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Letter

Possible mechanisms underlying transcriptional induction of metallothionein isoforms by tris(pentafluorophenyl) stibane, tris(pentafluorophenyl)arsane, and tris(pentafluorophenyl)phosphane in cultured bovine aortic endothelial cells

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ABSTRACT — Metallothionein (MT) is a low-molecular-weight, cysteine-rich, and metal-binding protein that protects cells from the cytotoxic effects of heavy metals and reactive oxygen species. Previously, we found that transcriptional induction of endothelial *MT-1A* was mediated by not only the metal-regulatory transcription factor 1 (MTF-1)-metal responsive element (MRE) pathway but also the nuclear factor-erythroid 2-related factor 2 (Nrf2)-antioxidant response element/electrophile responsive element (ARE) pathway, whereas that of *MT-2A* was mediated only by the MTF-1-MRE pathway, using the organopnictogen compounds tris(pentafluorophenyl)stibane, tris(pentafluorophenyl)arsane, and tris(pentafluorophenyl)phosphane as molecular probes in vascular endothelial cells. In the present study, we investigated the binding sites of MTF-1 and Nrf2 in the promoter regions of *MT*s in cultured bovine aortic endothelial cells treated with these organopnictogen compounds. We propose potential mechanisms underlying transcriptional induction of endothelial MT isoforms. Specifically, both MRE activation by MTF-1 and that of ARE in the promoter region of the MT-2A gene by Nrf2 are involved in transcriptional induction of *MT-1A*, whereas only MRE activation by MTF-1 or other transcriptional factor(s) is required for transcriptional induction of *MT-2A* in vascular endothelial cells.

Key words: Metallothionein, Organometallic compound, Endothelial cell, Metal response element, Antioxidant response element

INTRODUCTION

Metallothionein (MT) is a low-molecular-weight, cysteine-rich, and metal-binding protein (Kägi and Vallee, 1960). MT is induced by heavy metals and protects cells against the cytotoxicity of heavy metals and reactive oxygen species and is involved in zinc homeostasis (Davis and Cousins, 2000). Additionally, MT has anti-inflammatory effects and thus is considered a cytoprotective protein. Although inorganic zinc is a typical inducer

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of MT, we demonstrated that inorganic zinc cannot induce MT in vascular endothelial cells, which cover the luminal surface of blood vessels (Kaji *et al*., 1992; Fujie *et al*., 2016a).

It is thought that the metal response element (MRE)-binding transcription factor-1 (MTF-1) is crucially involved in MT induction. The transcriptional factor is activated when zinc ion binds to the zinc finger domains of the molecule (Bittel *et al*., 1998) and activated MTF-1 binds to the MRE, the consensus sequences of which are in MT gene promoter regions (Heuchel *et al*., 1994). In contrast, the antioxidant response element (ARE) contains consensus sequences and is activated by the transcription factor nuclear factor-erythroid 2-related factor 2 (Nrf2) (Itoh *et al*., 1997; Ohtsuji *et al*., 2008) in the MT gene promoter regions, although its role in MT isoform induction remains unclear.

We found that Nrf2 contributes in part to inducing MT by cadmium in cultured bovine aortic endothelial cells (Shinkai *et al*., 2016). However, because cadmium induces the expression of both isoforms of MT, MT-1 and MT-2, it is difficult to analyze the detailed mechanisms. Recently, in a study of bioorganometallics (Fujie *et al*., 2016b), we found that tris(pentafluorophenyl)stibane (Sb35), tris(pentafluorophenyl)arsane (As35), and tris(pentafluorophenyl)phosphane (P35) cause transcriptional induction of endothelial MT (Fujie *et al*., 2016c).

Specifically, both Sb35 and As35 induce the expression of *MT-1A* and *MT-2A*, whereas P35 increases the expression of only MT-2A in cells. Transcriptional induction of endothelial *MT-1A* was mediated by not only the MTF-1-MRE pathway but also the Nrf2- ARE pathway, whereas that of *MT-2A* was mediated only by the MTF-1-MRE pathway (Fujie *et al*., 2016c, 2016d). In the present study, we investigated the binding sites of MTF-1 and Nrf2 activated by Sb35, As35, and P35 in the promoter regions of MTs in cultured vascular endothelial cells.

MATERIALS AND METHODS

Materials

Bovine aortic endothelial cells were purchased from Cell Applications (San Diego, CA, USA). The following materials were also used: Dulbecco's modified Eagle's medium (DMEM) and calcium- and magnesium-free phosphate buffered saline (CMF-PBS) from Nissui Pharmaceutical (Tokyo, Japan); fetal bovine serum, Halt™ Protease Inhibitor Cocktail (100×), and Sheared Salmon Sperm DNA from Thermo Fisher Scientific (Waltham, MA, USA); Protein G beads from Tamagawa Seki (Nagano, Japan); and GeneAce SYBR qPCR Mixα from Nippon Gene (Tokyo, Japan). Other reagents were purchased from Nacalai Tesque (Kyoto, Japan).

Synthesis of Sb35, As35, and P35

Sb35, As35, and P35 were synthesized as described previously (Fild *et al*., 1964; Kant *et al*., 2008; Schäfer *et al*., 2011; Jiang *et al*., 2013).

Cell culture and treatment

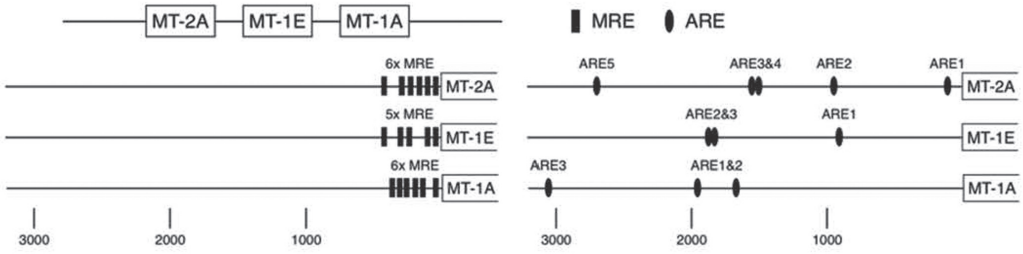
Bovine aortic endothelial cells were cultured at 37°C in 5% CO_2 in DMEM supplemented with 10% fetal bovine serum until confluence. The medium was removed, and the cells were washed twice with serum-free DMEM; the cells were exposed to Sb35 (50, 100, and 200 μM), As35 (5, 10, and 20 μM), or P35 (5, 10, and 20 μM) in serum-free DMEM for 3 hr at 37°C. Because the organopnictogen compounds used in this study were insoluble in water, they were dissolved in dimethyl sulfoxide and then added to the culture medium; the final concentration of dimethyl sulfoxide was less than 0.5%.

Chromatin immunoprecipitation (ChIP) assay

Bovine aortic endothelial cells were treated with Sb35, As35, or P35 for 3 hr. The DNA-proteins were cross-linked by incubating the cells with 1% formaldehyde at 37°C for 10 min. Excess formaldehyde was quenched with 0.125 M glycine at 37°C for 5 min. After washing with ice-cold CMF-PBS, the cells were scraped in ice-cold CMF-PBS and collected by centrifugation. The pellet was lysed in sodium dodecyl sulfate (SDS)-lysis buffer (50 mM Tris-HCl, 10 mM EDTA, 1% SDS, pH 8.1) supplemented with protease inhibitors and sonicated for 75 cycles (3-s pulse and 1-s rest, on ice) using an Ultrasonic Homogenizer (Microtec, Chiba, Japan). Sheared chromatin was diluted in ChIP dilution buffer (25 mM Tris-HCl, 150 mM sodium chloride, 1% Triton X-100, 0.1% sodium deoxycholate, 2 mM EDTA, pH 8.1) containing salmon sperm DNA (0.5 mg/mL) and a portion of the chromatin solution was stored as input DNA. The remaining chromatin was immunoprecipitated using protein G beads coated with normal rabbit IgG (#2729; Cell Signaling, Danvers, MA, USA), Nrf2 (NBP1-32822; Novus Biologicals, Littleton, CO, USA), or MTF-1 (NBP1-86380; Novus Biologicals) antibodies for 6 hr at 4°C. After washing, protein-DNA complexes were eluted from the antibody with elution buffer (50 mM Tris-HCl, 10 mM EDTA, 1% SDS, pH 8.1) and reverse cross-linked by heating overnight at 65°C. The DNA samples were purified using proteinase K treatment followed by phenol-extraction and ethanol precipitation, and then analyzed by real-time PCR using GeneAce SYBR qPCR

Table 1. Primers used for ChIP assay.

Target region		Forward primer (5′→ 3′)	Reverse primer (5′→ 3′)
MT-1A	MRE	ACAAAGTTCCTCCGTGTTGG	GTCGGATGGGAGGCGTGCTG
MT-1E	MRE	AGACTCTTGCGCTGGGCTC	CTCGCTCGCTGGGTTTGTAA
MT-2A	MRE	ACGTAGGGCGGCTTCTGGGA	GCAAAGGATGGCGAGGGTGTC
GCLM	ARE	GGGACGGGTAACGGTTAGCAA	CGGTAGAGTGGACTCCCAACTGA
MT-1A	ARE1&2	TCTCACCTCCTGGCCACACA	AGCACTGAGCTGGCAGTCAGAA
	ARE3	GGCTTGCCCAGGGTCAC	ACAATCACTTTGCTGCTTTCG
MT-1E	ARE1	ATGATGCTGCCACACCATTTG	GGCGAGTAGGTGAGACAGCTATAGA
	ARE2&3	CACTTCCTGCCACATATCCTGA	CCTGGTTAGGCCACAGTCCA
MT-2A	ARE1	CGTGTGCACAGCTCGGTGA	GGAGCTGGGACGAGTGCAAA
	ARE2	CCATCCTAGCCACGACTCTGGTA	CCAACAGATGCTGAAGTCCCTCTA
	ARE3&4	CACAGGAAGCACCAGGAAGGAA	TCTTCACAGTTCAGCTCACACATCA
	ARE5	TAAGGATACTGTCACAGGGGAAAGA	GTGTGAGTCATCAGTGTGAGGCAA



MRE consensus TGCA/GCNC

MT-2A	MRE1	-52	TTT**TGCACTC**GTC	-81
	MRE2	-126	CTG**TGCACAC**GGG	-138
	MRE3	-209	CTC**TGCGCCC**GGT	-197
	MRE4	-230	CCC**TGCACGC**GGT	-218
	MRE5	-260	ACC**TGCGCTC**AGC	-272
	MRE6	-282	CTC**TGCACAC**GCT	-270
MT-1E	MRE1	-71	CTC**TGCGCCC**GGG	-45
	MRE2	-68	GCC**TGCACCC**TCC	-80
	MRE3	-152	TCT**TGCGCTC**GGC	-140
	MRE4	-176	CTG**TGCACAC**GGA	-188
	MRE5	-460	CTC**TGCACAC**GAC	-448
MT-1A	MRE1	-54	CTC**TGCGCCC**GGC	-42
	MRE2	-66	GCC**TGCACCC**GCG	-78
	MRE3	-84	CGC**TGCGCCC**GGG	-96
	MRE4	-132	TCT**TGCGCTC**GGC	-120
	MRE5	-165	CGG**TGCACAC**GGA	-153
	MRE6	-435	CTC**TGCACAC**AAC	-423

ARE consensus TGAC/GNNNGC

MT-2A	ARE1	-103	CAG**TGAC**TCAG**C**GCG	-89
	ARE2	-957	CAG**TGAG**GCTG**C**CCC	-943
	ARE3	-1536	AGC**TGAC**CTAG**C**CGC	-1550
	ARE4	-1548	CGC**TGAC**TTAG**C**CTG	-1562
	ARE5	-2731	GGA**TGAC**TCAG**C**AGT	-2717
MT-1E	ARE1	-918	AGG**TGAG**ACA**GC**TAT	-932
	ARE2	-1848	ATA**TGAC**TGA**GC**GAT	-1862
	ARE3	-1869	AAG**TGAC**ATC**GC**TCA	-1865
MT-1A	ARE1	-1736	CAC**TGAG**CTGG**C**AGT	-1750
	ARE2	-1908	AGG**TGAG**ACA**GC**TGT	-1922
	ARE3	-3099	TGG**TGAG**TTGG**C**TTA	-3085

Fig. 1. Map of MRE and ARE regions in bovine MT promoter and corresponding MRE and ARE sequences.

Mixα on a StepOnePlus RT-PCR system (Thermo Fisher Scientific). The thermal cycling parameters were 50°C for 2 min, 95°C for 10 min, and 50 cycles of 95°C for 15 sec and 60°C for 1 min. The primers for ChIP analysis are listed in Table 1. Relative DNA levels were calculated from a standard curve using serial dilutions of input DNA and normalized with input DNA from a corresponding sample.

RESULTS AND DISCUSSION

A map of the MRE and ARE regions in the bovine *MT* isoforms and sequences of MRE and ARE in the promoter of the bovine *MT-1A*, *MT-1E*, and *MT-2A* genes are shown in Fig. 1. We tested bovine *MT-1A*, *MT-1E*, and *MT-2A* promoter regions containing six, five, and six, respective-

ly, MRE sequences and three, three, and five, respectively, ARE sequences.

The molecular structures of Sb35, As35, and P35 are shown in Fig. 2A. We found that MTF-1 was recruited to the MREs of the promoter regions of *MT-1A*, *MT-1E*, and *MT-2A* by Sb35 and As35 (Figs. 2B and 2C, respectively), whereas P35 failed to recruit MTF-1 to the MREs (Fig. 2D). Previously, we reported that P35 as well as Sb35 and As35 induce the expression of endothelial MT-2A by activating MRE (Fujie *et al*., 2016c). Therefore, our present and previous data suggest that Sb35 and As35 activate MTF-1, leading to the activation of MREs in the promoter region of *MT*, whereas P35 also activates the MREs by transcriptional factor(s) other than MTF-1 such as hypoxia inducible factor-1α (Dubé *et al*., 2011). As a result, these organopnictogen compounds induce the expression of *MT-2A* in vascular endothelial cells. Activation of MREs specifically by MTF-1 may be required for

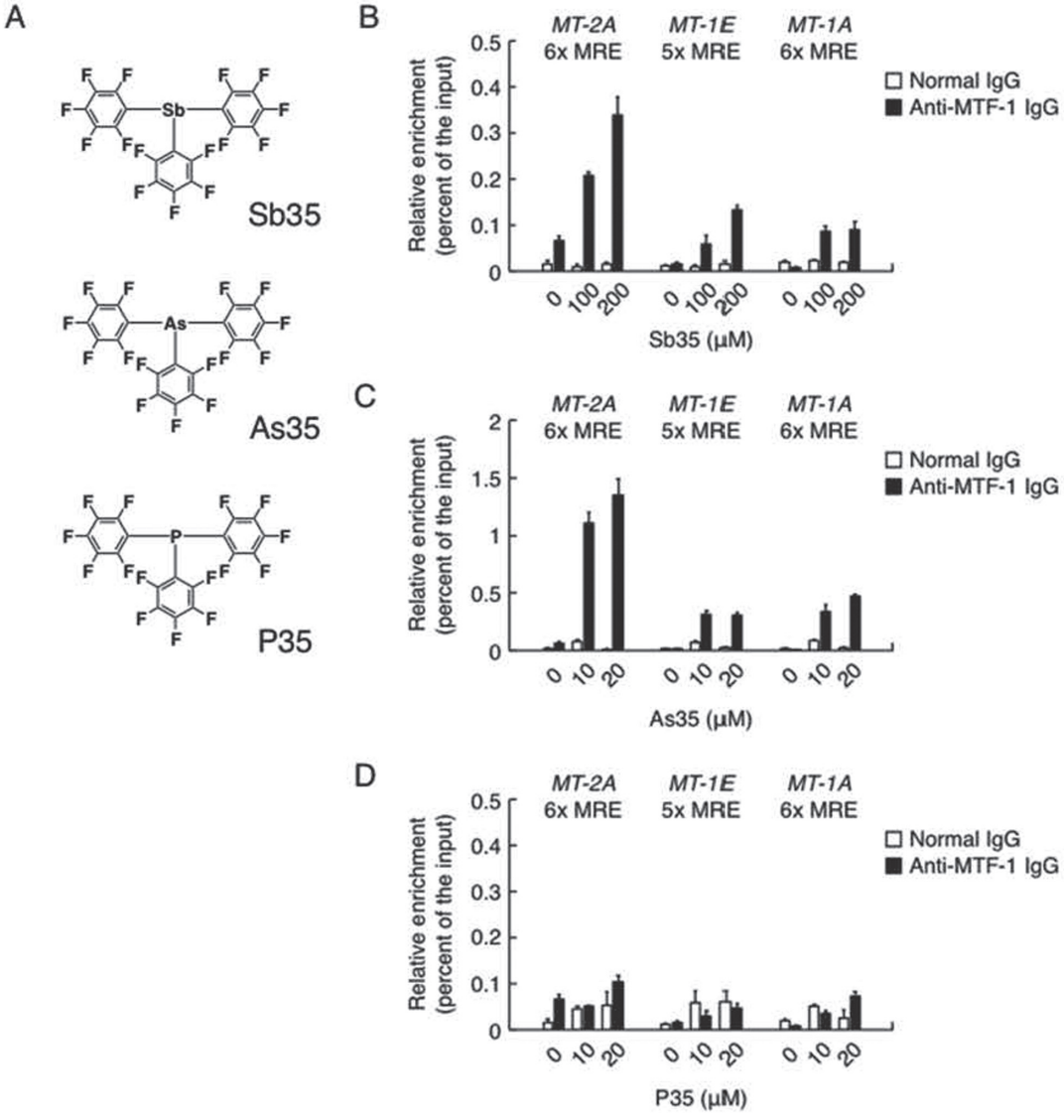


Fig. 2. Recruitment of MTF-1 to the promoter regions of *MT* genes in vascular endothelial cells. [A] Structures of organopnictogen compounds used in this study. Sb35, tris(pentafluorophenyl)stabane; As35, tris(pentafluorophenyl)arsane; P35, tris(pentafluorophenyl)phosphane. Bovine aortic endothelial cells were treated with or without [B] Sb35 (100 and 200 μM), [C] As35 (10 and 20 μM), or [D] P35 (10 and 20 μM) for 3 hr. Enrichment of the promoter DNA precipitated was determined by the ChIP assay with the indicated antibodies. Values represents the means ± SE of two independent experiments.

the expression of endothelial *MT-1A*, although P35 may promote the recruitment of MTF-1 to other MREs other than the *MT* promoter regions tested in this study.

Figure 3 shows the recruitment of Nrf2 to AREs in the promoter regions of *MT* genes in vascular endothelial cells. Treatment with Sb35, As35, and P35 significantly increased the recruitment of Nrf2 to AREs in the promoter region of *GCLM*, a target gene of Nrf2 in the cells (Fig. 3A), suggesting that these organopnictogen compounds activated Nrf2 as reported previously (Fujie *et al*.,

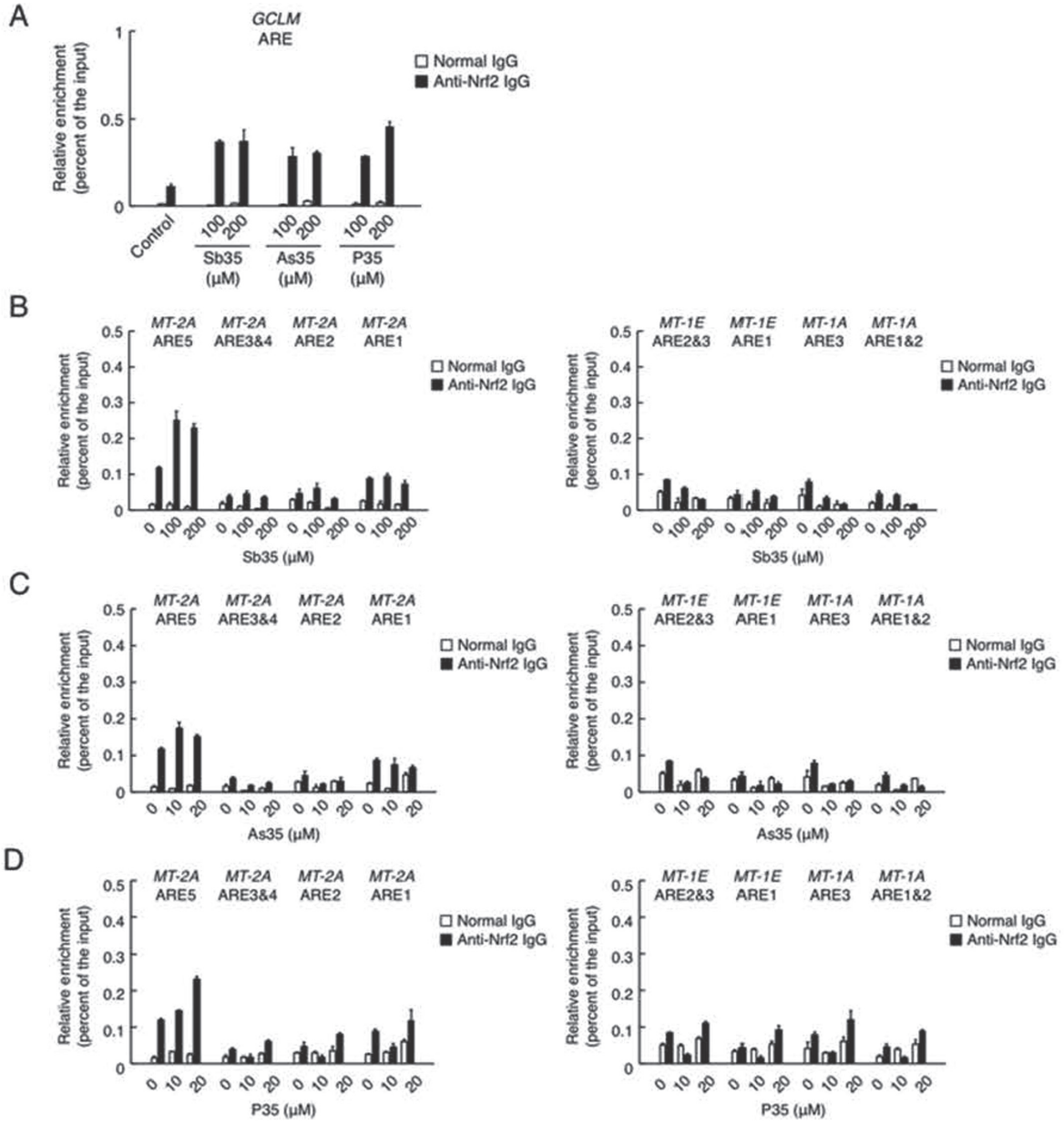


Fig. 3. Recruitment of Nrf2 to the promoter regions of *MT* genes in vascular endothelial cells. Bovine aortic endothelial cells were treated with or without [A] Sb35 (100 and 200 μM), As35 (10 and 20 μM), or P35 (10 and 20 μM), [B] Sb35 (100 and 200 μM), [C] As35 (10 and 20 μM), or [D] P35 (10 and 20 μM) for 3 hr. Enrichment of the promoter DNA precipitated was determined by the ChIP assay with the indicated antibodies. Values represents the means ± SE of two independent experiments.

2016c). It was demonstrated that Sb35 (Fig. 3B), As35 (Fig. 3C), and P35 (Fig. 3D) recruited Nrf2 to *MT-2A* ARE5 but not the other AREs of *MT-1A* and *MT-1E* in the *MT* promoter regions. Because transcriptional induction of endothelial *MT-1A* by Sb35 was mediated by the Nrf2-ARE pathway and MTF-1-MRE pathway (Fujie *et al*., 2016c), the involvement of the Nrf2-ARE pathway in inducing *MT-1A* may be mediated by the recruitment of Nrf2 to *MT-2A* ARE5 in vascular endothelial cells. P35, which activates MRE without recruiting MTF-1 to MREs and induces only *MT-2A* expression, also activates Nrf2 and promotes the recruitment of Nrf2 to *MT-2A* ARE5, supporting the hypothesis that endothelial *MT-1A* expression requires the activation of MREs specifically by MTF-1 but not the other transcriptional factors.

In conclusion, based on our previous and present data, we predicted the mechanisms underlying transcriptional induction of endothelial MT isoforms. Specifically, both MRE activation by MTF-1 and that of ARE in the promoter region of the MT-2A gene by Nrf2 are involved in the transcriptional induction of *MT-1A*, whereas only MRE activation by MTF-1 or other transcriptional factor(s) is required for transcriptional induction of *MT-2A* in vascular endothelial cells. It is possible that involvement of Nrf2 in inducing endothelial *MT-1A* is attributed to epigenetic regulation such as changes the chromatin structure around *MT*s, as it was reported that Nrf2 activation caused chromatin remodeling around the human heme oxygenase-1 promoter by Brahma-related gene 1 (Zhang *et al*., 2006; Maruyama *et al*., 2013). Although further studies are required to clarify the detailed mechanisms of endothelial MT isoform induction, our previous and present studies clearly showed that bioorganometallics, which can be used to analyze biological systems using organic-inorganic hybrid molecules (Fujie *et al*., 2016b), is useful for analyzing the mechanisms underlying MT induction in vascular endothelial cells.

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

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