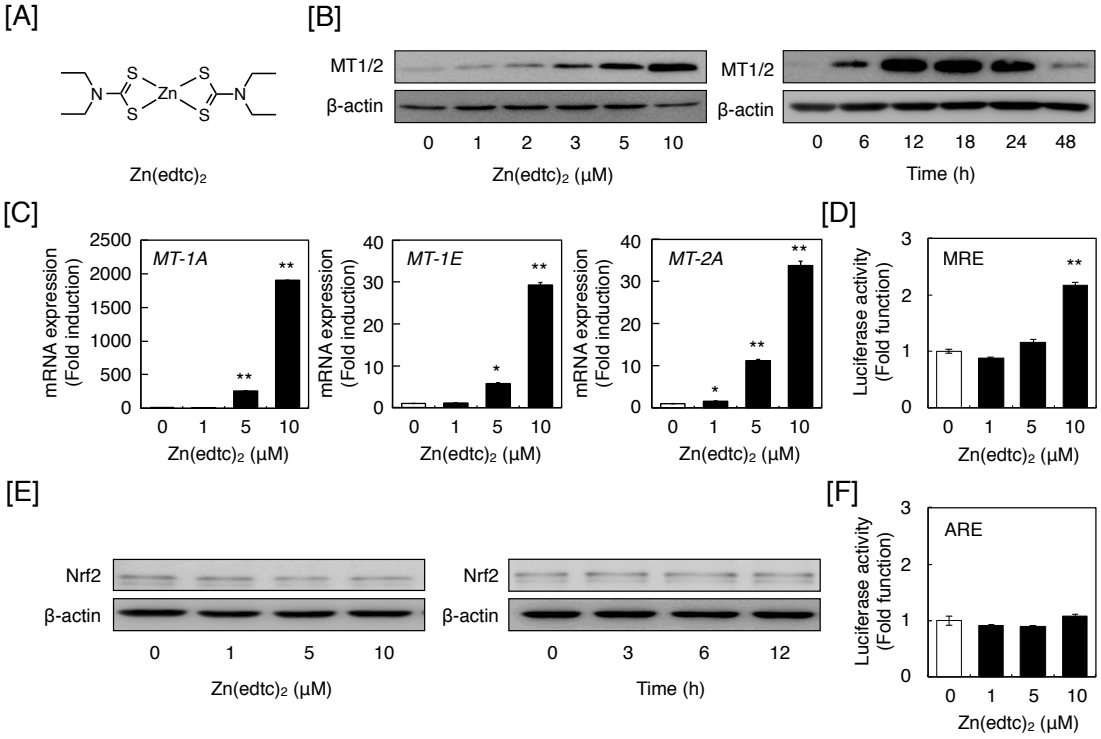

Fig. 1-3. Effects of $Zn(edtc)_2$ on the induction of MT in vascular endothelial cells. [A] Structure of $Zn(edtc)_2$. [B] Expression of MT proteins after treatment with $Zn(edtc)_2$ at 1, 2, 3, 5, or 10 μM for 12 h (left panel) or at 10 μM for 6, 12, 18, 24, or 48 h (right panel). [C] Expression of *MT-1A*, *MT-1E*, and *MT-2A* mRNAs after treatment with $Zn(cys)_2$ for 6 h. [D] Promoter activity of the MRE after treatment with $Zn(edtc)_2$ for 3 h. [E] Activation of Nrf2 after treatment with $Zn(edtc)_2$ at 1, 5, or 10 μM for 6 h (left panel) or at 10 μM for 3, 6, or 12 h (right panel). [F] Promoter activity of the ARE in the cells after treatment with $Zn(edtc)_2$ for 3 h.

Chapter 2. Cytotoxicity of organobismuth and organoantimony compounds

2. 1. The cytotoxicity of organobismuth compounds with certain molecular structures can be diminished by replaced the bismuth atom with an antimony atom in the molecules

2. 1. 1. Abstract

Organic-inorganic hybrid molecules, which are composed of an organic structure and metal(s), are indispensable for synthetic chemical reactions; however, their toxicity has been incompletely understood. In the present study, we discovered two cytotoxic organobismuth compounds whose cytotoxicity diminished upon replacement of the intramolecular bismuth atom with an antimony atom. The intracellular accumulation of the organobismuth compounds was much higher than that of the organoantimony compounds with the corresponding organic structures. We also showed that both the organic structure and bismuth atom are required for certain organobismuth compounds to exert their cytotoxic effect, suggesting that the cytotoxicity of such a compound is a result of an interaction between the organic structure and the bismuth atom. The present data suggest that organobismuth compounds with certain molecular structures exhibit cytotoxicity via an interaction between the molecular structure and the bismuth atom, and this cytotoxicity can be diminished by replacing the bismuth atom with an antimony atom, resulting in lower intracellular accumulation.

2. 1. 2. Introduction

Organic-inorganic hybrid molecules are composed of an organic structure and metal(s). Since the early work of pioneers such as Grignard and Wittig in using hybrid molecules as reagents for chemical synthetic reactions (Grignard, 1900; Wittig and Schöllkopf, 1954), these molecules have become indispensable for synthetic chemistry. However, the effects of incorporation of metal(s) into organic structures on biological systems have not been completely understood. An expansion of the biological applications of organic-inorganic hybrid molecules (bio-organometallics) is expected.

The toxicity of organic-inorganic hybrid molecules appears to depend on the interaction between the molecular structure and the intramolecular metal(s) as well as the intrinsic cytotoxicity of either the molecular structure or the metal(s). However, there have not been adequate model compounds to examine this hypothesis.

We investigated the cytotoxicity of organobismuth compounds using a cell culture system and found that tris(*N,N*-dimethylaminomethyl)phenylbismuthane exhibits selective cytotoxicity against vascular endothelial cells (Fujiwara et al., 2005). Recently, in our preliminary experiments, we found out that 2-(*N,N*-dimethylaminomethyl)phenylbis-(4-methylphenyl)bismuthane (DAPBi) was cytotoxic to vascular endothelial cells, but the cytotoxicity disappeared when the bismuth atom was replaced with an antimony atom (the organoantimony compound is named DAPSb). In addition, a similar relationship between *Bi*-phenyl-*N*-methyl-5,6,7,12-tetrahydro-dibenz[*c,f*][1,5]azabismocine (PMTABi) and *Sb*-phenyl-*N*-methyl-5,6,7,12-tetrahydro-dibenz[*c,f*][1,5]azastibocine (PMTAS) was observed in the cells.

In the present study, we investigated the relative cytotoxicity of DAPBi compared to DAPSb, and PMTABi compared to PMTAS in four cell types, including bovine aortic endothelial and smooth muscle cells, human fetal lung fibroblastic IMR-90 cells, and porcine

kidney epithelial LLC-PK_1 cells. In addition, intracellular accumulation of the organobismuth and organoantimony compounds was measured to examine whether the difference in the cytotoxicity between these compounds is owing to differences in their intracellular accumulation.

2. 1. 3. Results

Morphological observation (Fig. 2-1-2A) showed that DAPBi exhibited cytotoxicity in vascular endothelial and smooth muscle cells, fibroblastic IMR-90 cells, and epithelial LLC-PK_1 cells in a dose-dependent manner. In contrast, DAPSb did not show such cytotoxicity in these four cell types. DAPBi accumulated within these cell types, whereas the accumulation of DAPSb was very low in each cell type tested (Fig. 2-1-2B).

Fig. 2-1-3 shows the morphological observation of the four cell types after exposure to PMTABi or PMTAS and the intracellular accumulation of the organometallic compounds; similar to our previous observations, the organobismuth compound, PMTABi, accumulated extensively within the cells and exhibited cytotoxicity, whereas the organoantimony compound, PMTAS, accumulated to a lesser extent and demonstrated no cytotoxicity.

In order to examine whether the cytotoxicity of organobismuth compounds depends on the organic structure, bismuth atom, or interaction between the molecular structure and bismuth atom, the cytotoxicity of PMTABi was compared to dibenzylmethylamine, a compound in which the bridge containing the bismuth atom was removed, triphenyl bismuth, a compound in which the bridge containing the nitrogen atom was eliminated, and bismuth nitrate, an inorganic bismuth (Fig. 2-1-4). Among the compounds tested, only PMTABi exhibited cytotoxicity, suggesting that the cytotoxicity of the organobismuth compound requires the complete structure of the molecule.

2. 1. 4. Discussion

Although the toxicity of organic and inorganic compounds has been well investigated, the toxicity of organic-inorganic hybrid molecules has not been completely understood. In general, the toxicity of the hybrid molecules was believed to depend on the metal(s) rather than the organic molecular structure, even though this assertion is without reliable evidence. In spite of the knowledge that the cytotoxicity of inorganic bismuth compounds is low (Serfontein and Mekel, 1979) while that of inorganic antimony is high (Mann *et al.*, 2006), the present data demonstrated that there are cases where organobismuth compounds are cytotoxic but organoantimony compounds with the same organic structure are nontoxic. This suggests that the cytotoxicity of hybrid molecules does not necessarily depend on either the organic structure or metal atom(s) in the molecules.

The mechanisms underlying the toxicity of hybrid molecules appear to be complex. We have shown that PMTABi is toxic to vascular endothelial cells, but the structural components of the organobismuth compound by themselves are nontoxic, suggesting that the interaction of bismuth with the organic structure is important for the cytotoxicity of PMTABi. It is most likely that the difference in cytotoxicity between organic and inorganic bismuth compounds, and among the various organobismuth compounds, is based on this interaction. Not only chemical and biological approaches, but also physical approaches such as electronic state analysis may be required for understanding this interaction.

The cytotoxicity of DAPBi and PMTABi was much stronger than that of DAPSb and PMTABi, respectively, in all cell types tested, suggesting that the cytotoxicity of organobismuth compounds is independent of cell type and can be diminished by replacing the bismuth atom with an antimony atom. There may be common mechanisms underlying the cytotoxicity of organobismuth compounds. On the other hand, DAPBi and PMTABi accumulated within the cells whereas the accumulation of DAPSb and PMTABi was very less, suggesting that there may be a transport system for organobismuth compounds, which cannot

or very slightly, transport organoantimony compounds into the cells. Recently, transporters for divalent cations, including zinc, copper, manganese, iron, and cadmium have been identified (Gaither and Eide, 2001; Mackenzie *et al.*, 2007; Kambe, 2011) however, it is unlikely that there are selective transporters for organobismuth compounds. A biomolecule internalization system that has a high affinity for organobismuth compounds with a certain molecular structure may be involved in the transport of organobismuth compounds. Replacement of the bismuth atom with an antimony atom may result in lowering this affinity.

The present study elucidated several aspects of organobismuth compounds' cytotoxicity. Organobismuth compounds with certain molecular structures were shown to be cytotoxic, and this cytotoxicity can be diminished by replacing the bismuth atom with an antimony atom. This phenomenon was observed in various cell types including vascular endothelial and smooth muscle cells, fibroblastic cells, and epithelial cells. The intracellular accumulation of organobismuth compounds with certain molecular structures is high whereas that of organoantimony compounds with the corresponding organic structures is very low; this difference appears to be one of the mechanisms underlying the stronger cytotoxicity of the organobismuth compounds compared to the organoantimony compounds. Finally, we showed that the complete structure is required for organobismuth compounds to exhibit cytotoxicity; the interaction between the organic structure and the bismuth atom may be important for cytotoxicity. Although it is postulated that the cytotoxicity of hybrid molecules is dependent on the intramolecular metal(s), organic structure, or interaction between the metal(s) and the structure, further studies will be required to fully understand the toxicity of organic-inorganic hybrid molecules.

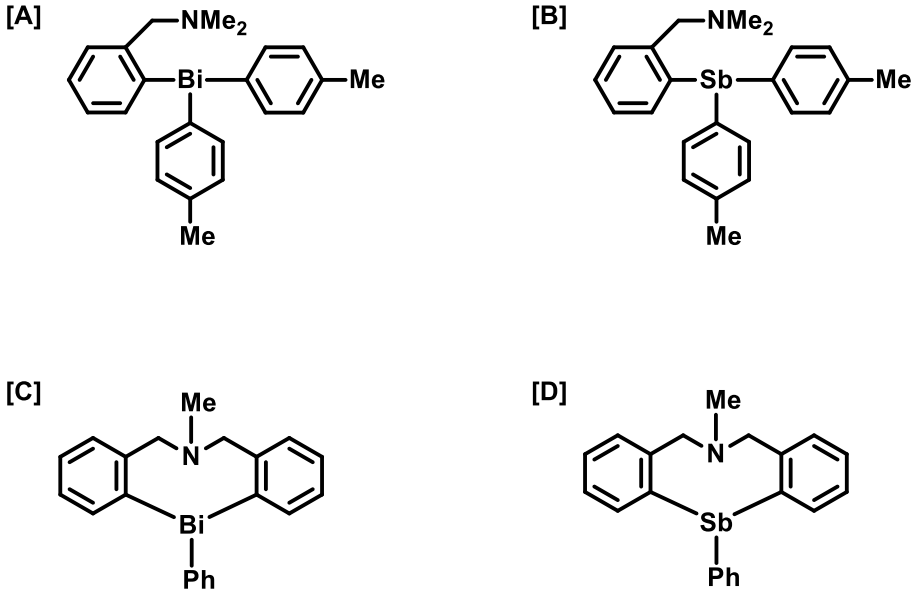

Fig. 2-1-1. Structures of organobismuth and organoantimony compounds. [A]: 2-(*N,N*-dimethylaminomethyl)phenylbis(4-methylphenyl)bismuthane (DAPBi); [B]: 2-(*N,N*-dimethylaminomethyl)phenylbis(4-methylphenyl)stibane (DAPSb); [C]: *Bi*-phenyl-*N*-methyl-5,6,7,12-tetrahydrodibenz[*c,f*][1,5]azabismocine (PMTABi); [D]: *Sb*-phenyl-*N*-methyl-5,6,7,12-tetrahydrodibenz[*c,f*][1,5]azastibocine (PMTAS).

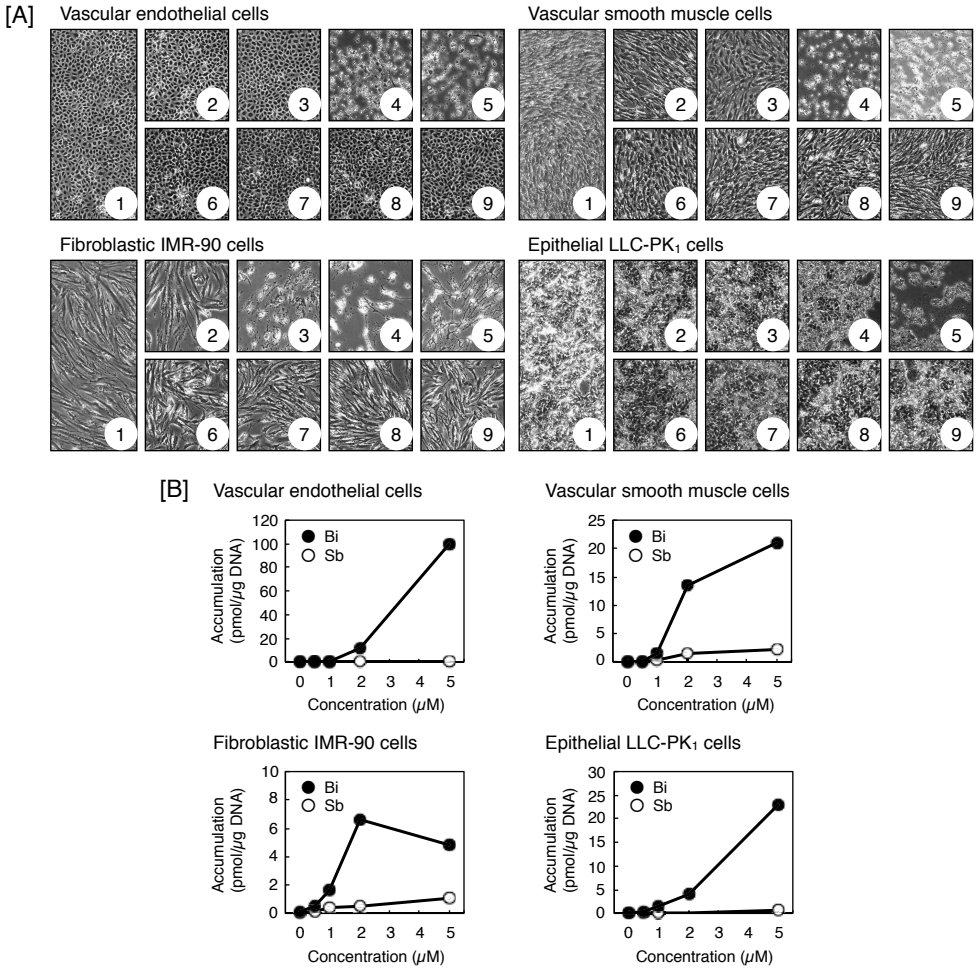

Fig. 2-1-2. Cytotoxicity and intracellular accumulation of DAPBi and DAPSb in vascular endothelial cells, vascular smooth muscle cells, fibroblastic IMR-90 cells, and epithelial LLC-PK_1 cells. Bovine aortic endothelial and smooth muscle cells, human fetal lung fibroblastic IMR-90 cells, and porcine kidney epithelial LLC-PK_1 cells were treated with DAPBi or DAPSb (0.5, 1, 2, and 5 μM) for 24 h. [A]: Morphological appearance after treatment with DAPBi or DAPSb. 1: Control; 2: DAPBi (0.5 μM); 3: DAPBi (1 μM); 4: DAPBi (2 μM); 5: DAPBi (5 μM); 6: DAPSb (0.5 μM); 7: DAPSb (1 μM); 8: DAPSb (2 μM); 9: DAPSb (5 μM). Intracellular accumulation of DAPBi and DAPSb. ●, DAPBi; ○, DAPSb

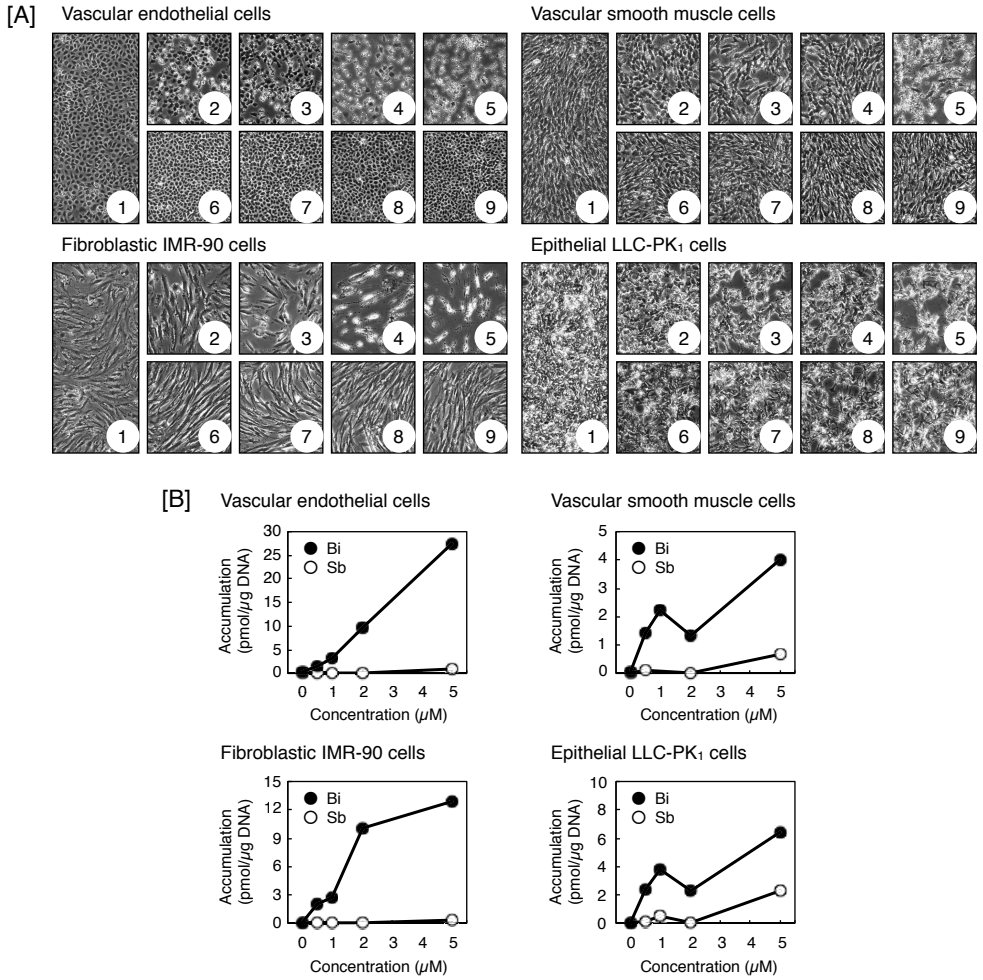

Fig. 2-1-3. Cytotoxicity and intracellular accumulation of PMTABi and PMTAS in vascular endothelial cells, vascular smooth muscle cells, fibroblastic IMR-90 cells, and epithelial LLC-PK_1 cells. Bovine aortic endothelial and smooth muscle cells, human fetal lung fibroblastic IMR-90 cells, and porcine kidney epithelial LLC-PK_1 cells were treated with PMTABi or PMTAS (0.5, 1, 2, and 5 μM) for 24 h. [A]: Morphological appearance after treatment with PMTABi or PMTAS. 1: Control; 2: PMTABi (0.5 μM); 3: PMTABi (1 μM); 4: PMTABi (2 μM); 5: PMTABi (5 μM); 6: PMTAS (0.5 μM); 7: PMTAS (1 μM); 8: PMTAS (2 μM); 9: PMTAS (5 μM). [B] Intracellular accumulation of PMTABi and PMTAS. ●, PMTABi; ○, PMTAS.

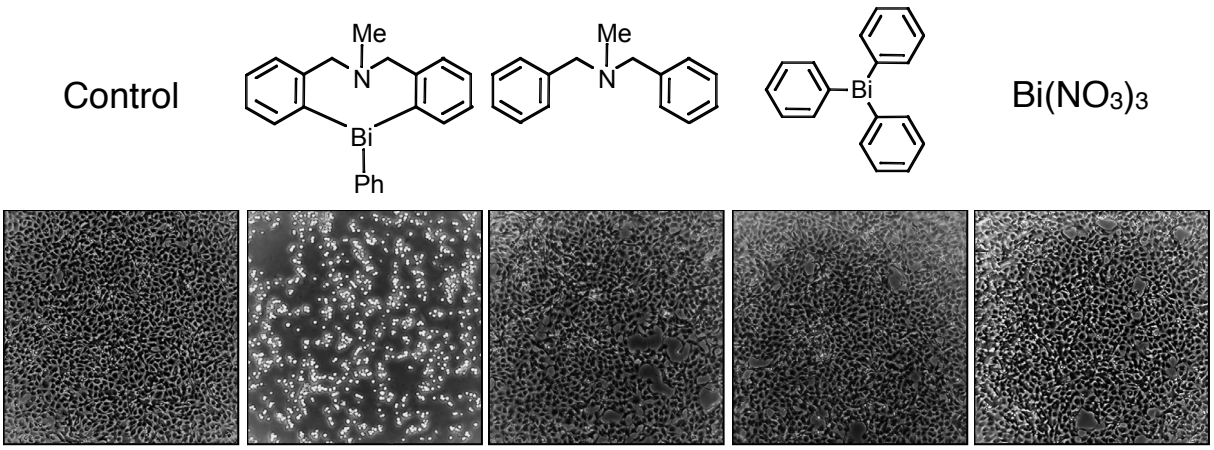

Fig. 2-1-4. Cytotoxicity of PMTABi, dibenzylmethylamine, triphenyl bismuth, and bismuth nitrate in vascular endothelial cells. Bovine aortic endothelial cells were treated with PMTABi, dibenzylmethylamine, triphenyl bismuth, or bismuth nitrate (10 μM each) for 24 h.

2. 2. Comparative cytotoxicity of triphenylstibane and fluorine- substituted triarylpnictogens in cultured vascular endothelial cells

2. 2. 1. Abstract

The toxicity of organic-inorganic hybrid molecules appears to depend on the toxicity of the organic structure, the metals, and their interaction. However, very little is known about the structure-activity relationship of these molecules. In the present study, we investigated the cytotoxicity of triphenylstibane (Sb25) and its fluorine-substituted derivatives the triarylstibanes, using a culture system of bovine aortic endothelial cells. The results showed that the cytotoxicity of tris(4-fluorophenyl)stibane (Sb33) and tris(3,4,5-trifluorophenyl)-stibane (Sb49) was higher than that of Sb25, suggesting that introduction of fluorine atoms into the benzene rings may potentiate the cytotoxicity of Sb25 in vascular endothelial cells. However, interestingly, tris(pentafluorophenyl)stibane (Sb35) was nontoxic. The pnictogen analogues tris(pentafluorophenyl)arsane (As35) and tris(pentafluorophenyl)phosphane (P35) showed a higher cytotoxicity than that of Sb35. In addition, the potentiation was much stronger with P35 than it was with As35. The intracellular accumulation of Sb35 was very low while the accumulation of As35 was higher than that of Sb25. These results collectively suggest that the hydrophobicity and metal of the organometallic compounds do not necessarily predict their cytotoxicity and intracellular accumulation in vascular endothelial cells.

2. 2. 2. Introduction

Organic-inorganic hybrid molecules are composed of an organic structure and metal(s). Although the molecules are widely used in synthetic chemical reactions, very little is known about their biological activities. Recently, we found that organobismuth compounds with certain organic structures exhibit cytotoxicity, which can be diminished by replacing the bismuth atom with an antimony atom (Kohri *et al.*, 2015). This suggests that the toxicological theory of organic and inorganic compounds cannot be applied to predict the cytotoxicity of organometallic compounds, in the more complex cases. A new toxicological approach to the biology of organic-inorganic hybrid molecules (bio-organometallics) may be required to enhance our understanding.

Antimony is a pnictogen element that is no longer used as an ingredient of pigments because of its irritant effects (Iavicoli *et al.*, 2006). After that, inorganic antimonial compounds such as sodium stibogluconate and meglumine antimoniate were medically used to treat parasitic infections such as leishmaniasis (Wyllie *et al.*, 2004). Antimony and arsenic belong to the same group of elements in the periodic table; however, the cytotoxicity of inorganic arsenic has been investigated more than that of inorganic antimony. Little is known about the cytotoxicity of organoantimony compounds, despite the fact that their biological activities have been elucidated (Jiang *et al.*, 2013; Chen *et al.*, 2014; Islam *et al.*, 2014).

In the present study, we investigated the cytotoxicity and intracellular accumulation of triphenylstibane (Sb25) and its fluorine-substituted triarylstibanes in vascular endothelial cells to determine their structure-activity relationships. In addition, the cytotoxicity and intracellular accumulation of tris(pentafluorophenyl)stibane (Sb35) were also compared with those of tris(pentafluorophenyl)arsane (As35) and tris(pentafluorophenyl)phosphane (P35). The results indicate that the hydrophobicity and metal of these compounds do not necessarily predict their cytotoxicity and intracellular accumulation in vascular endothelial cells.

2. 2. 3. Results and Discussion

Figs. 2-2-2 and 2-2-3 show the morphological appearance of and LDH leakage, respectively in vascular endothelial cells after treatment with Sb25, Sb33, Sb49, Sb35, As35, or P35 for 24 h. The cell damage evident from the morphological appearance was almost consistent with the leakage of LDH from the cells. The concentration of Sb49 that induced cell damage was 10 μM, which was lower than that of Sb25 and Sb33 (30 μM each). In addition, the cell damage caused by Sb33 was more severe than that caused by Sb25. These results indicate that the cytotoxicity of triarylstibanes might depend on the degree of fluorination of the benzene rings. However, Sb35, which has the most highly fluorinated benzene rings, did not exhibit cytotoxicity at 100 μM and less. Therefore, it is suggested that the cytotoxicity of triphenylstibane in vascular endothelial cells is influenced by fluorination of the benzene rings, but this does not necessarily correlate to the fluorination degree. When either an arsenic or a phosphorus atom replaced the antimony atom of Sb35, the cytotoxicity was intensified in the vascular endothelial cells, and the cytotoxicity of P35 was stronger than that of As35. Phosphorus is an essential element whereas inorganic arsenic is toxic and not beneficial. The cytotoxicity of an organometallic compound cannot be easily predicted from the cytotoxicity of the inorganic intramolecular metal, particularly in vascular endothelial cells.

The intracellular accumulation of Sb25, Sb33, Sb49, Sb35, As35, and P35 are shown in Fig. 4. Although triarylstibanes (Sb25, Sb33, Sb49, and Sb35) did not accumulate at concentrations of 10 μM and less, a higher accumulation of Sb25, Sb33, and Sb49 but not Sb35 was observed at 30 μM. The lower accumulation of Sb35 was consistent with the lower cytotoxicity of this triarylstibane. However, the degree of cytotoxicity of Sb25, Sb33, and Sb49 (Figs. 2-2-2 and 2-2-3) did not depend on the intracellular accumulation of these compounds (Fig. 2-2-4). The degree of fluorination of the benzene rings may influence the binding of these compounds to their molecular targets rather than their uptake in vascular

endothelial cells.

Hydrophobicity is one of the important characteristics that determine the toxicity of organic compounds. The cellular uptake of compounds with higher hydrophobicity is easier because of passive transport, and this results in higher intracellular accumulation and consequently higher toxicity. However, the principle that higher hydrophobicity results in higher intracellular accumulation and cytotoxicity did not appear to apply to Sb25, Sb33, Sb49, and Sb35. Since the intracellular accumulation of the organobismuth compounds can be much higher than that of the organoantimony compounds with the same organic structure (Kohri *et al.*, 2015), it is most likely that the intracellular accumulation of triphenylstibane and its related compounds is not necessarily predictable by the molecular characteristics including hydrophobicity.

Therefore, the present data shows that the hydrophobicity and the intramolecular metal of the molecules do not necessarily predict the cytotoxicity and intracellular accumulation of organometallic compounds in vascular endothelial cells. Further studies are required for a complete elucidation of the cytotoxicity of organometallic compounds.

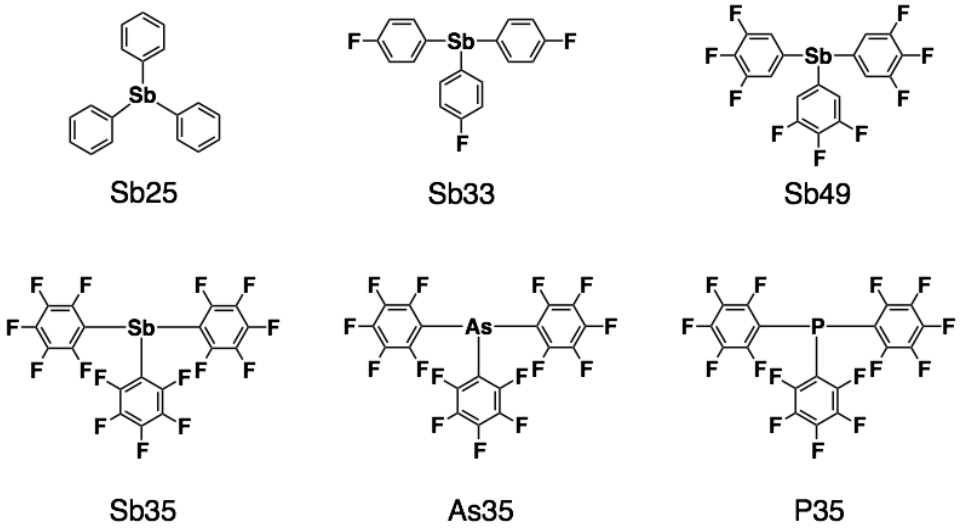

Fig. 2-2-1. Structures of the triphenylstibane and fluorine-substituted triarylpnictogens used in this study. Sb25, triphenylstibane; Sb33, tris(4-fluorophenyl)stibane; Sb49, tris(3,4,5-trifluorophenyl)stibane; Sb35, tris(pentafluorophenyl)stibane; As35, tris(pentafluorophenyl)-arsane; P35, tris(pentafluorophenyl)phosphane).

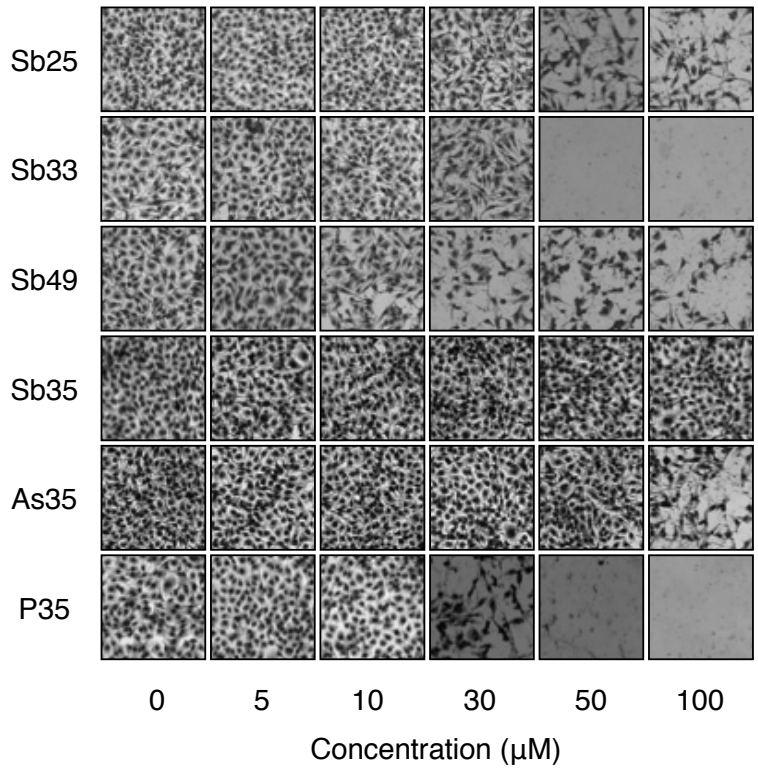

Fig. 2-2-2. Morphological appearance of vascular endothelial cells after treatment with triphenylstibane and fluorine-substituted triarylpnictogens. Bovine aortic endothelial cells were treated with Sb25, Sb33, Sb49, Sb35, As35, or P35 (5, 10, 30, 50, and 100 μM each) for 24 h.

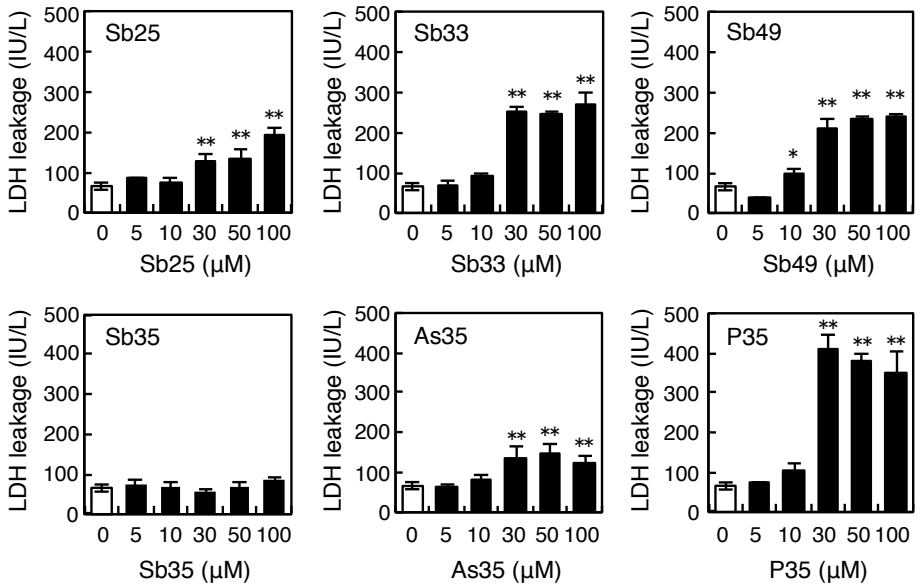

Fig. 2-2-3. The leakage of lactate dehydrogenase (LDH) from vascular endothelial cells after treatment with triphenylstibane and fluorine-substituted triarylpnictogens. Bovine aortic endothelial cells were treated with Sb25, Sb33, Sb49, Sb35, As35, or P35 (5, 10, 30, 50, and 100 μM each) for 24 h. Values are means ± SE of four samples. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control. Sb25, triphenylstibane; Sb33, tris(4-fluorophenyl)stibane; Sb49, tris(3,4,5-trifluorophenyl)stibane; Sb35, tris(pentafluorophenyl)stibane; As35, tris(pentafluorophenyl)arsane; P35, tris(pentafluorophenyl)phosphane.

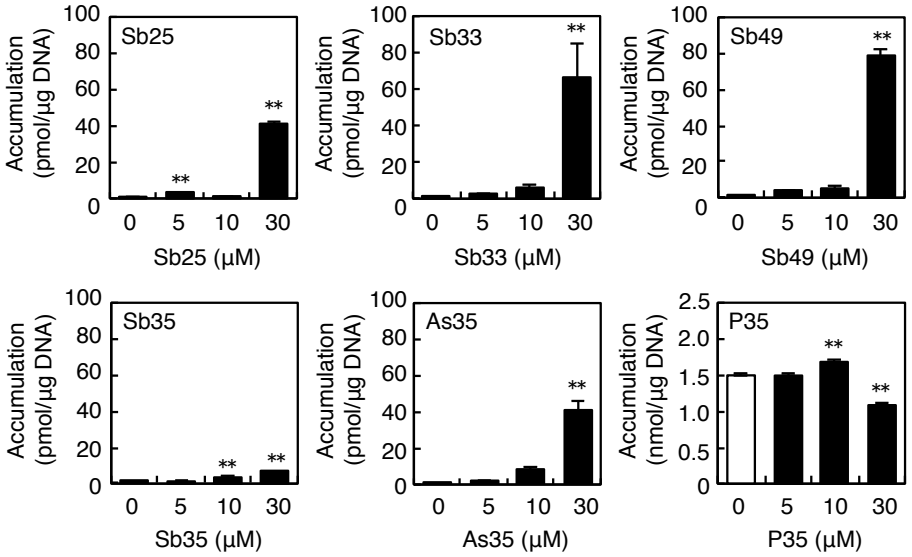

Fig. 2-2-4. The intracellular accumulation of triphenylstibane and fluorine-substituted triarylpnictogens in vascular endothelial cells. Bovine aortic endothelial cells were treated with Sb25, Sb33, Sb49, Sb35, As35, or P35 (5, 10, 30, 50, and 100 μM each) for 24 h. Values are means ± SE of three samples. $^{**}P < 0.01$ compared with control. Sb25, triphenylstibane; Sb33, tris(4-fluorophenyl)stibane; Sb49, tris(3,4,5-trifluorophenyl)stibane; Sb35, tris(pentafluorophenyl)stibane; As35, tris(pentafluorophenyl)arsane; P35, tris(pentafluorophenyl)-phosphane.

Chapter 3. Transcriptional induction of metallothionein by tris(pentafluorophenyl)stibane in cultured vascular endothelial cells

3. 1. Abstract

Vascular endothelial cells cover the luminal surface of blood vessels and contribute to the prevention of vascular disorders such as atherosclerosis. Metallothionein (MT) is a low molecular weight, cysteine-rich, metal containing, inducible protein, which protects cells from the toxicity of heavy metals and active oxygen species. Inorganic zinc does not induce MT in vascular endothelial cells. Adequate tools are required to investigate the mechanisms underlying endothelial MT induction. In the present study, we found that an organoantimony compound, tris(pentafluorophenyl)stibane, induces gene expression of MT-1A, MT-1E, and MT-2A, which are subisoforms of MT in bovine aortic endothelial cells. The data reveal that MT-1A/E is induced by activation of both the MTF-1–MRE and Nrf2–ARE pathways, whereas MT-2A expression requires only activation of the MTF-1–MRE pathway. These results suggest the original role of MT-1 is to protect cells from heavy metal toxicity and oxidative stress in the biological defense system, while that of MT-2 is to mainly regulates intracelluar zinc metabolism.

3. 2. Introduction

Metallothionein (MT) is a low molecular weight, cysteine-rich, metal-containing, inducible protein, which was found as a protein containing cadmium and zinc from equine renal cortex (Margoshes and Vallee, 1957). There are four isoforms of MT —MT-1, MT-2, MT-3, and MT-4— (Quaife et al., 1994), and MT-1 consists of several subisoforms: seven in human tissue but only two —MT-1A and MT-1E— in bovine tissue. Among MT isoforms, MT-3 and MT-4 exist in specific tissue: MT-3 is in the neural tissue (Yeiser et al., 1999) and MT-4 is in stratified squamous epithelia (Quaife et al., 1994). MT-1 and MT-2 ubiquitously exist in the liver, kidney, and other organs and are induced by heavy metals such as cadmium and zinc, oxidative stress, and other physiological factors including cytokines and growth factors (Kägi, 1991; Sato and Bremner, 1993). MT-1 and MT-2 are considered to have the same functions and induction mechanisms (Searle et al., 1984; Yagle et al., 1985). However, several reports show the induction level is different between MT-1 and MT-2 (Kobayashi and Sayato-Suzuki, 1988; Kershaw et al., 1990; Jahroudi et al., 1990; Cavigelli et al., 1993; Somji et al., 2001; Lim et al., 2006), suggesting that these MT isoforms may be induced differently.

Mechanisms underlying transcriptional activation of MT genes are not completely understood. Metal-responsive transcriptional factor 1 (MTF-1) mediates heavy metal signaling and is essentially required for MT gene expression (Hauchel et al., 1994). MTF-1 is activated by zinc and binds the sequences termed metal responsive element (MRE) that exist in the upstream region of the MT gene (Stuart et al., 1985; Culotta and Hamer, 1989). Additionally, the MT gene includes sequences termed antioxidant response element (ARE) in the promoter region (Palmiter, 1987), which are activated by the transcriptional factor nuclear factor-erythroid 2-related factor 2 (Nrf2) and regulate the expression of antioxidant genes (Ohtsuji et al., 2008). Although ARE is involved in the transcriptional activation of MT genes by hydrogen peroxide (Dalton et al., 1994), there is little information about the role of ARE in

the MT induction. We hypothesize that the MTF-1–MRE and Nrf2–ARE pathways cooperatively regulate transcription of MT.

Vascular endothelial cells cover the luminal surface of blood vessels and prevent vascular disorders such as atherosclerosis by regulating the blood coagulation-fibrinolytic system and vascular tonus (Harker et al., 1981). Since MT protects cells from heavy metal toxicity (Kägi and Schaffer, 1988) and oxidative stress (Thornalley and Vasák, 1985), MT is considered a multifunctional protein involved in defense mechanisms. We previously studied the toxicity of heavy metals in cell culture vascular endothelial cells and found that cadmium induces MT in vascular endothelial cells and in other cell types whereas zinc— a representative MT inducer— does not induce MT in vascular endothelial cells (Kaji et al., 1992). In addition, endothelial MT is not induced only by activation of the MTF-1–MRE pathway (Fujie et al., 2016b). Therefore, inorganic zinc is not a good tool to clarify the mechanisms underlying endothelial MT induction.

Organic-inorganic hybrid molecules are composed of an organic structure and metal(s) and are in general used as reagents in chemical synthetic reactions, since pioneers such as Grignard and Wittig used the molecules as organic synthesis reagents (Grignard, 1900; Wittig and Schöllkopf, 1954). Studies on organopnictogen compounds, a type of organic-inorganic hybrid molecules, indicate that their cytotoxicity depends on intracellular accumulation and is influenced by intramolecular metal(s), organic structure, and the interaction between the metal(s) and the structure (Kohri et al., 2015; Murakami et al., 2015). Bio-organometallics, the biological study of organic-inorganic hybrid molecules may be used to analyze the mechanisms underlying endothelial MT induction. In the present study, we constructed a library of 28 organoantimony compounds and tested the effect on transcriptional activation of MT genes and found that tris(pentafluorophenyl)stibane (termed Sb35) induces the gene expression of the subisoforms MT-1A, MT-1E, and MT-2A in bovine aortic endothelial cells. We analyzed the intracellular pathways involved in endothelial MT induction using Sb35.

3. 3. Results

Transcriptional induction of MT isoforms by Sb35

First, we constructed a library of 28 organoantimony compounds (Table 1). In human brain microvascular endothelial cells, we tested the induction of expression of MT-1X—the major MT isoforms in HeLa cells (Miura and Koizumi, 2007). As shown in Fig. 3-1A, it was found that Sb35 induces high MT-1X gene expression.

Fig. 3-1B depicts the locations of MREs and AREs in the upstream regions of MT genes of the bovine cells. Bovine cells express three MT subisoforms—MT-1A, MT-1E, and MT-2A—and each of their genes has MRE and ARE consensus sequences in the promoter region. In bovine aortic endothelial cells, Sb35 (Fig. 3-2A) induced MT-1A and MT-2A gene expression in a concentration-dependent manner when treated for 12 h (Fig. 3-2B, upper panels). We observed maximum induction of MT-1A and MT-2A by 100 μM Sb35 at 12 and 24 h, respectively (Fig. 3-2B, lower panels), indicating that Sb35 stimulates the transcriptional induction of MT-1 and MT-2 isoform genes in the cells. However, induction of MT protein by Sb35 was not observed in western blot analysis (not shown), suggesting that Sb35 compound is a tool to analyze the transcriptional induction of endothelial MT.

Involvement of the MTF-1–MRE pathway

We investigated the involvement of the MTF-1–MRE pathway in the transcriptional induction of MT isoforms by Sb35. Sb35 only slightly increased the MRE-driven transcriptional activity in a concentration-dependent manner (Fig. 3-3A). In the MTF-1 knockdown (Fig. 3-3B), Sb35-induced expression of MT-1A and MT-2A was suppressed (Fig. 3-3C). Sb35 did not induce the transcription of MT-1E, although siRNA-mediated knockdown of MTF-1 suppressed the constitutive expression. As reported previously (Gunther et al., 2012), the MTF-1–MRE pathway plays a central role in Sb35 induction of endothelial MT-1A and MT-2A.

Involvement of the Nrf2–ARE pathway

Sb35 increased the intracellular accumulation of Nrf2 in a concentration- and time-dependent manner and upregulated Nrf2 target proteins such as heme oxygenase-1 (HO-1) and glutamate-cysteine ligase, modifier subunit (GCLM) (Fig. 3-4A). Sb35 significantly increased the ARE-driven transcriptional activity in a concentration-dependent manner (Fig. 3-4B), confirming that it activates the Nrf2–ARE pathway in vascular endothelial cells. It was shown that Sb35 exhibited proteasome inhibitory activity in a concentration-dependent manner (Fig. 3-4C), suggesting that activation of Nrf2 by Sb35 is at least partly due to the proteasome inhibition that stabilizes Nrf2.

Nrf2 knockdown (Fig. 3-5A) resulted in a markedly lower expression of MT-1A in the presence of Sb35 (Fig. 3-5B, upper panel). In contrast, MT-2A expression in the Nrf2 knockdown and in the presence of Sb35 was unaffected (Fig. 3-5B, lower panel). Sb35 did not induce the transcription of MT-1E (Fig. 3-5B, middle panel) Thus, the transcriptional induction of MT-1A is regulated by both the MTF-1–MRE and Nrf2–ARE pathways whereas that of MT-2A is stimulated by only the MTF-1–MRE pathway in vascular endothelial cells.

Determination of the pathway involved in the transcriptional induction of endothelial MT using tris(pentafluorophenyl)phosphane (P35)

We next investigated the effects of pnictogen analogues —tris(pentafluorophenyl)arsane (As35) and tris(pentafluorophenyl)phosphane (P35) (Fig. 3-6A)— on the transcriptional induction of MT-1A, MT-1E, and MT-2A in bovine aortic endothelial cells. As35 significantly increased MT-1A and MT-2A expression similar to Sb35 (Fig. 3-6B, upper panels). The transcriptional induction of MT-1A and MT-1E by P35 was very weak; however, P35 strongly induced transcription of MT-2A (Fig. 3-6B, lower panels).

Therefore, we investigated the involvement of the MTF-1–MRE and Nrf2–ARE pathways in

the transcriptional induction of MT-2A by P35. P35 significantly increased MRE-driven transcription in a concentration-dependent manner in vascular endothelial cells (Fig. 3-7A). The transcriptional induction of MT-2A by P35 was suppressed in an siRNA-mediated knockdown of MTF-1 (Fig. 3-7B).

Similarly, P35 activated Nrf2 (Fig. 3-8A) and significantly increased ARE-driven transcription in a concentration-dependent manner (Fig. 3-8B). The transcriptional induction of MT-2A by P35 was, however, unaffected in an siRNA-mediated knockdown of Nrf2 (Fig. 3-8C), indicating that the Nrf2–ARE pathway does not transactivate MT-2A induction.

3. 4. Discussion

MT protects cells from heavy metal toxicity and oxidative stress; however, the mechanisms underlying MT induction are not fully understood. Vascular endothelial cells regulate the blood coagulation-fibrinolytic system and vascular tone while MT likely protects the cells from functional damage, thereby preventing vascular disorders. Since zinc is a weak inducer of endothelial MT synthesis, we used a library of organoantimony compounds to analyze the mechanisms of endothelial MT induction. We found that the organoantimony compound Sb35 causes transcriptional induction of MT isoforms —MT-1A and MT-2A— in bovine aortic endothelial cells. Among these MT isoforms, the transcriptional induction of MT-1A is regulated by either the MTF-1–MRE or Nrf2–ARE pathways whereas only the MTF-1–MRE pathway regulates MT-2A expression. Since sulforaphane, an Nrf2 activator, weakly induces MT isoform expression (data not shown), the MTF-1–MRE pathway is likely essential for transcriptional induction of all endothelial MT isoforms while the Nrf2–ARE pathway enhances the induction of MT-1A isoform by MTF-1. However, the increase in MRE-driven promoter activity by Sb35 was not so marked. Although the details are not clear, an assumption can be made that Sb35 may activate other regulatory pathways that induce transcription genes (Katsutani et al., 1998) in vascular endothelial cells. Since Nrf2 is a transcriptional factor that induces the expression of antioxidant proteins such as HO-1 (Alam et al., 1999) and GCLM (Erickson et al., 2002), the original role of MT-1A may be induced as a cytoprotection against oxidative damage by injury or inflammation. Alternatively, that of MT-2A may be to regulate zinc metabolism. Specifically, MT-1 and MT-2 do not share these roles but the original roles may be different. Involvement of the Nrf2–ARE pathway in the transcriptional induction of MT-2A but not MT-1A is a new finding of the present study.

Recently, we found that the Nrf2–ARE pathway is involved in the cadmium mediated induction of endothelial MT isoforms including MT-1E and MT-2A (Shinkai et al., 2016). Cadmium modifies Kelch-like ECT-associated protein 1 (Keap1), a negative regulator of Nrf2,

and activates Nrf2, which is recruited to ARE1 and ARE5 of the promoter region of endothelial MT-2A. However, induction of both MT-2A and MT-1E is reinforced by ARE activation. Since MT-1E is located next to MT-2A in the genome, we speculated that recruitment of Nrf2 to the AREs of the MT-2 promoter region stimulates the transcriptional activity of MT-1 and MT-2A. In the present study, the Nrf2–ARE pathway increases the induction of MT-1A and MT-1E but not MT-2A. Although we have not identified AREs to which Nrf2 is recruited in the presence of Sb35, we speculate that Sb35 modifies Nrf2 and alters AREe to be recruited. For example, Nrf2 might be recruited to the promoter region of MT-1 rather than that of MT-2 in the presence of Sb35. In fact, Nrf2 is regulated by acetylation (Sun et al., 2009). Proteasome inhibition results in the Nrf2 stabilization (Stewart et al., 2003) and acetylation (Mercado et al., 2011). We confirmed that in bovine aortic endothelial cells after 12 h Sb35 (>50 μM) inhibits proteasome activity whereas cadmium (<10 μM) does not (Fujie et al., 2016a). Likely the degree of acetylation of Nrf2 is responsible for the differences in involvement of the Nrf2–ARE pathway in the MT isoform transcriptional induction between Sb35 and cadmium, although other mechanisms might also be involved.

In vascular endothelial cells P35 induces MT-2A transcription but not that of MT-1A/E and the induction depends on the MTF-1–MRE pathway but is unaffected by the Nrf2–ARE pathway. Therefore, this suggests that activation of Nrf2 by P35 is not involved in MT induction in these cells. If P35-activated Nrf2 is recruited to AREs in the promoter regions of MT-1A/E genes, transcription of MT-1A/E would be stimulated, as is the case when Sb35 is present. Even if Nrf2 is recruited to MT-2A AREs, transcription of MT-1A/E would be stimulated, as is the case for cadmium. Therefore, P35 induction of MT-2A transcription depends on the activity of the MTF-1–MRE pathway, although P35 can activate Nrf2. Although it is not known why P35-activated Nrf2 does not stimulate MT-1A/E transcription, the present data suggest that there is a mechanism by which only MT-2A is induced in

vascular endothelial cells In other words, both Sb35 and P35 activate Nrf2–ARE pathway but only Sb35 may be able to act on the mechanism that is required for MT-1A gene expression. On the other hand, As35 increased the transcriptional induction of MT-1A and MT-2A similarly to Sb35, but at concentrations lower than that of Sb35. The properties of arsenic as an element are very similar to those of antimony. In our previous study, however, it was shown that intracellular accumulation of As35 was higher than that of Sb35 in cultured vascular endothelial cells. It is suggested that the mechanisms underlying transcriptional induction of MT-1A and MT-2A by As35 and Sb35 are similar, but the induction by As35 can occur at lower concentrations because of the higher intracellular accumulation.

In the present study, we demonstrate the intracellular signaling pathways for transcriptional induction of endothelial MT isoforms using the organic-inorganic hybrid molecules Sb35 and P35. We found that there are two signaling pathways —the MTF-1–MRE and Nrf2–ARE pathways— that regulate the transcription of endothelial MT. The MTF-1–MRE pathway is essential for induction of all isoforms and the Nrf2–ARE pathway reinforces this induction. In addition, there is a mechanism by which only MT-2A is induced in vascular endothelial cells. It is suggested that MT-1A/E and MT-2A could have different functions. Additionally, proteasome inhibition could be involved in transcriptional regulation by modulating Nrf2 recruitment to AREs in the promoter regions of MT genes via Nrf2 acetylation. In addition, MTF-1 is activated by zinc; zinc binds to the zinc finger domain of MTF-1 to recruit transcription factors to the MREs (Chen et al., 1998; Bittel et al., 2000). Zinc is the only heavy metal that can induce MTF-1 binding to MREs (Koizumi et al., 1992; Bittel et al., 1998). The zinc ion to activate MTF-1 is supplied from intracellular proteins that are binding zinc ion nonspecifically. Recently, a family of zinc transporters was identified (Grotz et al., 1998), which are expressed on the transmembrane (Fukada et al., 2011), Golgi apparatus (Taniguchi et al., 2013), and endoplasmic reticulum (Taylor et al., 2004) and may regulate the intracellular concentration of zinc and therefore may be involved in the regulation of MT

transcription. Although the mechanisms for endothelial MT induction remain to be elucidated, our present study revealed the exact role of the Nrf2–ARE pathway in endothelial MT transcriptional induction by using an organometallic compound as an endothelial MT inducer. This suggests that the strategy of bio-organometallics, in which organic-inorganic hybrid molecules are used as tolls for analysis of biological functions, is effective to reveal unknown biological mechanisms.

Table 1. Organoantimony compounds used in this study

No.	Molecular formula	
Sb13	$C_{23}H_{20}NSb$	*N*-Methyl-*Sb*-phenylethynyl-5,6,7,12-tetrahydrodibenz[*c,f*][1,5]azastibocine
Sb14	$C_{24}H_{22}NSb$	*N*-Ethyl-*Sb*-phenylethynyl-5,6,7,12-tetrahydrodibenz[*c,f*][1,5]azastibocine
Sb15	$C_{25}H_{24}NSb$	*N-iso*-propyl-*Sb*-Phenylethynyl-5,6,7,12-tetrahydrodibenz[*c,f*][1,5]azastibocine
Sb16	$C_{26}H_{26}NSb$	*N*-2-Methylpropyl-*Sb*-phenylethynyl-5,6,7,12-tetrahydrodibenz[*c,f*][1,5]azastibocine
Sb17	$C_{28}H_{28}NSb$	*N*-Cyclohexyl-*Sb*-phenylethynyl-5,6,7,12-tetrahydrodibenz[*c,f*][1,5]azastibocine
Sb18	$C_{28}H_{22}NSb$	*N*-Phenyl-*Sb*-phenylethynyl-5,6,7,12-tetrahydrodibenz[*c,f*][1,5]azastibocine
Sb19	$C_{21}H_{20}NSb$	*N*-Methyl-*Sb*-phenyl-5,6,7,12-tetrahydrodibenz[*c,f*][1,5]azastibocine
Sb20	$C_{24}H_{26}NSb$	*N-t*-Butyl-*Sb*-phenyl-5,6,7,12-tetrahydrodibenz[*c,f*][1,5]azastibocine
Sb22	$C_{16}H_{18}NSb$	*N*-Methyl-*Sb*-methyl-5,6,7,12-tetrahydrodibenz[*c,f*][1,5]azastibocine
Sb23	$C_{19}H_{26}NSb$	*N*-Methyl-*Sb*-trimethylsilylmethyl-5,6,7,12-tetrahydrodibenz[*c,f*][1,5]azastibocine
Sb24	$C_{19}H_{24}NSb$	*N-t*-Butyl- *Sb*-methyl-5,6,7,12-tetrahydrodibenz[*c,f*][1,5]azastibocine
Sb26	$C_{21}H_{21}O_3Sb$	Tris(4-methoxylphenyl)stibane
Sb29	$C_{21}H_{21}Sb$	Tris(4-methylphenyl)stibane
Sb30	$C_{21}H_{21}Sb$	Tris(3-methylphenyl)stibane
Sb31	$C_{21}H_{21}Sb$	Tris(2-methylphenyl)stibane
Sb32	$C_{27}H_{33}Sb$	Tris(2,4,6-trimethylphenyl)stibane
Sb33	$C_{18}H_{12}F_3Sb$	Tris(4-fluorophenyl)stibane
Sb34	$C_{18}H_{12}Cl_3Sb$	Tris(4-chlorophenyl)stibane
Sb35	$C_{18}F_{15}Sb$	Tris(pentafluorophenyl)stibane
Sb37	$C_{24}H_{15}S_3Sb$	Tris(1-benzothiophen-2-yl)stibane
Sb38	$C_{24}H_{15}O_3Sb$	Tris(2-benzofuranyl)stibane
Sb40	$C_{21}H_{12}F_9Sb$	Tris[(4-trifluoromethyl)phenyl]stibane
Sb41	$C_{27}H_{27}O_6Sb$	Tris(4-ethoxycarbonylphenyl)stibane
Sb42	$C_{27}H_{36}N_3Sb$	Tris[2-(*N,N*-dimethylaminomethyl)phenyl]stibane
Sb43	$C_{24}H_{27}O_3Sb$	Tris[2-(methoxymethyl)phenyl]stibane
Sb44	$C_{24}H_{27}S_3Sb$	Tris[2-(methylsulfanylmethyl)phenyl]stibane
Sb46	$C_{18}H_{15}Cl_2Sb$	Triphenylantimony dichloride
Sb48	$C_{22}H_{21}O_4Sb$	Triphenylantimony diacetate

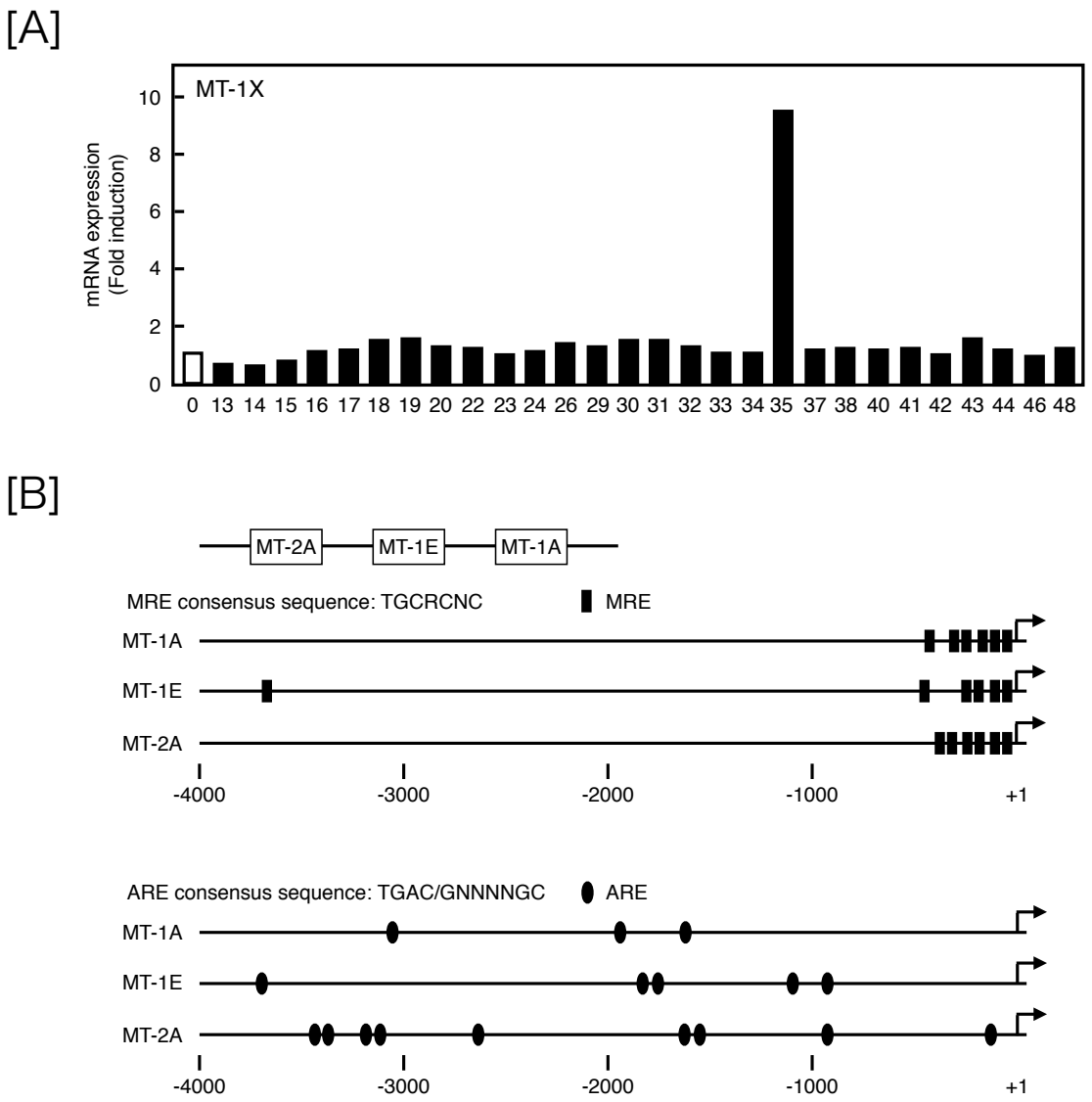

Fig. 3-1. [A] Transcriptional induction of MT-1X in vascular endothelial cells after treatment with organoantimony compounds shown in Table 1. Human brain microvascular endothelial cells were incubated with or without organoantimony compounds at 10 μM each for 3 h, and the expression of MT-1X mRNA was determined by real-time RT-PCR. [B] The map of MRE and ARE regions in the bovine MT promoter.

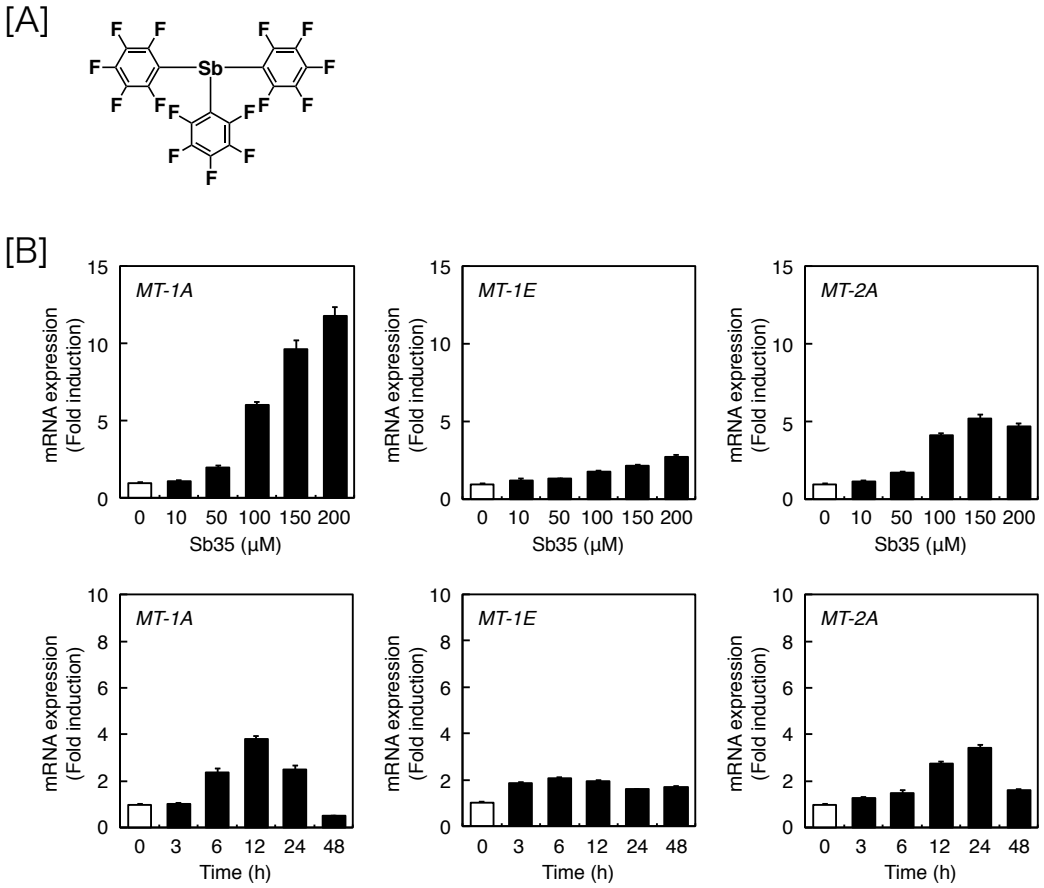

Fig. 3-2. Transcriptional induction of MT-1A, MT-1E, and MT-2A in vascular endothelial cells after treatment with Sb35. [A] The structure of Sb35. [B] Transcriptional induction of MT by Sb35. Bovine aortic endothelial cells were incubated with or without 10, 50, 100, 150, or 200 μM Sb35 for 12 h (upper panels) or for 3, 6, 12, 24, or 48 h with or without 100 μM Sb35 (lower panels), and the expression of MT-1A, MT-1E, and MT-2A mRNAs was determined by real-time RT-PCR. Data are represented as mean ± SE of three samples. Statistical significance when compared to corresponding control: **P*<0.05, ***P*<0.01.

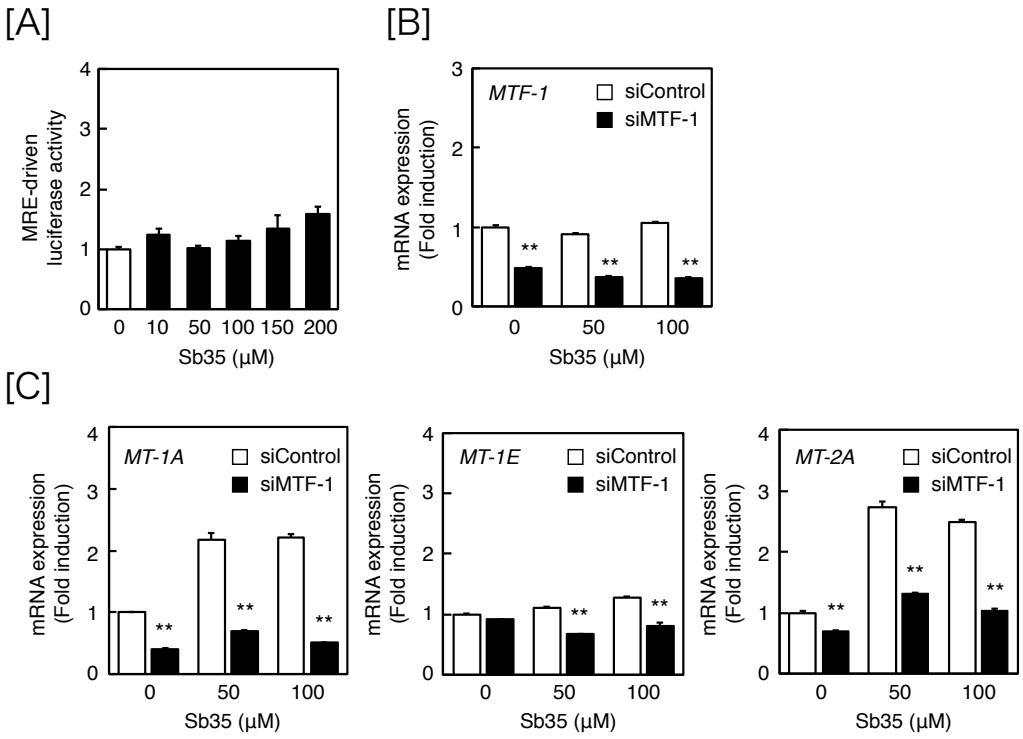

Fig. 3-3. Involvement of the MTF-1–MRE pathway in the transcriptional induction of MT-1A, MT-1E, and MT-2A by Sb35 in vascular endothelial cells. [A] MRE-driven transcriptional activity. Bovine aortic endothelial cells were transfected with an MRE reporter vector and incubated with or without 10, 50, 100, 150, or 200 μM Sb35 for 12 h, and the MRE-driven transcriptional activity was determined by the MRE-driven reporter assay. [B] Expression of MTF-1 after siRNA-mediated knockdown of MTF-1. Bovine aortic endothelial cells were transfected with control or MTF-1 siRNA and incubated with or without 50, or 100 μM Sb35 for 12 h, and the expression of MTF-1 mRNA was determined by real-time RT-PCR. Data are represented as mean ± SE of three samples. Statistical difference when compared to the corresponding "siControl": $^{**}P<0.01$. [C] Transcriptional induction of MT-1A, MT-1E, and MT-2A by Sb35 after knockdown of MTF-1. Bovine aortic endothelial cells were transfected with control or MTF-1 siRNA and incubated with or without 50, or 100 μM Sb35 for 12 h, and the expression of MT-1A, MT-1E, and MT-2A mRNAs was determined by real-time RT-PCR. Data are represented as mean ± SE of three samples. Statistical difference when compared to the corresponding "siControl": $^{**}P<0.01$

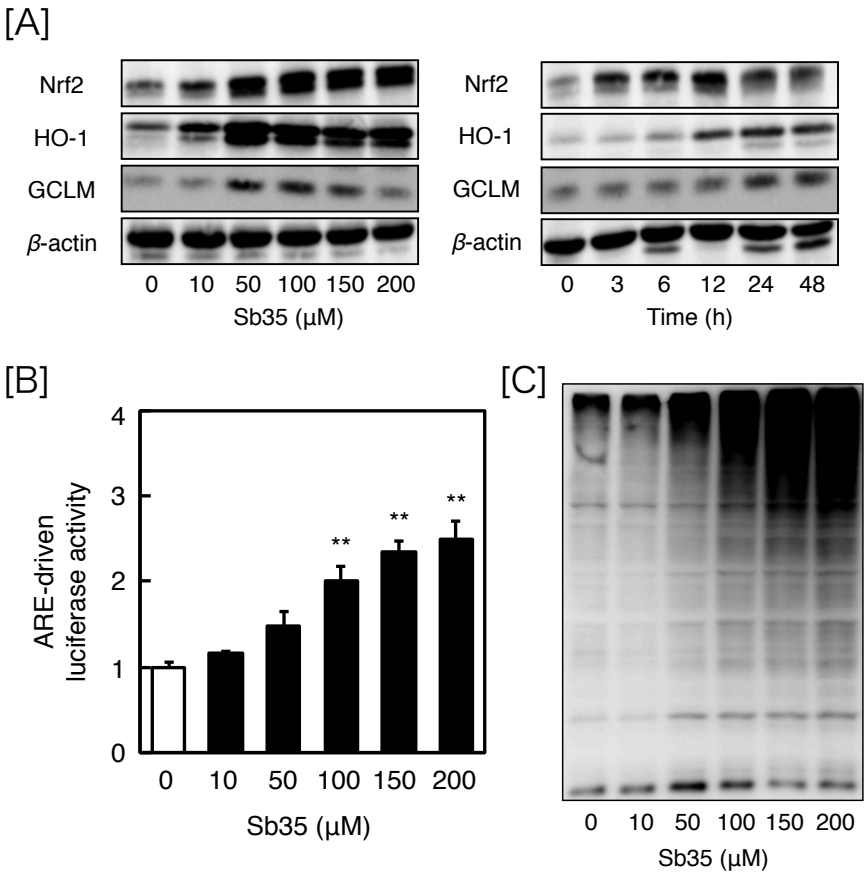

Fig. 3-4. Expression of Nrf2, its downstream proteins, and ARE-driven transcriptional activity by Sb35, and proteasome inhibition by Sb35 in vascular endothelial cells. [A] Expression of Nrf2, HO-1, and GCLM. Bovine aortic endothelial cells were incubated with or without 10, 50, 100, 150, or 200 μM Sb35 for 12 h (left panels) or for 3, 6, 12, 24, or 48 h with or without 100 μM Sb35 (right panels), and the expression of Nrf2, HO-1, and GCLM proteins were determined by western blot analysis. [B] ARE-driven transcriptional activity. Bovine aortic endothelial cells were transfected with an ARE reporter vector and incubated with or without 10, 50, 100, 150, or 200 μM Sb35 for 12 h, and the ARE-driven transcriptional activity was determined by the ARE-driven reporter assay. Data are represented as mean ± SE of three samples. Statistical significance when compared to corresponding control: $^{**}P<0.01$. [C] Proteasome inhibition by Sb35. Bovine aortic endothelial cells were incubated with or without 10, 50, 100, 150, or 200 μM Sb35 for 12 h, and ubiquitinated proteins were determined by western blot analysis.

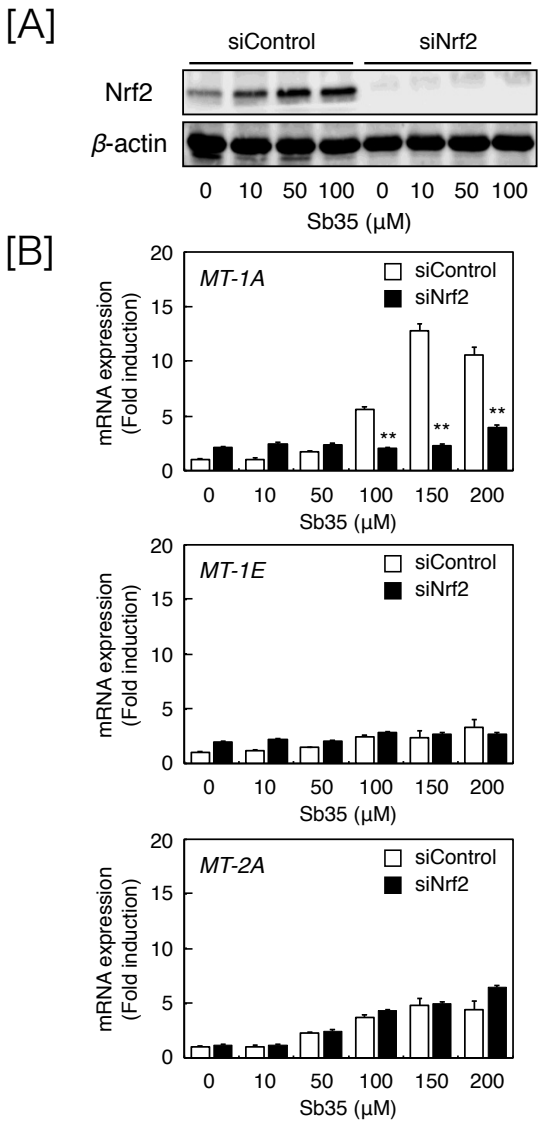

Fig. 3-5. Involvement of the Nrf2–ARE pathway in the transcriptional induction of MT-1A, MT-1E, and MT-2A by Sb35 in vascular endothelial cells. [A] Expression of Nrf2. siRNA-mediated knockdown of Nrf2. Bovine aortic endothelial cells were transfected with control or Nrf2 siRNA and incubated with or without 10, 50, or 100 μM Sb35 for 24 h, and the expression of Nrf2 protein was determined by western blot analysis. [B] Transcriptional induction of MT-1A, MT-1E, and MT-2A by Sb35 after knockdown of Nrf2. Bovine aortic endothelial cells were transfected with control or Nrf2 siRNA and incubated with or without 10, 50, 100, 150, or 200 μM Sb35 for 12 h, and the expression of MT-1A, MT-1E, and MT-2A mRNAs was determined by real-time RT-PCR. Data are represented as mean ± SE of three samples. Statistical difference when compared to the corresponding "siControl": $^{**}P<0.01$.

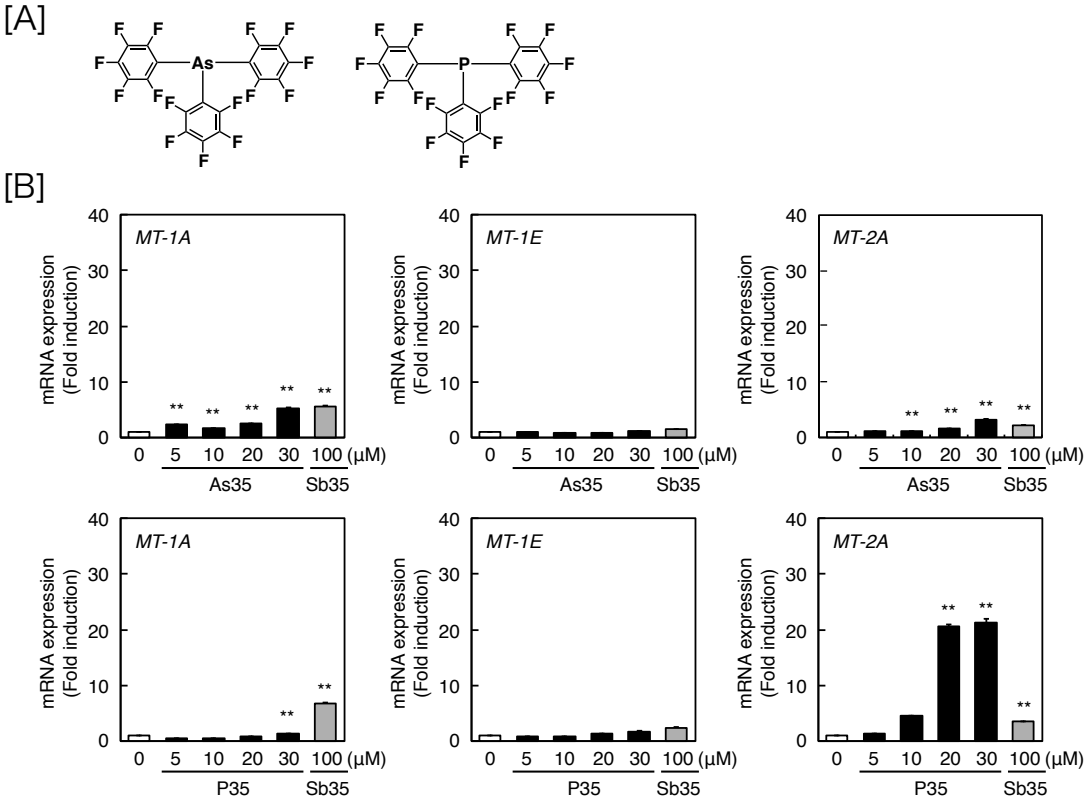

Fig. 3-6. Transcriptional induction of MT-1A, MT-1E, and MT-2A in vascular endothelial cells after treatment with As35 or P35. [A] The structures of As35 and P35. [B] Transcriptional induction of MT-1A, MT-1E, and MT-2A by As35 (upper panels) or P35 (lower panels). Bovine aortic endothelial cells were incubated with or without,10, 20, 50, or 100 μM As35 for 12 h (upper panels) or 5, 10, 20, or 30 μM P35 at (lower panels),. The cells were also treated with 100 μM Sb35 as the comparative control. The expression of MT-1A, MT-1E, and MT-2A mRNAs was determined by real-time RT-PCR. Data are represented as mean ± SE of three samples. Statistical significance when compared to corresponding control: **P*<0.05, ***P*<0.01.

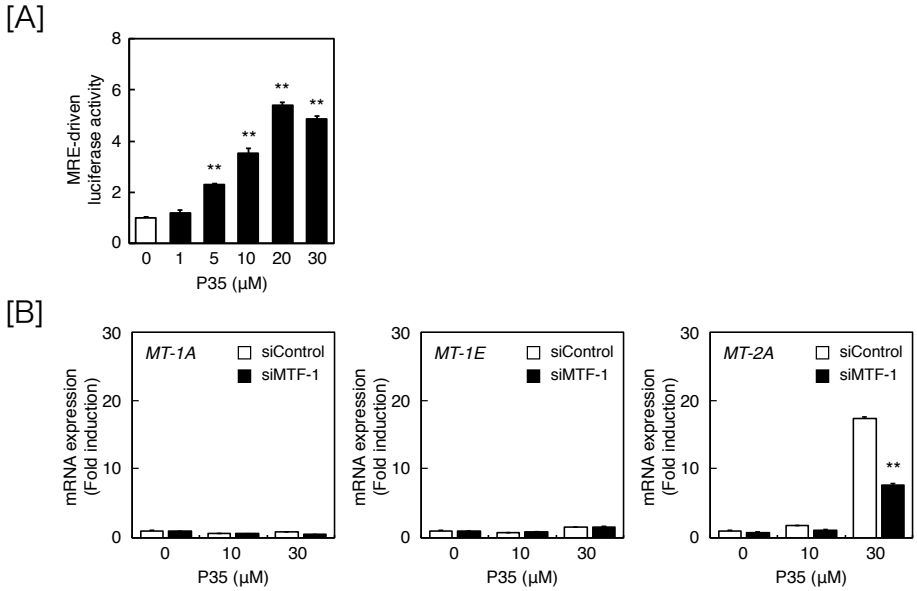

Fig. 3-7. Involvement of the MTF-1–MRE pathway in the transcriptional induction of MT-2A by P35 in vascular endothelial cells. [A] MRE-driven transcriptional activity. Bovine aortic endothelial cells were transfected with an MRE reporter vector and incubated with or without 1, 5, 10, 20, or 30 μM P35 for 12 h, and the MRE-driven transcriptional activity was determined by the MRE-driven reporter assay. Statistical significance when compared to corresponding control: $^{**}P<0.01$. [B] Transcriptional induction of MT-1A, MT-1E, and MT-2A by P35 after knockdown of MTF-1. Bovine aortic endothelial cells were transfected with control or MTF-1 siRNA and incubated with or without 10, or 30 μM P35 for 12 h, and the expression of MT-1A, MT-1E, and MT-2A mRNAs was determined by real-time RT-PCR. Data are represented as mean ± SE of three samples. Statistical difference when compared to the corresponding "siControl": $^{**}P<0.01$.

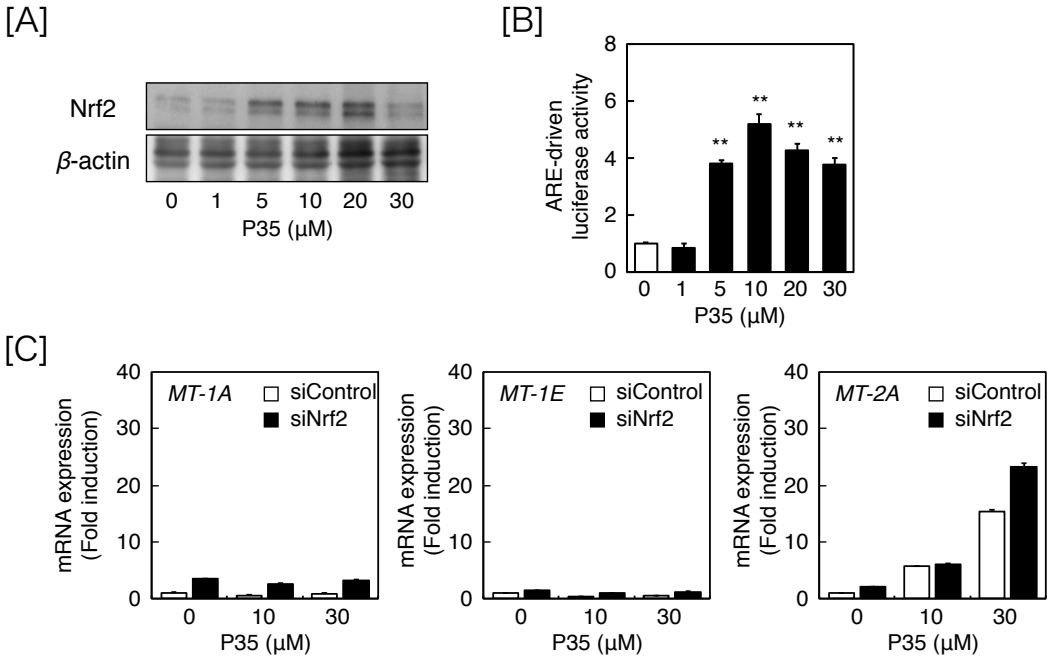

Fig. 3-8. Involvement of the Nrf2–ARE pathway in the transcriptional induction of MT-2A by P35 in vascular endothelial cells. [A] Expression of Nrf2. Bovine aortic endothelial cells were incubated with or without 1, 5, 10, 20, or 30 μM P35 for 12 h, and the expression of Nrf2, HO-1, and GCLM proteins were determined by western blot analysis. [B] ARE-driven transcriptional activity. Bovine aortic endothelial cells were transfected with an ARE reporter vector and with or without 1, 5, 10, 20, or 30 μM P35 for 12 h, and the ARE-driven transcriptional activity was determined by the ARE-driven reporter assay. Data are represented as mean ± SE of three samples. Statistical significance when compared to corresponding control: $^{**}P<0.01$. [C] Transcriptional induction of MT-1A, MT-1E, and MT-2A by P35 after knockdown of Nrf2. Bovine aortic endothelial cells were transfected with control or Nrf2 siRNA and incubated with or without 10, or 30 μM P35 for 12 h, and the expression of MT-1A, MT-1E, and MT-2A mRNAs was determined by real-time RT-PCR. Data are represented as mean ± SE of three samples. Statistical difference when compared to the corresponding "siControl": $^{**}P<0.01$.

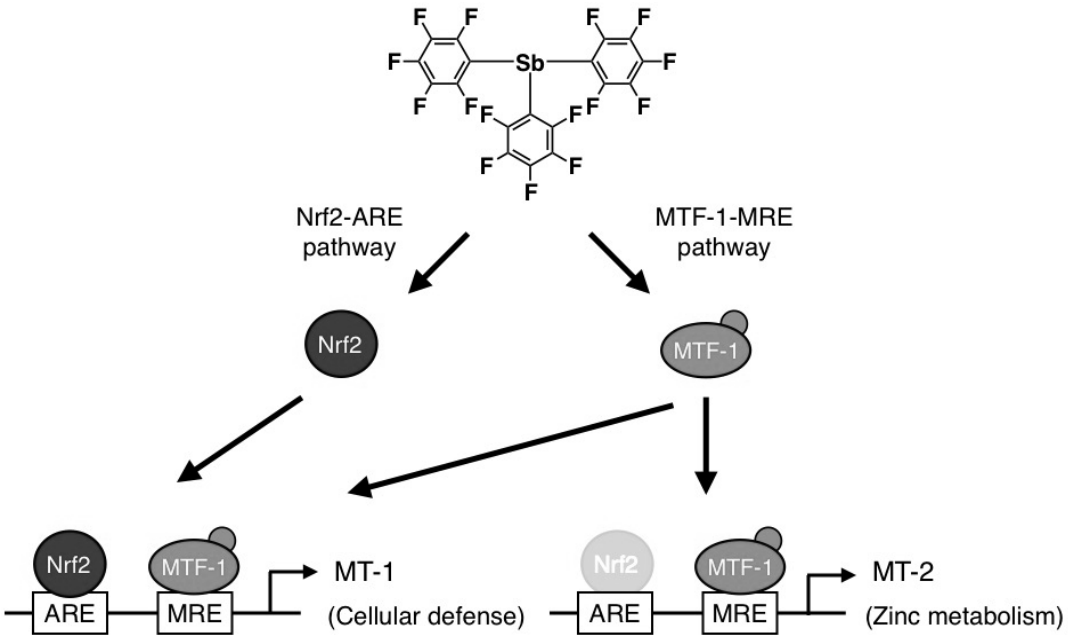

Fig. 3-9. A possible mechanism of the transcriptional induction of MT-1 and MT-2 genes by Sb35 in vascular endothelial cells

Chapter 4. Copper diethyldithiocarbamate as an activator of Nrf2 in cultured vascular endothelial cells

4. 1. Abstract

The interest in organic-inorganic hybrid molecules as molecular probes for biological systems has been growing rapidly. Such hybrid molecules with discrete metal–ligand coordination structures exhibit unique biological activities. Herein, copper(II) bis(diethyldithiocarbamate) (Cu10) was found to activate the transcription factor NF-E2-related factor 2 (Nrf2), which is responsible for regulating antioxidant and phase II xenobiotic enzymes, in vascular endothelial cells. The copper complex rapidly accumulated within cells and induced nuclear translocation of Nrf2, leading to upregulation of the expression of downstream proteins without cytotoxic effects. However, while copper bis(2-hydroxyethyl)dithiocarbamate activated Nrf2, copper ion, diethyldithiocarbamate ligand with or without zinc or iron failed to exhibit this activity. The intracellular accumulation of Cu10 was much higher than that of Cu(I) and Cu(II). Although the accumulation of Copper(II) bis(dimethyldithiocarbamate) was reduced by siRNA-mediated knockdown of the copper transporter CTR1, the knockdown did not affect the accumulation of Cu10, indicating Cu10 rapidly enter vascular endothelial cells via CTR1-independent mechanisms. In addition, copper and iron complexes with other ligands tested could not activate Nrf2, suggesting that the intramolecular interaction between copper and dithiocarbamate ligand is important for the activation of the transcription factor. Cu10 induced the expression of heme oxygenase-1 and γ-glutamylcyine synthetase, downstream proteins of Nrf2, more strongly than sulforaphane, a typical activator of Nrf2; however, the induction of NAD(P)H quinone oxidoreductase 1 was low in the cells treated with Cu10. It was suggested that Cu10-induced activation of Nrf2 was due to proteasome inhibition as well as binding to Kelch-like ECH-associated protein 1. Since the effects of Cu10 on vascular endothelial cells are unique and diverse, the copper complex may be a good

molecular probe to analyze the functions of vascular endothelial cells.

4. 2. Introduction

NF-E2-related factor 2 (Nrf2) is a transcription factor that belongs to Cap‘n’Collar transcription factor family and has a basic leucine zipper domain (Itoh et al., 1997). Under basal conditions, Nrf2 is bound to Kelch-like ECH-associated protein 1 (Keap1), which is an adapter protein to Cullin3-based E3 ubiquitin ligase, to prevent the proteasomal degradation of Nrf2 in the cytoplasm (Kobayashi et al., 2004). Keap1 also functions as a sensor protein against electrophiles and reactive oxygen species. Modification of the reactive thiols of Keap1 by electrophiles results in the dissociation of Nrf2 from Keap1 and its nuclear translocation, allowing it to bind antioxidant response element (ARE) of the genes, thereby forming a heterodimerized complex of Nrf2 with co-activators such as small Maf (Motohashi et al., 2002).

Nrf2 mainly regulates the gene expression of antioxidant and phase II xenobiotic metabolizing enzymes such as heme oxygenase-1, NAD(P)H quinone oxidoreductase 1, and γ-glutamylcysteine synthetase, by binding to the ARE of the promoter region of the genes. Induction of heme oxygenase-1 protects cells from oxidative injury by catalyzing heme to biriverdin, carbon monoxide, and iron (Baumer and Baumer, 2002). NAD(P)H quinone oxidoreductase 1 catalyzes the detoxification of quinones and their derivatives (Riley and Workman, 1992). γ-Glutamylcysteine synthetase is a rate-limiting enzyme in glutathione synthesis and consists of two subunits, the modifier subunit and the catalytic subunit. We postulate that low-molecular-weight molecular probes that activate Nrf2 and regulate cellular functions will be useful in analyzing the involvement of the transcription factor in the regulation of vascular endothelial cell functions.

Organic-inorganic hybrid molecules—organometallic compounds and metal coordination compounds—consist of metals and organic ligands in a common feature. These compounds can exhibit unique biological activities, different from those of organic and inorganic

compounds; their activities are attributable to their unique three-dimensional structures and electronic states (Kimura et al., 2012; Fujiwara et al., 2005; Kohri et al., 2015). It is most likely that organic-inorganic hybrid molecules exhibit their activities by modifying the activities of ligand, those of metal, or interaction between ligand and metal. We found that bis(L-cysteinato)zincate(II) serves as a specific zinc donor to the metal response element-binding transcription factor-1, a transcription factor containing six C_2H_2 zinc finger domains (Kimura et al., 2012). We have reported that an organobismuth compound—tris[2-(*N,N*-dimethylaminomethyl)phenyl]bismuthane—exhibits vascular endothelial cell-specific toxicity (Fujiwara et al., 2005) and the cytotoxicity disappears when the bismuth atom is replaced with an antimony atom (Kohri et al., 2015). Recently, it was found that an organoantimony compound—tris(pentafluorophenyl)stibane—causes transcriptional induction of metallothionein (submitted).

There are many reports on low-molecular-weight compounds including toxic metal(loid)s that activate Nrf2; for example, sulforaphane, curcumin, tert-butylhydroquinone, 1,2-naphtoquinone, methylmercury, and arsenite (Eggler et al., 2008; Cortese-Krott et al., 2009; Miura et al., 2011; Toyama et al., 2007; Abiko et al., 2011). However, little is known about organic-inorganic hybrid molecules. In the present study, to obtain a good molecular probe for analysis of vascular endothelial cell functions that are regulated by Nrf2, we searched for organic-inorganic hybrid molecules that activate Nrf2 without cytotoxicity in cultured vascular endothelial cells. We found that copper(II) bis(diethyldithiocarbamate) (Cu10) exerts such a biological activity via proteasome inhibition as well as Keap1 modification in the cells.

4. 3. Results

Cu10 activates Nrf2 in vascular endothelial cells

Fig. 4-1 shows the activation of Nrf2 by Cu10 (the structure is shown in Fig. 4-1A) in vascular endothelial cells. Cu10 at 10 μM or less increased the expression of Nrf2 in a concentration-dependent manner (Fig. 4-1B). Cu10 at 10 μM increased the expression of Nrf2 after 2 h or longer in a time-dependent manner; the highest expression was observed at 8 h and gradually reduced thereafter (Fig. 4-1C). Nrf2 was detected in the nuclear fraction after 3 and 6 h in vascular endothelial cells treated with Cu10 at 5 and 10 μM (Fig. 4-1D). After a 24-h treatment with Cu10 at 0.1 μM or higher, the expression of downstream proteins of Nrf2—heme oxygenase-1, NAD(P)H quinone oxidoreductase 1, and γ-glutamylcysteine synthetase modifier subunit—significantly increased in a concentration-dependent manner (Fig. 1E).

Role of copper in the Cu10 molecule in Nrf2 activation

In order to examine whether copper in the Cu10 molecule is critical to the activation of endothelial Nrf2, the effects of zinc and iron complexes with the same ligand of Cu10 on Nrf2 activation were investigated. In this experiment, copper sulfate, Na01 as the ligand of Cu10, and Cu09 were investigated. The structures of the tested metal complexes and Na01 are shown in Fig. 4-2A. As shown in Fig. 4-2B, Cu10 and Cu09 increased the expression of Nrf2; however, copper sulfate, Na01, Zn01, and Fe05 failed to exhibit such an activity (Fig. 4-2B), indicating that copper is required for diethyldithiocarbamate complexes to activate Nrf2 in vascular endothelial cells. Copper sulfate, Zn01, and Fe05 did not accumulate within the cells after a 3 h treatment, whereas significant accumulation of Cu10 and Cu09 was observed (Fig. 4-2C), suggesting that Nrf2-activating activity of the copper complexes may depend on their high intracellular accumulation.

Since Cu10 and Cu09 highly accumulated within vascular endothelial cells and increased

the expression Nrf2, it is possible that Cu(II) was reduced to Cu(I) by thiol groups in the thiocarbamate ligands and Cu(I) ion released from the Cu10 molecule efficiently entered the cells through the copper transporter CTR1 that efficiently mediates the Cu(I) uptake (Lee et al., 2002). To examine this possibility, we compared the intracellular accumulation of Cu(II), Cu(I), and Cu10 in vascular endothelial cells and determined the involvement of CTR1 in the uptake of copper complexes with thiol groups. The structures of tested copper complexes are shown in Fig. 4-3A. Cu10 highly accumulated in vascular endothelial cells compared to Cu(II) and Cu(I) (Fig. 4-3B). The accumulation of copper(II) bis(dimethyl-dithiocarbamate)(Cu17) was significantly reduced by siRNA-mediated knockdown of CTR1; however, that of Cu10, copper(II) bis(dibutyldithiocarbamate)(Cu18), and copper(II) bis(dibenzyldithiocarbamate)(Cu19) was unaffected by the knockdown (Figs. 4-3C and 4-3D). Cu17 as well as Cu10 activated Nrf2 regardless of whether CTR1 was knocked down or not (Fig. 4-3E). However, Cu18 and Cu19 highly accumulated within the cells (Fig. 4-3D) regardless of CTR1 expression but failed to activate Nrf2 in vascular endothelial cells.

Role of the ligand in the Cu10 molecule in Nrf2 activation

It is possible that copper complexes in general activate endothelial Nrf2. To examine this possibility, we evaluated copper complexes with various ligands. In this experiment, compounds containing iron were also examined. The compounds used in this experiment are shown in Fig. 4-4A. Among the tested copper complexes, only Cu10 and Cu09 increased the expression of Nrf2 (Fig. 4-4B), suggesting that the ligand as well as copper ion is important for the activation of Nrf2 by Cu10. Other compounds could not activate the transcription factor.

Characterization of Nrf2 activation by Cu10

The activation of Nrf2 by Cu10 was compared with that by sulforaphane (the structure is

shown in Fig. 4-5A), an isothiocyanate that modifies Keap1 and activates Nrf2 (Kobayashi et al., 2009). It was shown that the Nrf2-activating activity of Cu10 and sulforaphane is almost comparable (Fig. 4-5B); however, the expression of Nrf2 downstream proteins induced by Cu10 and sulforaphane differed. Specifically, Cu10 markedly increased the expression of heme oxygenase-1 and γ-glutamylcysteine synthetase modifier subunit, whereas the expression of NAD(P)H quinone oxidoreductase 1 was markedly upregulated by sulforaphane (Fig. 4-5C), suggesting that the mechanisms underlying Nrf2 activation by Cu10 may be different from that of sulforaphane.

Activation mechanisms of Nrf2 by Cu10

To examine whether Cu10 can bind Keap1, the BPM-labeling assay was performed. Incubation of recombinant mouse Keap1 with Cu10 decreased the signal of biotinylated Keap1 protein (Fig. 4-6), indicating that Cu10 was bound to Keap1.As shown in the previous study (Toyama et al., 2013), binding fo $CdCl_2$ to Keap1 was observed. Since Keap1 binds Nrf2 and protects it from proteasomal degradation, inhibition of proteasome can be a mechanism through which Nrf2 is activated. To examine this possibility, the proteasome inhibitory activity of Cu10 was investigated. In this experiment, Zn01 and nickel(II) bis(diethyldithiocarbamate) (Ni06) were also evaluated (the structures are shown in Fig. 4-7A). It was shown that Cu10 increased the ubiquitinated proteins in a concentration- and time-dependent manner (Fig. 4-7B), indicating that the copper complex inhibits proteasome; MG132, a typical proteasome inhibitor also increased the level of ubiquitinated proteins. Zn01 and Ni06 failed to increase both Nrf2 expression and level of ubiquitinated proteins (Fig. 4-7C). Since cadmium, a toxic heavy metal in vascular endothelial cells (Fujiwara et al., 2014), did not display proteasome inhibitory activity, it is suggested that the proteasome inhibition by Cu10 was not a nonspecific effect of the copper complex (Fig. 4-7D).

4. 4. Discussion

Dithiocarbamates are metal-chelating compounds that affect the activities of various metal-binding proteins such as nuclear factor-kappa B and superoxide dismutase-1 (Schreck et al., 1992; Heikkila et al., 1976). The present study revealed the previously unrecognized biological activities of metal diethyldithiocarbamate coordination compounds. The following results were obtained: (1) copper(II) bis(diethyldithiocarbamate), Cu10, activates Nrf2 in vascular endothelial cells without cytotoxicity, (2) the combination of copper with the diethyldithiocarbamate ligand is essential for the effect of Cu10, (3) Cu10 rapidly enters the cells and induces nuclear translocation of Nrf2, resulting in the induction of downstream proteins such as heme oxygenase-1, NAD(P)H quinone oxidoreductase 1, and γ-glutamylcysteine synthetase modifier subunit, (4) Cu10 highly accumulated within the cells compared to Cu(II) and Cu(I) regardless of CTR1 expression and the Nrf2 activation activity of copper complexes with thiol groups depend on the structures but not the intracellular accumulation, (5) both proteasome inhibition and binding to Keap1 are mechanisms through which Cu10 activates Nrf2. In general, the relationship between the biological activity and the structure of a certain organic-inorganic hybrid molecule could follow any of the following three patterns. First, the ligand has biological activity and intramolecular metal intensifies the activity. Second, the metal has biological activity and the ligand intensifies the activity. Third, the biological activity of either the ligand or the metal is only slight but the hybrid molecule exhibits activity owing to their discrete molecular structures that originate from metal–ligand coordination (i.e. intramolecular interaction). Since neither copper sulfate nor Na01 could activate Nrf2, we postulate that the activation of Nrf2 by Cu10 is induced by the intramolecular interaction between copper atom and the diethyldithiocarbamate ligand. It is also suggested that this interaction is required for rapid accumulation of Cu10 within vascular endothelial cells.

The mechanisms of the higher intracellular accumulation of Cu10 appears to be important

for understanding the mechanisms by which Cu10 activates Nrf2 in vascular endothelial cells. Copper complexes with thiol groups such as Cu10 and Cu09 highly accumulated within the cells and activated Nrf2 whereas copper complexes without thiol groups failed to activate Nrf2, suggesting a possibility that Cu(II) was reduced to Cu(I) by thiol groups in the thiocarbamate ligands and Cu(I) ion released from the Cu10 molecule efficiently entered the cells through the copper transporter CTR1 (Lee et al., 2002). In other words, Cu10 served as a donor of Cu(I) to CTR1 and, consequently, the copper ion but not Cu10 molecule activated Nrf2 through modification of Keap1. However, intracellular accumulation of Cu10 was much higher than Cu(II) and Cu(I) and unaffected by siRNA-mediated knockdown of CTR1. On the other hand, the accumulation of Cu17 was significantly reduced by the knockdown, suggesting that CTR1 is at least partly involved in the uptake of Cu17. Therefore, the possibility that CTR1 partly mediate the uptake of copper complexes like cisplatin [26] cannot be excluded, it is suggested that CTR1 expression is not the major mechanisms of Cu10 uptake. In addition, Cu18 and Cu19 also highly accumulated within the cells but failed to activate Nrf2, suggesting that activation of Nrf2 by copper complexes depends on the ligand structure rather than copper ion released from the molecule. Thus, although the detail remains to be elucidated, an assumption can be made that Cu10 was transported as the molecule, modified Keap1, and activated Nrf2 in vascular endothelial cells

Ubiquitin-proteasome system is responsible for the degradation of numerous proteins, including Nrf2. The 26S proteasome consists of two complexes—the 20S proteolytic core and the 19S regulatory complex (Gerards et al., 1998). Previously, it was reported that Cu10 inhibits proteasomal function by inhibiting both 20S chymotrypsin-like activity and 19S complex in human breast cancer MBA-MD-231 cells (Cvek et al., 2008). In this report, Zn01 but not Ni06 showed proteasome inhibitory activity, suggesting that the type of metal complexes that have similar biological activities may depend on cell types. While the reason underlying this cell type-dependency is unclear, it is certain that Cu10 exhibits proteasome

inhibitory activity and inhibits the degradation of Nrf2 in vascular endothelial cells. Proteasome inhibition would be one of the major mechanisms by which Cu10 activates endothelial Nrf2. Conversely, it was shown that Cu10 binds Keap1 and this led to the release of Nrf2 from Keap1 followed by nuclear translocation of Nrf2. There are several reactive cysteine residues in the Keap1 molecule and the residues that are involved in Nrf2 activation are Cys151 in the BTB domain and Cys273/Cys288 in the IVR domain (Zhang and Hannink, 2003; Wakabayashi et al., 2003). The modified cysteine residues that are employed for Nrf2 activation depend on the compounds that activate Nrf2 (Kumagai et al., 2013; Wang et al., 2008). For example, zinc ion binds to both Cys273 and Cys288 and activates Nrf2 (Dinkova-Kostova et al., 2005). It is suggested that Cu10 binds to at least one of the three reactive cysteine residues of the Keap1 molecule. While it is unclear as to which cysteine residue(s) are modified by Cu10, it is postulated that modification of Keap1 is one of the major mechanisms by which Cu10 activates endothelial Nrf2. It was shown that, among the downstream proteins of Nrf2, heme oxygenase-1 and γ-glutamylcysteine synthetase modifier subunit were markedly induced by Cu10, whereas sulforaphane, which is bound to Keap1 and activates Nrf2, strongly induced NAD(P)H quinone oxidoreductase 1. This difference in the induction of downstream protein between Cu10 and sulforaphane may be attributable to the difference of Nrf2 activation mechanisms. However, this remains to be elucidated.

A compound that has a specific target biomolecule is an excellent tool to analyze the role of the biomolecule in the regulation of some biological systems. However, a compound that has multiple targets is also useful to analyze the relationship among the targets. Cu10 appears to belong to the latter case. In fact, we have analyzed vascular endothelial cell functions using Cu10. Metallothionein is a low-molecular-weight, cysteine-rich, metal-containing, inducible protein, which protects cells from heavy metals and oxidative stress (Kägi et al., 1991). Since cadmium and zinc induce metallothionein, they have been used as tools to analyze mechanisms underlying metallothionein induction. However, the metals cannot be good tools

because vascular endothelial cells are sensitive to cadmium (Kaji et al., 1996) and zinc does not induce metallothionein in the cells (Kaji et al., 1992; Fujie et al., 2016). Recently, we found that this copper complex induces metallothionein in vascular endothelial cells; activation of Nrf2 and consequent activation of ARE in the promoter region of metallothionein genes contribute to the induction of specific metallothionein isoform (Fujie et al., 2016). Further studies on Cu10 as a tool to analyze vascular endothelial cell functions are ongoing.

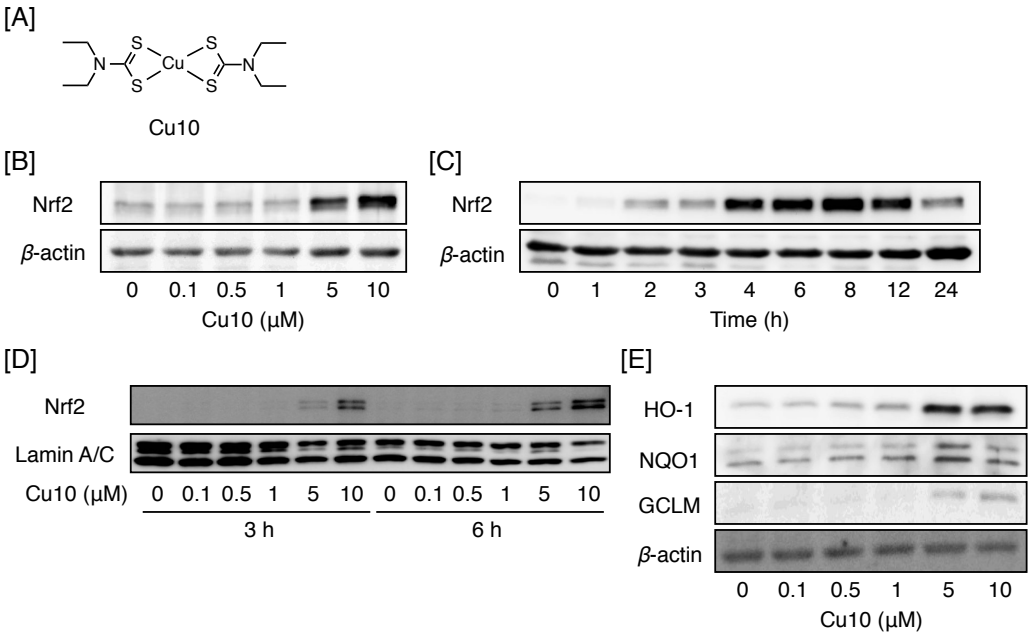

Fig. 4-1. Activation of Nrf2 by Cu10 in vascular endothelial cells. [A] The structure of Cu10. [B] The expression of Nrf2. Confluent cultures of bovine aortic endothelial cells were incubated at 37°C for 3 h in the presence or absence of Cu10 (0.1, 0.5, 1, 5, or 10 μM). [C] Time course of the effect of Cu10 on the expression of Nrf2. Confluent cultures of bovine aortic endothelial cells were incubated at 37°C for 1, 2, 3, 4, 6, 8, 12, and 24 h in the presence or absence of Cu10 (10 μM). [D] The expression of Nrf2 in the nuclei. Confluent cultures of bovine aortic endothelial cells were incubated at 37°C for 3 and 6 h in the presence or absence of Cu10 (0.1, 0.5, 1, 5, or 10 μM). [E] The expression of downstream proteins of Nrf2. Confluent cultures of bovine aortic endothelial cells were incubated at 37°C for 24 h in the presence or absence of Cu10 (0.1, 0.5, 1, 5, or 10 μM). HO-1, heme oxygenase-1; NQO1 (upper bands), NAD(P)H quinone oxidoreductase 1; GCLM, γ-glutamylcysteine synthetase modifier subunit.

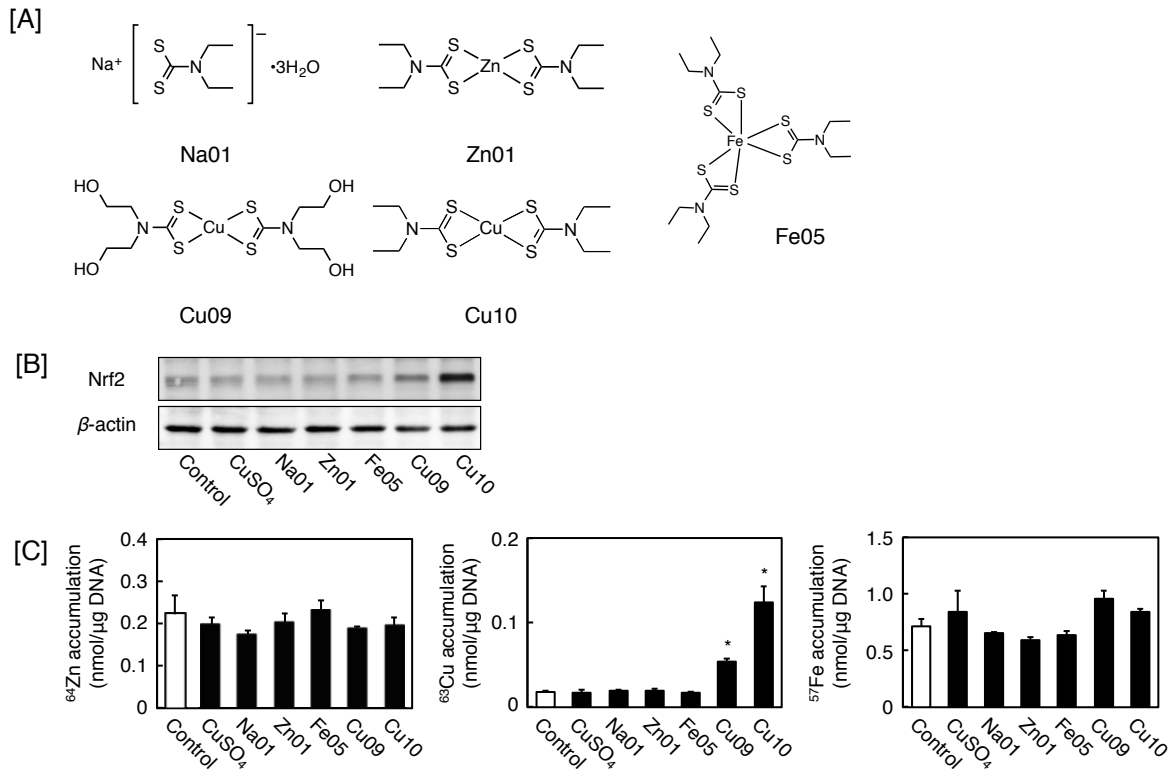

Fig. 4-2. Role of copper in the Cu10 molecule in Nrf2 activation in vascular endothelial cells. [A] The structures of Na01, Zn01, Fe05, Cu09, and Cu10. [B] The expression of Nrf2. Confluent cultures of bovine aortic endothelial cells were incubated at 37°C for 3 h in the presence or absence of $CuSO_4$, Na01, Zn01, Fe05, Cu09, and Cu10 (10 μM each). c Intracellular accumulation of $CuSO_4$, Na01, Zn01, Fe05, Cu09, and Cu10. Confluent cultures of bovine aortic endothelial cells were incubated at 37°C for 3 h in the presence or absence of $CuSO_4$, Na01, Zn01, Fe05, Cu09, and Cu10 (10 μM each). Values are means ± S.E. of three samples. *Significantly different from the control, $P < 0.01$.

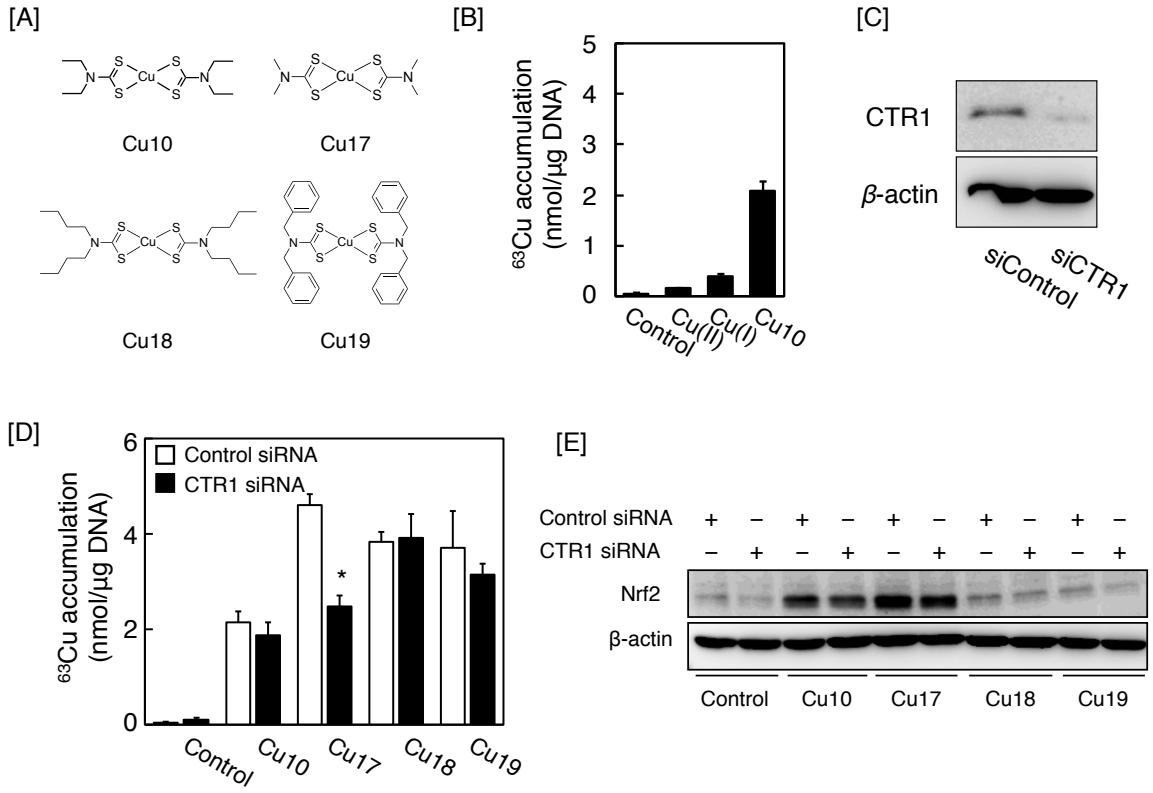

Fig. 4-3. Intracellular accumulation of Cu(II), Cu(I), and Cu10 in vascular endothelial cells and involvement of CTR1 in the accumulation of copper complexes. [A] The structures of Cu10, Cu17, Cu18, and Cu19. [B] Intracellular accumulation of Cu(II), Cu(I), and Cu10. Confluent cultures of bovine aortic endothelial cells were incubated at 37°C for 3 h in the presence or absence of $CuSO_4$ [Cu(II)], $CuSO_4$ with 1 mM ascorbate [Cu(I)], and Cu10 (10 μM each). Values are means ± S.E. of four samples. [C] CTR1 protein expression after siRNA-mediated knockdown of CTR1. Subconfluent cultures of bovine aortic endothelial cells were transfected with control or CTR1 siRNA and incubated at 37°C in the presence or absence of Cu10, Cu17, Cu18, and Cu19 (10 μM each) for 3 h. [D] Intracellular accumulation of Cu10, Cu17, Cu18, and Cu19. Subonfluent cultures of bovine aortic endothelial cells were transfected with control or CTR1 siRNA and incubated at 37°C in the presence or absence of Cu10, Cu17, Cu18, and Cu19 (10 μM each). Values are means ± S.E. of four samples. *Significantly different from the control, $P < 0.01$. [E] The expression of Nrf2. Subconfluent cultures of bovine aortic endothelial cells were transfected with control or CTR1 siRNA and incubated at 37°C in the presence or absence of Cu10, Cu17, Cu18, and Cu19 (10 μM each).

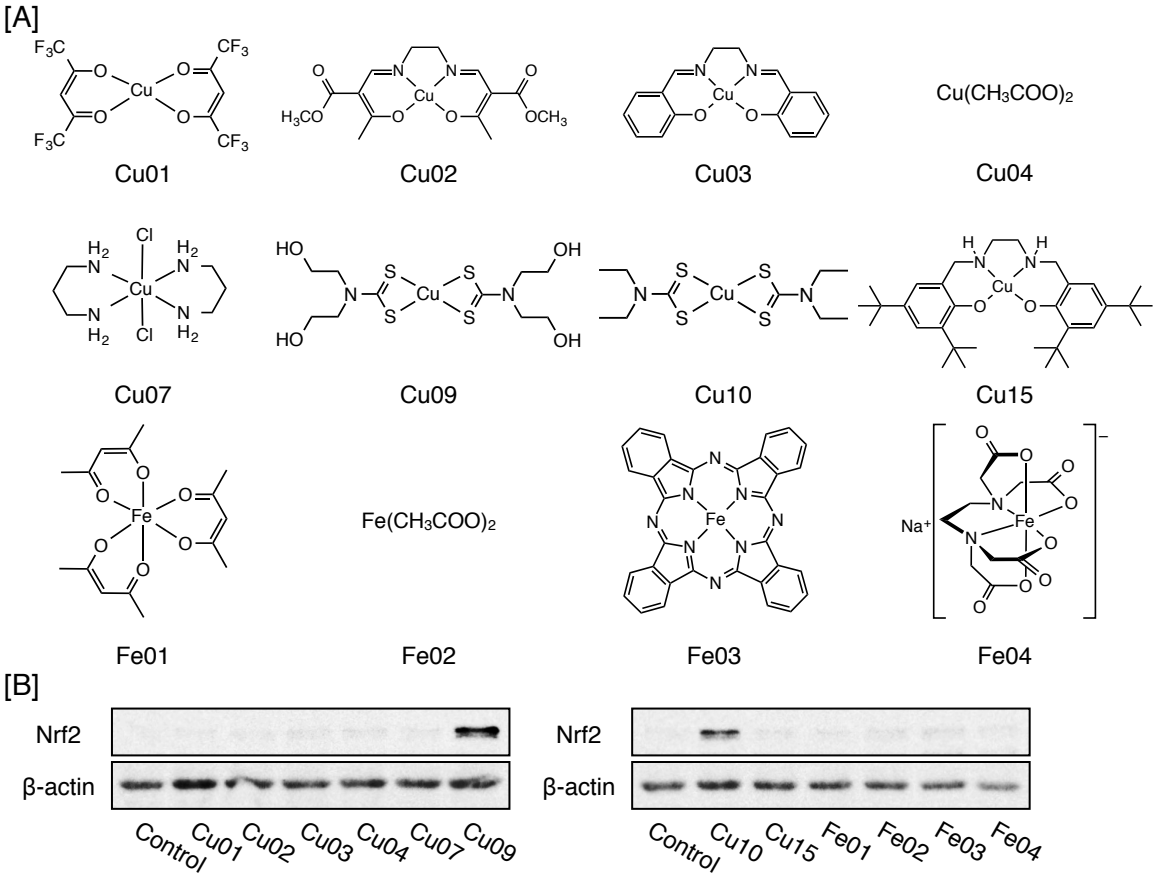

Fig. 4-4. Role of the ligand in Cu10 molecule in Nrf2 activation in vascular endothelial cells. [A] The structures of Cu01, Cu02, Cu03, Cu04, Cu07, Cu09, Cu10, Cu15, Fe01, Fe02, Fe03, and Fe04. [B] The expression of Nrf2. Confluent cultures of bovine aortic endothelial cells were incubated at 37°C for 3 h in the presence or absence of Cu01, Cu02, Cu03, Cu04, Cu07, Cu09, Cu10, Cu15, Fe01, Fe02, Fe03, and Fe04 (10 μM each).

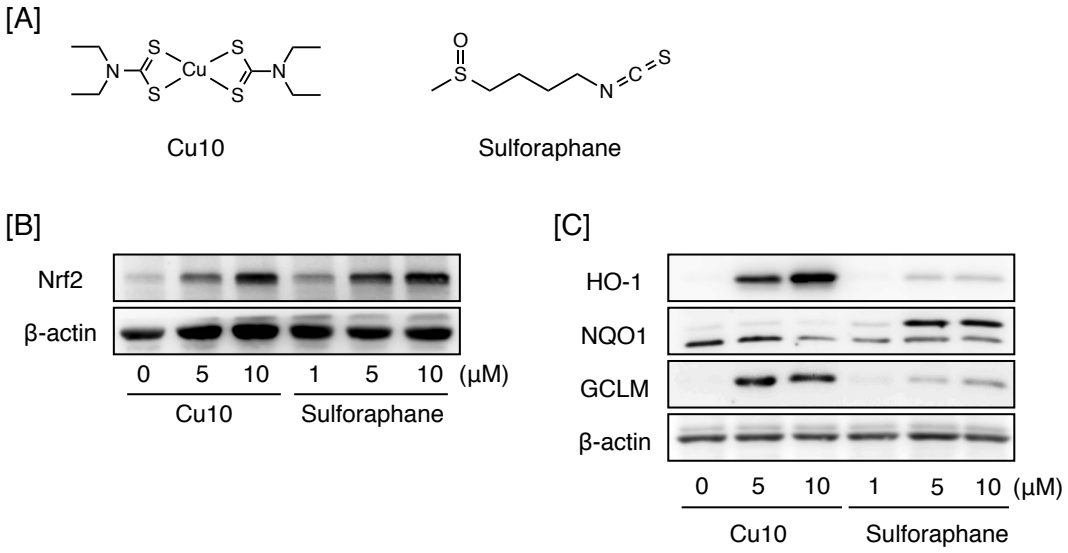

Fig. 4-5. Characterization of Nrf2 activation by Cu10 compared with sulforaphane. [A] The structures of Cu10 and sulforaphane. [B] The expression of Nrf2. Confluent cultures of bovine aortic endothelial cells were incubated at 37°C for 3 h in the presence or absence of Cu10 (5 or 10 μM) or sulforaphane (1, 5, or 10 μM). [C] The expression of downstream proteins of Nrf2. Confluent cultures of bovine aortic endothelial cells were incubated at 37°C for 24 h in the presence or absence of Cu10 (5 or 10 μM) or sulforaphane (1, 5, or 10 μM). HO-1, heme oxygenase-1; NQO1, NAD(P)H quinone oxidoreductase 1; GCLM, γ-glutamylcysteine synthetase modifier subunit.

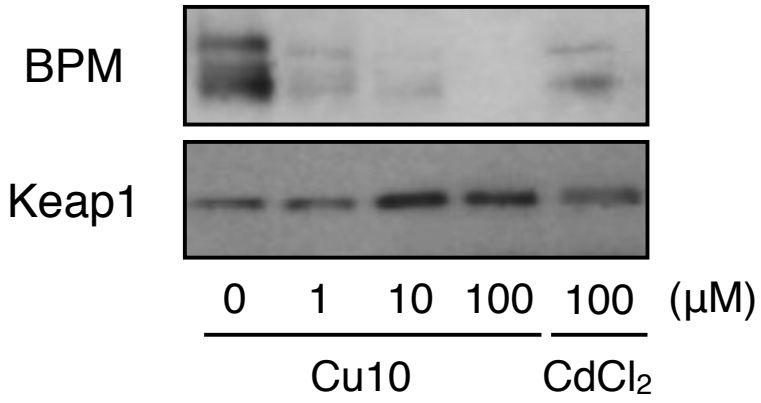

Fig. 4-6. Binding of Cu10 to Keap1. Recombinant mouse Keap1 protein (2 μg) was incubated with Cu10 (1, 10, or 100 μM) at 37°C for 30 min in 100 mM Tris-HCl (pH 7.5) and then further incubated at 37°C for 30 min after addition of 25 μM biotin-$PEAC_5$-maleimide. The samples were subjected to western blotting, which was performed using anti-biotin antibody (BPM) and anti-Keap1 antibody (Keap1). Cadmium chloride ($CdCl_2$) was used as positive control.

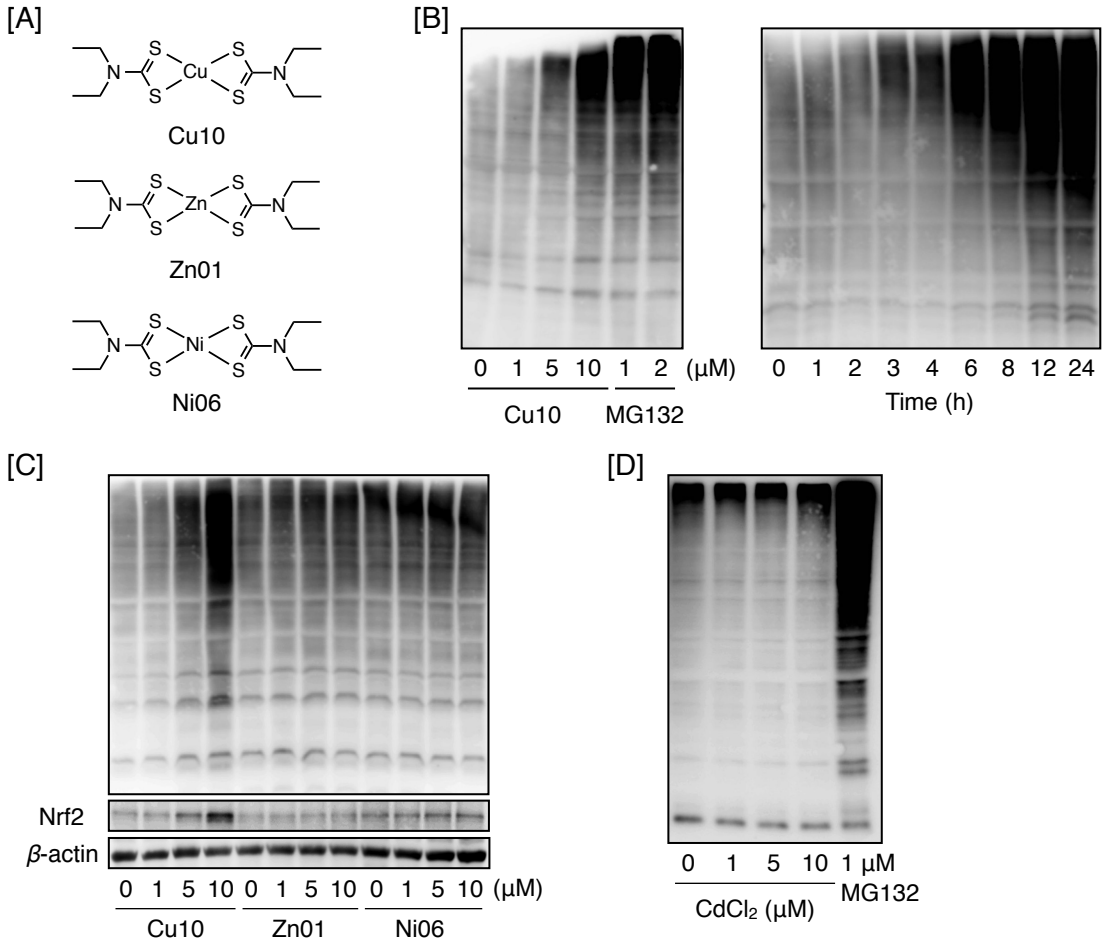

Fig. 4-7. Proteasome inhibition by Cu10, Zn01, Ni06, and $CdCl_2$ in vascular endothelial cells. [A] The structures of Cu10, Zn01, and Ni06. [B] Proteasome inhibitory activity. Confluent cultures of bovine aortic endothelial cells were incubated at 37°C for 8 h in the presence or absence of Cu10, Zn01, Ni06, or Cadmium chloride ($CdCl_2$) (1, 5, 10 μM each). MG132 was used as positive control. The total cell lysates were subjected to western blotting, which was performed using an anti-ubiquitin antibody.

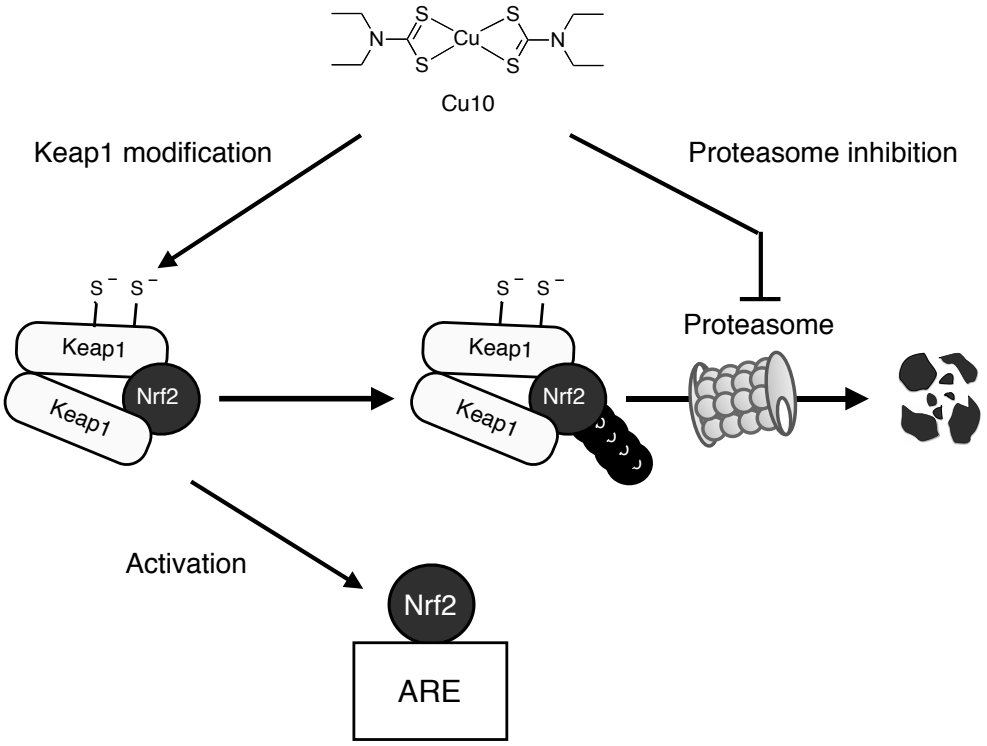

Fig. 4-8. A possible mechanism of Nrf2 activation by Cu10 in vascular endothelial cells.

Chapter 5. Induction of metallothionein isoforms by copper diethyldithiocarbamate in cultured vascular endothelial cells

5. 1. Abstract

Metallothionein (MT) plays a central role in cellular defense against heavy metals and oxidative stress. Since the induction of MT requires the activation of metal response element (MRE)-binding transcription factor-1 (MTF-1) by binding of zinc ions, inorganic zinc is regarded as a typical MT inducer. However, in a previous report, we showed that inorganic zinc could not induce MT in vascular endothelial cells. While it is suggested that endothelial MT presents mechanisms different from those of other cell types, these remain unclear. In this study, we investigated whether the induction of endothelial MT expression involves the Nrf2–ARE pathway using copper(II) bis(diethyldithiocarbamate), termed Cu10. Cu10 induced MT-1/2 protein expression and increased the expression of mRNAs for MT-1A, MT-1E, and MT-2, MT isoforms expressed in vascular endothelial cells. Cu10 activated not only the MTF-1-MRE, but also the Nrf2-ARE pathway. MTF-1 knockdown resulted in the repression of Cu10-induced MT-1 and -2 expression. Cu10-induced MT-1 expression was down-regulated by Nrf2 knockdown. However, MT-2 expression was not affected by Nrf2 knockdown. These results suggest that the expression of endothelial MT is up-regulated by the Nrf2–ARE pathway as well as by the MTF-1–MRE pathway. Moreover, MT-1 regulation mechanisms differ from that of MT-2. Specifically, the present data support the hypothesis that MT-1 participates in the biological defense system, while MT-2 mainly regulates intracelluar zinc metabolism.

5. 2. Introduction

Organic-inorganic hybrid molecules—organometallic compounds and metal coordination compounds—consist of metals and organic ligands in a common feature. These compounds are expected to exhibit unique biological activities, different from those of organic and inorganic compounds, which are based on their unique three-dimensional structures and electronic states (Fujiwara et al., 2005; Murakami et al., 2015; Kohri et al., 2015). We previously reported that copper(II) bis(diethyldithiocarbamate), termed Cu10, activates Nrf2, while copper ion, diethyldithiocarbamate ligand with or without zinc or iron complex failed to exhibit this activity (Fujie et al., 2016a). It is also likely that organic-inorganic hybrid molecules exhibit their activities by modifying the activities of ligands, those of metals, or interaction between ligands and metals.

Metallothionein (MT) was isolated from equine tissue as a cadmium-binding protein (Margoshes and Vallee, 1957). They belong to a family of low molecular weight and cysteine-rich proteins with high affinity for heavy metal ions (Margoshes and Vallee, 1957). Human MT genes are located on chromosome 16 and are classified as four isoforms: MT-1 and -2 are expressed ubiquitously, MT-3 is expressed in neural tissues, and MT-4 is expressed in the stratified squamous epithelia (Stennard et al., 1994; Yeiser et al., 1999; Quaife et al., 1994). MT-1 and MT-2 expression is induced by cadmium, zinc, and copper, and these proteins protect against cytotoxicity induced by these metals (Kägi, 1991; Sato and Bremmer, 1993).

Metal response element (MRE)-binding transcription factor-1 (MTF-1) is essential for the induction of MT (Heuchel et al., 1994). MTF-1 requires zinc ions for its binding to the MRE in the promoter region of MT (Redtke et al., 1993; Zhang et al., 2001). This mechanism is essential for the induction of MT isoforms (Bittel et al., 1998). However, in vascular endothelial cells, inorganic zinc is unable to induce endothelial MT (Kaji et al., 1992;

submitted). Moreover, we previously showed that the expression of endothelial MT is not induced only by activation of the MTF-1–MRE pathway using a zinc complex, bis(L-cysteinato)zincate(II) (submitted). Thus, the mechanisms of MT induction in vascular endothelial cells remain unclear.

NF-E2 related factor 2 (Nrf2), a transcription factor, is regarded as a major regulator of numerous phase 2 detoxification and antioxidant enzymes (Itoh et al., 1997). Nrf2 regulates its downstream genes by binding to the antioxidant response element (ARE) in the promoter region of the genes (Motohashi et al., 2002). AREs exist in the promoter region of MTs (Ohtsuji et al., 2008). We previously showed that Cu10 increases Nrf2 expression by proteasome inhibition as well as binding to Kelch-like ECH-associated protein 1, which induces heme oxygenase-1, NAD(P)H quinone oxidoreductase 1, and gamma-glutamate-cysteine ligase, modifier subunit (Fujie et al., 2016a). We postulate that the induction of endothelial MT expression involves the Nrf2–ARE pathway as well as the MTF-1–MRE pathway and that Cu10 is a good agent to analyze the relathionship between Nrf2 activation and MT induction. In the present study, we examined whether Cu10 induces endothelial MT or not. Since Cu10 induces the expression of mRNAs for MT-1A, MT-1E, and MT-2, we investigated the involvement of the MTF-1–MRE and Nrf2-ARE pathways in MT isoform expression in vascular endothelial cells. It was found that the induction mechanism depends on the MT isoform.

5. 3. Results

Cu10 induces MT-1/2 protein expression in vascular endothelial cells

The structure of Cu10 is shown in Fig. 5-1A. When vascular endothelial cells were treated with Cu10 at 10 μM and below, the expression of MT-1/2 protein was increased in a concentration-dependent manner (Fig. 5-1B). Cu10 at 10 μM increased the expression of MT-1/2 protein in a time-dependent manner (Fig. 5-1C). However, copper sulfate and Na01, which is the ligand of Cu10, at 50 μM and below did not affect MT-1/2 expression (Fig. 5-1D), indicating that the formation of a complex between copper and diethyldithiocarbamate is required for the induction of MT-1/2 expression by Cu10 and that copper ion or diethyldithiocarbamate ligand alone is unable to induce MT-1/2 protein expression in vascular endothelial cells. Since Cu10 induces MT-1/2 protein expression in vascular endothelial cells, *MT-1/2* mRNA expression was examined. Cu10 at 10 μM and below increased the expression of *MT-1A, MT-1E*, and *MT-2A* mRNAs in a concentration-dependent manner (Fig. 5-2A).

Activation of the MTF-1−MRE and Nrf2-ARE pathway

We investigated the effect of Cu10 on MRE- and ARE-driven transcriptional activity in vascular endothelial cells. Cu10 increased MRE-driven transcriptional activity in a concentration-dependent manner (Fig. 5-3A). Under the same conditions, Cu10 increased ARE-driven transcriptional activity (Fig. 5-3B), suggesting that Cu10 activates the Nrf2-ARE pathway as well as the MTF-1−MRE pathway.

MTF-1−MRE pathway-mediated MT-1/2 expression by Cu10

In order to examine whether activation of the MTF-1−MRE pathway affects MT-1/2 protein expression induced by Cu10 in vascular endothelial cells, we analyzed the mRNA expression levels of MT isoforms induced by Cu10 in cells transfected with MTF-1 siRNA.

In the MTF-1 knocked down cells (Fig. 5-4A), the Cu10-induced MT-1/2 protein expression was suppressed (Fig. 5-4B). The expression of *MT-1A*, *MT-1E,* and *MT-2A* mRNA was also attenuated in MTF-1 knocked down cells (Fig. 5-4C). Cu10 induced the expression of endothelial MT-1/2 isoforms via the MTF-1−MRE pathway.

Nrf2–ARE pathway-mediated MT-1 induction by Cu10

When Nrf2 expression was silenced by the transfection of an Nrf2 siRNA in vascular endothelial cells (Fig. 5-5A), the expression of MT-1/2 protein was suppressed (Fig. 5-5B). In Nrf2 knocked down cells, Cu10-increased *MT-1A/1E* mRNA expression was inhibited (Fig. 5-5C). However, *MT-2A* mRNA expression was not affected by Nrf2 knockdown. These data suggest that Cu10-induced MT-1 expression is regulated by the Nrf2–ARE pathway as well as the MTF-1–MRE pathway, whereas MT-2 is not regulated by the Nrf2–ARE pathway in vascular endothelial cells.

5. 4. Discussion

The MTF-1-MRE pathway is essential for the transcriptional induction of MT expression (Heuchel et al., 1994). However, we recently reported that bis(L-cysteinato)zincate(II), a specific zinc ion donor for MTF-1 (Kimura et al., 2012), activates MTF-1 in vascular endothelial cells, but does not induced endothelial MT expression (Fujie et al., 2016c). In this study, the following results were obtained: (1) Cu10 induces MT-1/2 expression in vascular endothelial cells; (2) Cu10 activates both the MTF-1–MRE and Nrf2–ARE pathways; and (3) the MTF-1–MRE pathway regulates both MT-1 and -2 expression, whereas the Nrf2–ARE pathway only regulates MT-1, not but MT-2 expression. These results suggest that the expression of all isoforms of endothelial MT is upregulated by the MTF-1–MRE pathway, while the Nrf2–ARE pathway is involved in the upregulation of MT-1A and MT-1E expression in cooperation with the MTF-1–MRE pathway. Since the induction mechanism is different for each isoform, it is postulated that the original function of MTs is also isoform-dependent. For example, MT-1, of which expression is regulated by Nrf2, mainly plays a cytoprotective role against heavy metals or oxidative stress, whereas the original function of MT-2 regulates zinc metabolism.

In a recent study, we demonstrated that zinc(II) bis(diethyldithiocarbamate), which is a zinc complex in which the copper in Cu10 is substituted by zinc, also induces endothelial MT expression independently of the Nrf2-ARE pathway (Fujie et al., 2016a), suggesting that Nrf2 activation may not be necessary for the induction of endothelial MT expression by the zinc complex. On the other hand, sulforaphane, a specific activator of Nrf2, alone did not induce MT-1/2 protein expression and sulforaphane-increased *MT-1A/1E/2A* mRNA expression was low compared to that induced by Cu10 in vascular endothelial cells (data not shown). Taken together, these data suggest that the activation of the Nrf2-ARE pathway alone is not sufficient to induce endothelial MT-1 expression. It was recently reported that Nrf2 directly interacts with histone acetyltransferases (e.g., p300), histone deacetylases (HDACs), and

chromatin remodeling factors (e.g., brahma regulated gene 1) (Sun et al., 2009; Martin et al., 2014; Zhang et al., 2006). Nrf2 may form a multiple complex with other proteins. We postulate that AREs are necessary to maintain the chromatin structure in the *MT-1* promoter region, and that Nrf2 binding to AREs, accompanied with the enzyme for (de)acetylation of histone or the chromatin remodeling factor, enhanced the activity of the *MT-1* promoter.

MTF-1 consists of six C_2H_2 zinc finger motifs and only binds zinc ions (Bittel et al., 1998). Since Cu10 activated the MTF-1-MRE pathway in vascular endothelial cells, Cu10 should supply zinc ions for the zinc finger domains of MTF-1. However, the source of zinc ions remains unclear. A family of zinc transporters (ZIPs), classified as fourteen isoforms, was identified (Grotz et al., 1998). ZIP9 and ZIP13 are expressed in the Golgi apparatus (Taniguchi et al., 2013). ZIP7 is expressed in the endoplasmic reticulum (Taylor et al., 2004 and the others are expressed in the cellular membrane (Fukada et al., 2011). Since they are responsible for upregulating the concentration of zinc ions in the cytoplasm, ZIPs may be involved in the induction of MT expression via MTF-1 activation. In conclusion, we partially identified the mechanisms underlying endothelial MT expression using a copper complex. The present data support the hypothesis that there may be a functional differentiation between MT-1 and MT-2. Organic-inorganic hybrid molecules may be useful tools to identify unknown biological mechanisms.

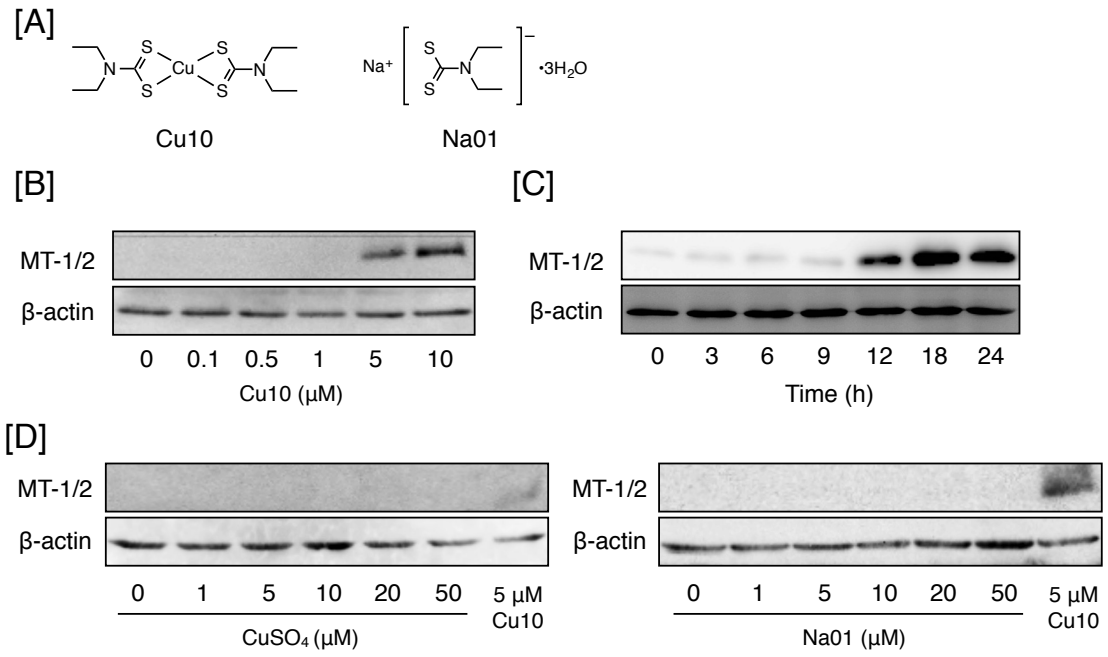

Fig. 5-1. Induction of MT-1/2 protein expression by Cu10 in vascular endothelial cells. [A] Structures of Cu10 and Na01. [B] Induction of MT-1/2 protein expression. Confluent cultures of bovine aortic endothelial cells were incubated at 37°C for 24 h in the presence or absence of Cu10 (0.1, 0.5, 1, 5, or 10 μM). [C] Time course of Cu10 induction of MT-1/2 protein expression. Confluent cultures of bovine aortic endothelial cells were incubated at 37°C for 3, 6, 9, 12, 18, and 24 h in the presence or absence of Cu10 (10 μM). [D] Role of copper in the Cu10 molecule in the induction of MT-1/2 expression. Confluent cultures of bovine aortic endothelial cells were incubated at 37°C for 24 h in the presence or absence of Copper sulfate (left panel) or Na01 (right panel) (1, 5, 10, 20, or 50 μM).

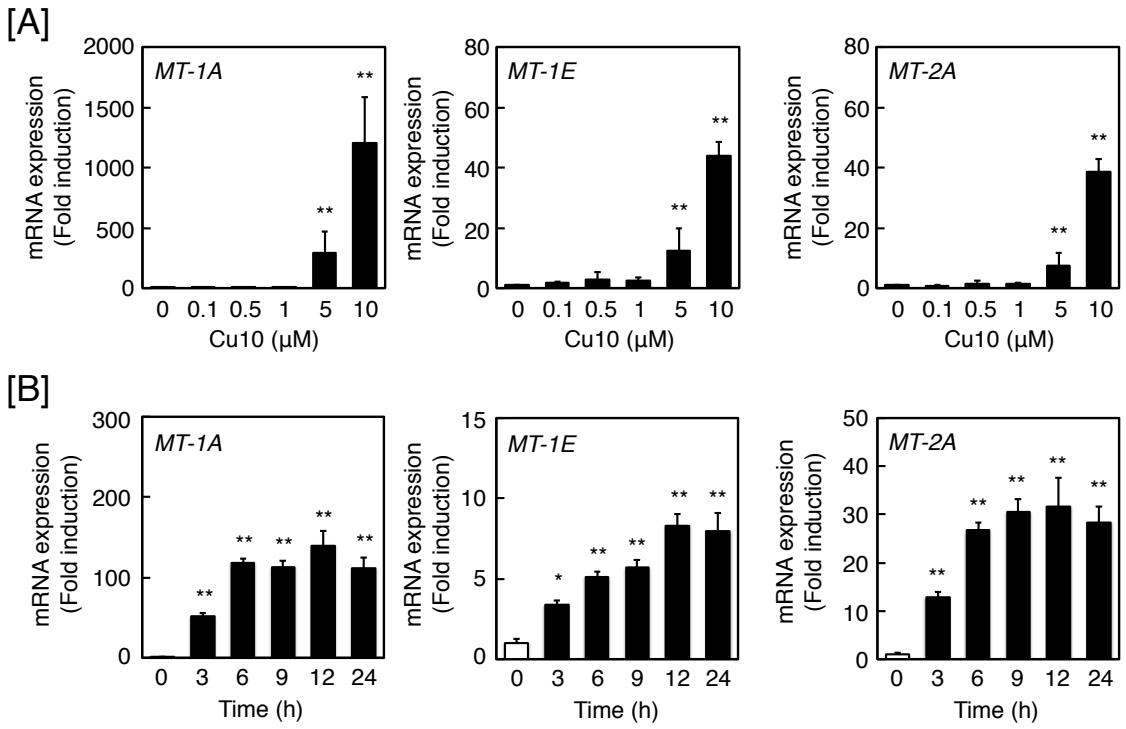

Fig. 5-2. Transcriptional induction of *MT-1A, MT-1E,* and *MT-2A* mRNA expression in vascular endothelial cells after treatment with Cu10. Bovine aortic endothelial cells were incubated [A] with or without 0.1, 0.5, 1, 5, or 10 μM Cu10 for 24 h or [B] for 3, 6, 9, 12, or 24 h with or without 10 μM Cu10. Data are represented as mean ± S.E. of three samples. Statistical significance when compared to the corresponding control: * $P < 0.05$, ** $P < 0.01$.

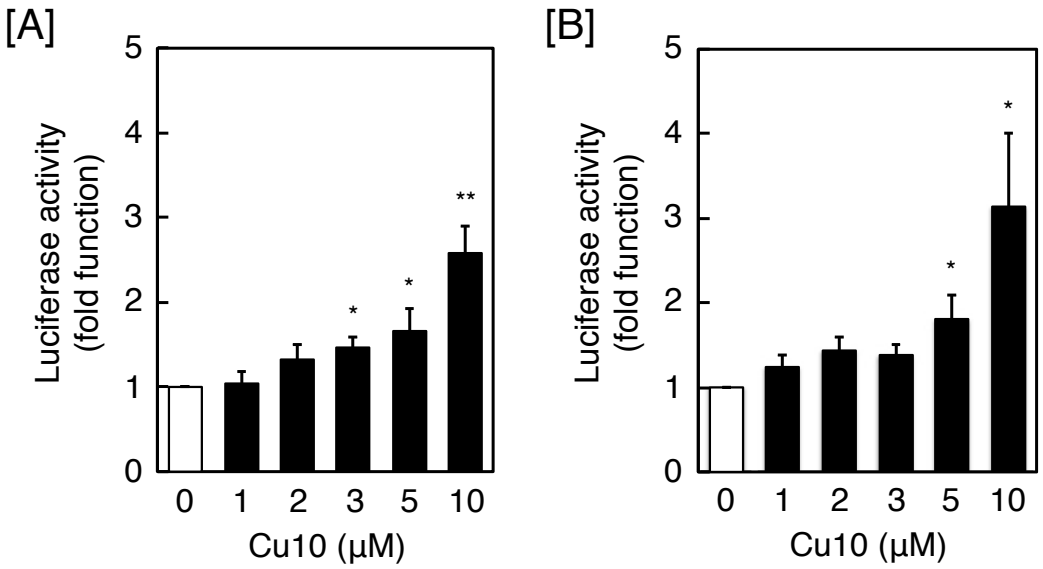

Fig. 5-3. Activation of the MTF-1–MRE and Nrf2-ARE pathways by Cu10 in vascular endothelial cells. Bovine aortic endothelial cells were transfected with [A] an MRE or [B] ARE reporter vector and incubated with or without 1, 2, 3, 5, or 10 μM Cu10 for 8 h. Data are represented as mean ± S.E. of four samples. Statistical significance when compared to corresponding control: * $P < 0.05$, ** $P < 0.01$.

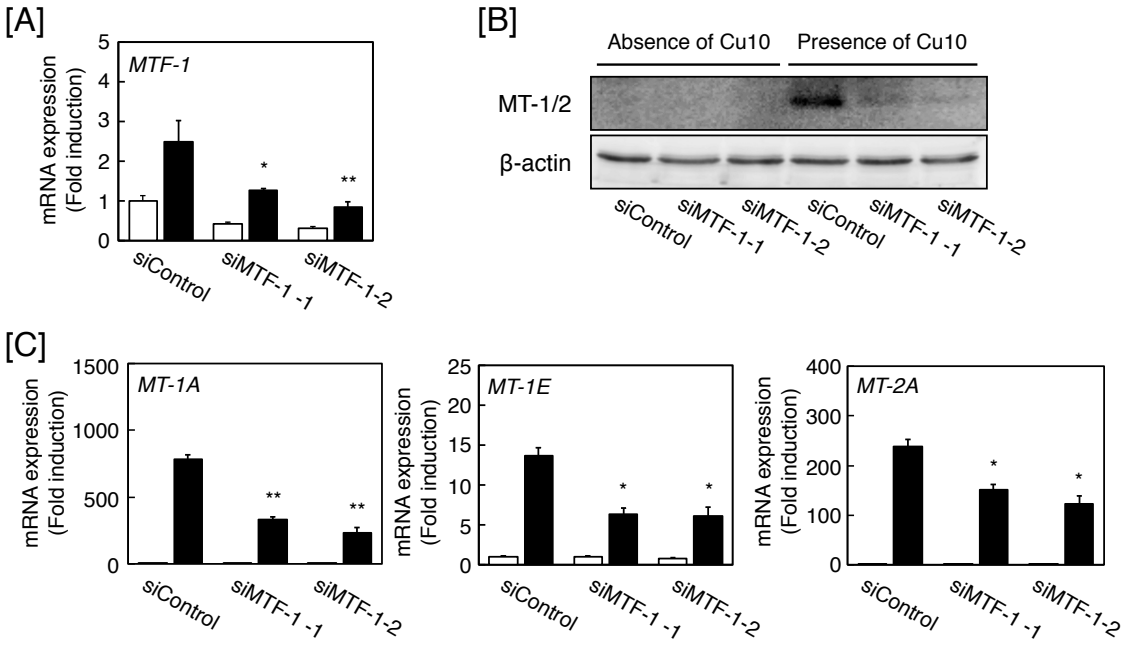

Fig. 5-4. Involvement of the MTF-1–MRE pathway in the induction of MT-1/2 protein expression by Cu10 in vascular endothelial cells. [A] *MTF-1* mRNA expression after siRNA-mediated knockdown of MTF-1. Bovine aortic endothelial cells were transfected with control or MTF-1 siRNA and incubated with (white bars) or without (black bars) 5 μM Cu10 for 12 h. [B] Induction of MT-1/2 protein expression by Cu10 after MTF-1 knockdown. Bovine aortic endothelial cells were transfected with control or MTF-1 siRNA and incubated with or without 5 μM Cu10 for 24 h. [C] Transcriptional induction of *MT-1A*, *MT-1E*, and *MT-2A* mRNA expression by Cu10 after MTF-1 knockdown. Bovine aortic endothelial cells were transfected with control or MTF-1 siRNA and incubated with (white bars) or without (black bars) 5 μM Cu10 for 12 h. Data are represented as mean ± S.E. of three samples. Statistical difference when compared to the corresponding "siControl": ** $P < 0.01$.

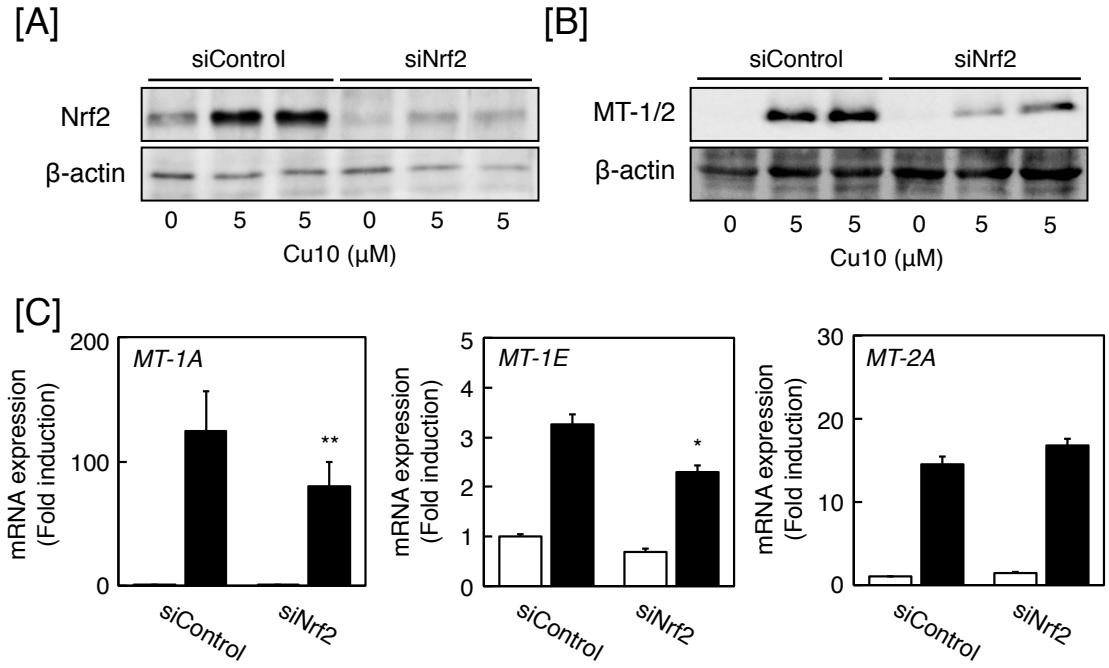

Fig. 5-5. Involvement of the Nrf2–ARE pathway in the induction of MT-1/2 protein expression by Cu10 in vascular endothelial cells. [A] Nrf2 protein expression after siRNA-mediated knockdown of Nrf2. Bovine aortic endothelial cells were transfected with control or Nrf2 siRNA and incubated with or without 5 μM Cu10 for 3 h. [B] Induction of MT-1/2 protein expression by Cu10 after Nrf2 knockdown. Bovine aortic endothelial cells were transfected with control or Nrf2 siRNA and incubated with or without 5 μM Cu10 for 24 h. [C] Transcriptional induction of *MT-1A*, *MT-1E*, and *MT-2A* mRNA expression by Cu10 after Nrf2 knockdown. Bovine aortic endothelial cells were transfected with control or Nrf2 siRNA and incubated with (white bars) or without (black bars) 5 μM Cu10 for 12 h. Data are represented as mean ± S.E. of three samples. Statistical difference when compared to the corresponding "siControl": ** $P < 0.01$.

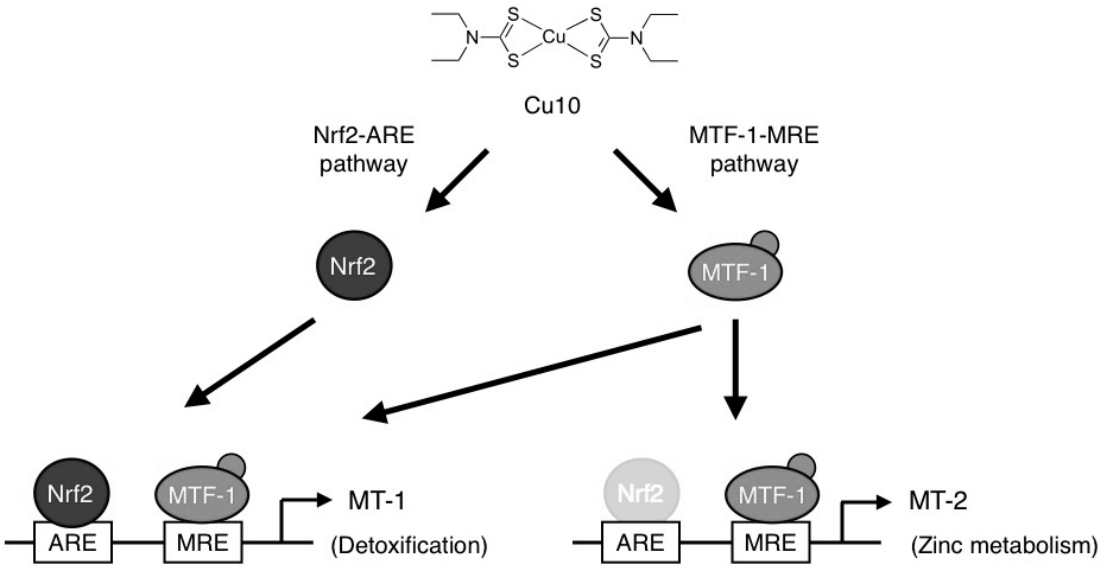

Fig. 5-6. A possible mechanism of MT-1 and MT-2 induction by Cu10 in vascular endothelial cells.

Genereal Conclusion

MT plays a central role in zinc metabolism and the cellular protection against the cytotoxicity of heavy metals and oxidative stress. However, the mechanisms underlying MT induction are not fully understood. In this study, organic-inorganic hybrid molecules were used to analyze the signaling pathway that is involved in the induction of MT subisoforms and the following results were obtained.

1. $Zn(edtc)_2$ as an inducer of endothelial MT

Inorganic zinc, in general a representative MT inducer, did not alter the expression of MT protein and gene, and did not affected MTF-1–MRE and Nrf2–ARE pathway, suggesting that inorganic zinc does not act on any steps of endothelial MT induction and is not a good tool to analyze the mechanisms underlying endothelial MT induction. $Zn(cys)_2$, a specific donor of zinc ion to MTF-1, activated MRE promoter activity but did not induce MT, suggesting that endothelial MT is not induced only by activation of the MTF-1–MRE pathway. Another zinc complex $Zn(edtc)_2$ as well as $Zn(cys)_2$ activated the MTF-1–MRE pathway without Nrf2–ARE pathway; however, the zinc complex induced endothelial MT protein. These results suggested that organic-inorganic hybrid molecules are useful to analyze the regulation of MT induction in vascular endothelial cells.

2. Cytotoxicity of organobismuth/antimony compounds

To obtain orhanic-inorganic hybrid molecules as good tools to analyze endothelial MT induction, the cytotoxicity of the compounds was first investigated. Organobismuth compounds, 2-(*N,N*-dimethylaminomethyl)phenylbis(4-methylphenyl)- bismuthane (DAPBi) and *Bi*-phenyl-*N*-methyl-5,6,7,12-tetrahydrodibenz[*c,f*][*1,5*]aza- bismocine (PMTABi) exhibited cytotoxicity. The cytotoxicity was diminished upon replacing the intramolecular bismuth atom with an antimony atom with a lower intracellular accumulation. These results

suggested that organobismuth compound with certain molecular structures exhibit cytotoxicity via interaction between the molecular structure and the bismuth atom. Next, an characteristicsof of cytotoxicity of organoantimony compounds were investigated but no distinct structure-cytotoxicity relationship was seen. However, we could obtain an organoantimony compound, tris(pentafluorophenyl)stibane (Sb35) that cause a transcriptional induction of MT without cytotoxicity in vascular endothelial cells from a library of 48 organoantimony compounds.

3. Transcriptional induction of endothelial MT by Sb35

Sb35 induced gene expression of all MT-1/2 isoforms and activated both MTF-1–MRE and Nrf2–ARE pathways in vascular endothelial cells. Sb35-increased expression of MT-1/2 genes was suppressed by siRNA-mediated knockdown of MTF-1, suggesting that MTF-1–MRE pathway is essentially involved in the induction of both MT-1 and MT-2. When Nrf2 was knocked down, Sb35-increased expression of MT-1 gene was reduced, while that of MT-2 gene was not changed, indicating that the Nrf2–ARE pathway is involved in the induction of only MT-1. Similar results were obtained when transcriptional induction of endothelial MT-2A isoform was induced by Tris(pentafluorophenyl)phosphane. Since Nrf2 is a transcription factor for cellular defense mechanisms, these results suggest that MT-1participates in the biological defense system, while MT-2 mainly regulates intracellular zinc metabolism.

4. Cu10 as an activator of Nrf2 in vascular endothelial cells

Since the Nrf2–ARE pathway is importantly involved in the regulation of endothelial MT induction, we sought organic-inorganic hybrid molecules that activate Nrf2 and found copper(II) bis(diethyldithiocarbamate) (Cu10) as an activator Nrf2 in vascular endothelial cells. Neither copper ion nor diethyldithiocarbamate ligand exhibited such an activity. In addition, copper and iron complexes with other structures of the ligand could not activate

Nrf2, suggesting that the intramolecular interaction between copper and the dithiocarbamate ligand is important for the activation of the transcription factor. It was revealed that Cu10 activates Nrf2 through two mechanisms: one is binding to Keap1 and the other is inhibiting proteasome, both of which stabilize Nrf2.

5. Induction of endothelial MT isoforms by Cu10

Cu10 not only activated Nrf2 but also induced the expression of endothelial MT protein and mRNAs of all MT isoforms. In addition, the copper complex activated both MTF-1–MRE and Nrf2–ARE pathways in vascular endothelial cells. It was demonstrated that Cu10-increased MT-1 expression was up-regulated via activation of both MTF-1–MRE and Nrf2–ARE pathways, while only the MTF-1–MRE pathway participated in MT-2 gene expression, as did Sb35, supporting that hypothesis that MT-1 participated in the biological defense system, while MT-2 mainly regulates intracellular zinc metabolism.

In this study, the signaling pathways that participate in the induction of MT isoforms were clarified using organic-inorganic hybrid molecules. The results suggest that there may be a functional differentiation between MT-1 and MT-2 as stated above. Further studies should be performed to clarify the detailed mechanisms for endothelial MT induction including the source of zinc ion that activates MTF-1 and mechanisms underlying the recruitment of Nrf2 to specific ARE(s). Also in such studies, a biological research strategy using organic-inorganic hybrid molecules —bio-organometallics— appears to be effective to analyze unknown biological mechanisms.

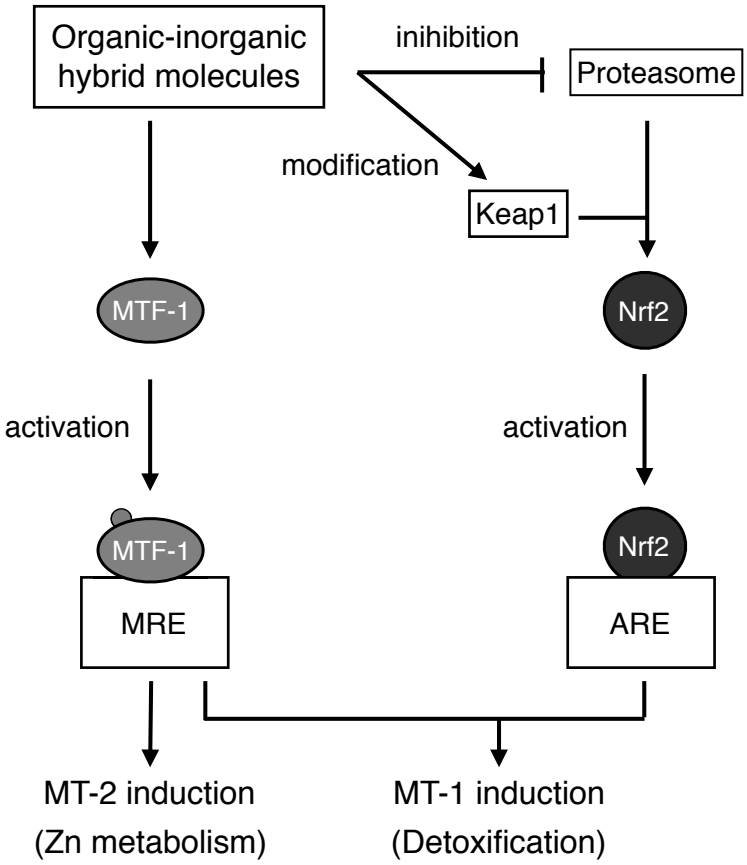

Fig. 6. A possible mechanisms of metallothionein isoforms induction by organic-inorganic hybrid molecules in vascular endothelial cells.

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