DOCTORAL THESIS

Study on effects and mechanisms of methylmercury toxicity on neuronal and endothelial cells

(神経および血管内皮細胞に対するメチル水銀毒性の影響と作用機序に関する研究)

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ABSTRACT

The present thesis was designed to study the effects and mechanisms of methylmercury (MeHg) toxicity on neuronal and endothelial cells.

The first chapter report a study entitled“MARCKS is involved in MeHg-induced decrease in cell viability and nitric oxide production in EA.hy926 cells”.MeHg is a persistent environmental contaminant that has been reported worldwide. MeHg exposure has been reported to lead to increased risk of cardiovascular diseases; however, the mechanisms underlying the toxic effects of MeHg on the cardiovascular system have not been well elucidated. We have previously reported that mice exposed to MeHg had increased blood pressure along with impaired endothelium-dependent vasodilation. In this study, we investigated the toxic effects of MeHg on a human endothelial cell line, EA.hy926.Although it has been reported that the alteration in MARCKS expression or phosphorylation affects MeHg-induced neurotoxicity in neuroblastoma cells, the relationship between MeHg toxicity and MARCKS has not yet been determined in vascular endothelial cells. Therefore, in this study, we investigated the role of MARCKS in MeHg-induced toxicity in the EA.hy926 endothelial cell line. Cells exposed to MeHg (0.1–10 μM) for 24 hr showed decreased cell viability in a dose-dependent manner. Treatment with submaximal concentrations of MeHg decreased cell migration in the wound healing assay, tube formation on Matrigel and

spontaneous nitric oxide (NO) production of EA.hy926 cells. MeHg exposure also elicited a decrease in MARCKS expression and an increase in MARCKS phosphorylation. MARCKS knockdown or MARCKS overexpression in EA.hy926 cells altered not only cell functions, such as migration, tube formation and NO production, but also MeHg-induced decrease in cell viability and NO production. These results suggest the broad role played by MARCKS in endothelial cell functions and the involvement of MARCKS in MeHg-induced toxicity.

In the second chapter, the author report a study entitled"MARCKS protein amount is differently regulated by calpain during toxic effects of methylmercury between SH-SY5Y and EA.hy926 cells". We previously reported that amount of MARCKS protein in SH-SY5Y neuroblastoma and EA.hy926 vascular endothelial cell lines is decreased by treatment of MeHg, however, the mechanisms are not known. While, calpain, a Ca^{2+}-dependent protease, is suggested to be associated with the MeHg toxicity. Since MARCKS is known as a substrate of calpain, we investigated relationship between calpain activation and cleavage of MARCKS, and its role in MeHg toxicity. In SH-SY5Y cells, MeHg induced a decrease in cell viability accompanying calcium mobilization, calpain activation, and a decrease in MARKCS expression. However, pretreatment with calpain inhibitors attenuated the decrease in cell viability and MARCKS expression only induced by 1 μM but not by 3 μMMeHg. In cells with MARCKS-knockdown, calpain inhibitors failed to attenuate the decrease in cell

viability by MeHg. In EA.hy926 cells, although MeHg caused calcium mobilization and a decrease in MARCKS expression, calpain activation was not observed. These results indicated that involvement of calpain in the regulation of MARCKS was dependent on the cell type and concentration of MeHg. In SH-SY5Y cells, calpain-mediated proteolysis of MARCKS was involved in cytotoxicity induced by low concentration of MeHg.

Together, the present thesis revealed that 1) characteristics of MeHg toxicity on endothelial cells, 2) involvement of MARCKS on its toxicity, and 3) different toxic mechanism of MeHg between neuronal and endothelial cells. The results of our study suggest the broad role of MARCKS in endothelial cell functions and show that MARCKS is involved in MeHg-induced toxicity in endothelial cells. The results also indicated that the participation of calpain in the regulation of MARCKS amounts is dependent on the cell type and concentration of MeHg. These findings will stimulate and support further progress in research on toxic mechanisms of MeHg in central nervous system and cardiovascular system.

GENERAL INTRODUCTION

Inorganic mercury (Hg) is a heavy metal contaminant with potential for global mobilization following its give off from anthropogenic activities or natural processes [25]. In anaerobic environments, elementary mercury (Hg^0) can be biotransformed and methylated to methylmercury (MeHg) by sulphate and iron reducing bacteria, which is the most toxic form of Hg in the environment [12, 16, 18, 51]. From this microbial starting point, MeHg readily bioaccumulates up the food chain, with increased levels found at each trophic level [16]. As such, all seafood contains some MeHg, while apex predators; such as marine mammals, sharks and swordfish; generally have the highest (>0.5 mg Hg/kg body weight) MeHg levels [50, 90].

The studies about MeHg toxicity became ubiquitous and diversified since the outbreak of environmental catastrophes such as those in Minamata Bay in Kumamoto Prefecture in 1956, and later it occurred in the Agano River basin in Niigata Prefecture in the 1960s in Japan. Minamata disease is a neurotoxic syndrome caused by daily consumption of large quantities of fish/shellfish heavily contaminated with MeHg that had been discharged from chemical factory into rivers and seas [29]. In such episodes, as a consequence of MeHg exposure, the exposed individuals exhibit severe forms of neurological disease which include a collection of cognitive, sensory, and motor disturbance [20, 83]. The studies on MeHg toxicity

have tried to evaluate its impact on several ecosystems around the world, including places in Japan, Iraq, Canada, Africa, including Brazilian Amazon, and India [1, 30, 51], as well as to understand its toxicological effect on biological systems.

More than 90% of Hg in fish is presented as MeHg [3, 47]. MeHg in fish is largely bound with a ratio of 1:1 ratio to thiol groups (R-SH) of mainly protein incorporated cysteine (Cys) residues, and in the form of complex termed methylmercury-L-cysteinate (MeHg-Cys) [31, 47]. This MeHg-Cys is transported into cells and across membranes by the L-Type amino acid transporters, LAT1 and LAT2 [78], found throughout the body [67, 72]. It is hypothesized that MeHg-Cys is transported by the LAT's occurs as MeHg-Cys, which structurally mimics another LAT substrate, methionine, however, this mimicry hypothesis is in controversion [5, 34]. Irrespectively, MeHg-Cys is efficiently absorbed (>95%) [61, 79] in the intestine [13] and transported throughout the body; even acrossing the placental [82] and blood-brain barriers [42],with a concentration-dependent manner [59].

MeHg is a ubiquitous and potent environmental toxic pollutant [22] that is generated by bacterial methylation of inorganic mercury in an aquatic environment [85].The central nervous system is the main target of MeHg toxicity [19, 20, 21, 91] in humans and experimental animal models [10]. For example, prenatal MeHg intoxication has been implicated in neurodevelopmental disorders such as mental retardation and motor and cognitive dysfunction [39]. The cardiovascular system has

also been reported as a target of MeHg [11, 69]. In humans, MeHg exposure has been reported to cause cardiovascular dysfunctions, including myocardial infarction [68], heart rate variability, atherosclerosis, coronary heart disease and hypertension [74, 95]. In animal experimental models, *in vivo* treatment of MeHg has been reported to induce hypertension [28, 92, 93]. We recently showed that mice exposed to MeHg *in vivo* develop high blood pressure and impaired endothelium dependent vasodilation [37].However, the exact mechanism by which MeHg induces a toxic effect on the cardiovascular system is not yet fully understood.

The myristoylated alanine-rich C kinase substrate (MARCKS) is a major protein kinase C substrate that is expressed in many tissues [2], including brain and endothelial cells [40, 53, 80]. Homozygous mutant mice with targeted deletion of the Marcks gene showed morphological abnormalities in the central nervous system and perinatal death [81], suggesting the essential role of MARCKS in brain development. In neurons, the functions of MARCKS in dendrite branching, dendritic-spine morphology, growth cone guidance, neurite outgrowth, and higher brain functions, such as learning and memory, have been reported [9, 24, 48, 54, 76]. MARCKS plays roles in cellular functions, such as adhesion, migration, proliferation and fusion in multiple types of cells through its interaction with the membrane phospholipids and actin, which is regulated by phosphorylation at the central polybasic region of MARCKS called the effector domain [4, 8, 58, 100]. In

vascular smooth muscle and endothelial cells, MARCKS has been shown to regulate proliferation [96], cell migration [40, 57, 87, 97] and endothelial cell permeability [38]. These studies have shown that MARCKS also plays an important role in the cardiovascular system. Our group has previously reported that in human neuroblastoma and endothelial cell lines, MeHg induces a significant decrease in MARCKS amount, and that the decrease in cell viability induced by MeHg is accelerated in MARCKS knockdown cells [77, 87], suggesting that MARCKS plays an important role in MeHg cytotoxicity. However, the precise mechanisms underlying the regulation of MARCKS content by MeHg exposure remain unclear.

Calpain is a cytosolic, Ca^{2+}-activated, neutral cysteine protease. The well-studied calpain isoforms, calpain 1 (μ-calpain) and calpain 2 (m-calpain), are ubiquitously expressed and regulate important functions of neuronal [6] and endothelial cells [23]. MeHg induces calpain activation, which is involved in MeHg cytotoxicity *in vitro* [14, 49, 73, 86] and *in vivo* [7, 94, 99]. Furthermore, regulation of MARCKS function by calpain proteolytic cleavage has been suggested [17, 46, 84].

Therefore, in the first study, we investigated the characteristics of MeHg toxicity on EA.hy926 endothelial cells and involvement of MARCKS on its toxicity. We observed that MeHg exposure induced decrease in cell viability, migration in wound healing assay, tube formation on Matrigel® and nitric oxide (NO) production,

and this was accompanied by an increase in MARCKS phosphorylation in EA.hy926 cells. Furthermore, the involvement of MARCKS in MeHg toxicity was studied by using cells with MARCKS knockdown or MARCKS overexpression. In the second study, we determined the contribution of MeHg-induced calpain activation to the regulation of full-length MARCKS content in a human neuroblastoma cell line, SH-SY5Y, and in a human endothelial cell line, EA.hy926, by means of different concentrations of MeHg, potent cell-permeating calpain I and II inhibitors, or MARCKS small interfering RNA (siRNA) knockdown cells. Our results indicated that the participation of calpain in the regulation of MARCKS protein content was dependent on the cell type and concentration of MeHg. In SH-SY5Y cells, MARCKS proteolysis by calpain was found to be involved in cytotoxicity induced by a low concentration of MeHg. These findings add to our understanding of the distinct molecular mechanisms of MeHg-induced cytotoxicity toward different types of cells.

Chapter 1

Study 1

MARCKS is involved in methylmercury-induced decrease in cell viability and nitric oxide production in EA.hy926 cells

1. ABSTRACT

Methylmercury (MeHg) is a persistent environmental contaminant that has been reported worldwide. MeHg exposure has been reported to lead to increased risk of cardiovascular diseases; however, the mechanisms underlying the toxic effects of MeHg on the cardiovascular system have not been well elucidated. We have previously reported that mice exposed to MeHg had increased blood pressure along with impaired endothelium-dependent vasodilation. In this study, we investigated the toxic effects of MeHg on a human endothelial cell line, EA.hy926. In addition, we have tried to elucidate the role of myristoylated alanine-rich C kinase substrate (MARCKS) in the MeHg toxicity mechanism in EA.hy926 cells. Cells exposed to MeHg (0.1–10 μM) for 24 hr showed decreased cell viability in a dose-dependent manner. Treatment with submaximal concentrations of MeHg decreased cell migration in the wound healing assay, tube formation on Matrigel and spontaneous nitric oxide (NO) production of EA.hy926 cells. MeHg exposure also elicited a decrease in MARCKS expression and an increase in MARCKS phosphorylation. MARCKS knockdown or MARCKS overexpression in EA.hy926 cells altered not only cell functions, such as migration, tube formation and NO production, but also MeHg-induced decrease in cell viability and NO production. These results suggest the broad role played by MARCKS in endothelial cell functions and the involvement of MARCKS in MeHg-induced toxicity.

Keywords: EA.hy926 cells, endothelium, MARCKS, methylmercury, nitric oxide

2. INTRODUCTION

The myristoylated alanine-rich C kinase substrate (MARCKS) is a major protein kinase C substrate that is expressed in many tissues [2], including brain and endothelial cells [40, 53, 80]. Homozygous mutant mice with targeted deletion of the Marcks gene showed morphological abnormalities in the central nervous system and perinatal death [81], suggesting the essential role of MARCKS in brain development. MARCKS plays roles in cellular functions, such as adhesion, migration, proliferation and fusion in multiple types of cells through its interaction with the membrane phospholipids and actin, which is regulated by phosphorylation at the central polybasic region of MARCKS called the effector domain [4, 8, 58, 100]. In vascular smooth muscle and endothelial cells, MARCKS has been shown to regulate proliferation [96], cell migration [40, 57, 97] and endothelial cell permeability [38]. These studies have shown that MARCKS also plays an important role in the cardiovascular system.

Methylmercury (MeHg) is a ubiquitous and potent environmental pollutant [22]. The central nervous system is the main target of MeHg toxicity [19, 21, 91]. The cardiovascular system has also been reported as a target of MeHg [11, 69]. In humans, MeHg exposure has been reported to cause cardiovascular dysfunctions, including myocardial infarction [68], heart rate variability, atherosclerosis, coronary

heart disease and hypertension [74, 95]. In animal experimental models, *in vivo* treatment of MeHg has been reported to induce hypertension [28, 92, 93]. However, the exact mechanism by which MeHg induces a toxic effect on the cardiovascular system is not yet fully understood.

We recently demonstrated that mice exposed to MeHg *in vivo* developed increased blood pressure and impaired endothelium-dependent vasodilation [37]. Although it has been reported that the alteration in MARCKS expression or phosphorylation affects MeHg-induced neurotoxicity in neuroblastoma cells [77], the relationship between MeHg toxicity and MARCKS has not yet been determined in vascular endothelial cells. Therefore, in this study, we investigated the role of MARCKS in MeHg-induced toxicity in the EA.hy926 endothelial cell line. We observed that MeHg exposure induced decrease in cell viability, migration in wound healing assay, tube formation on Matrigel and nitric oxide (NO) production, and this was accompanied by an increase in MARCKS phosphorylation in EA.hy926 cells. Furthermore, the involvement of MARCKS in MeHg toxicity was studied by using cells with MARCKS knockdown or MARCKS overexpression.

3. MATERIALS AND METHODS

3.1. Cell viability assay

A human endothelial cell line, EA.hy926 cells (ATCC, Manassas, VA, U.S.A.), was grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, U.S.A.) containing 10% fetal bovine serum at 37°C in a humidified atmosphere with 5% CO_2. To evaluate MeHg cytotoxicity, cell viability was measured using the WST-8 assay Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) in accordance with the manufacturer's instructions. Two days before experiments, the cells were seeded at a density of 1×10^4cells/cm^2 in a 96-well plate. Cells were serum-starved for 4 hr before the addition of MeHg chloride (Kanto Chemical, Tokyo, Japan) dissolved in distilled water. The absorbance of formazan dye solution in the WST-8 assay was measured using an Infinite M200 FA plate reader (TECAN, Männedorf, Switzerland).

3.2. Cell cycle analysis by flow cytometry

One day before the experiments, cells were seeded on 35-mm dishes at a density of 2.5×10^4 cells/cm^2. After 4 hr of serum starvation, the cells were treated with MeHg for 24 hr. Then, the cells were harvested by using Accumax (Innovative Cell Technologies, San Diego, CA, U.S.A.) and then fixed with 4% paraformaldehyde. The cell cycle was analyzed by flow cytometry (FACSCalibur, bD biosciences, San Jose, CA, U.S.A.) by using cells stained with propidium iodide.

3.3. Wound healing assay

Two days before the experiments, cells were seeded on 35-mm dishes at a density of 1.5×10^4 cells/cm^2. After 4 hr of serum starvation, confluent cells were scraped with sterile 200-µl pipette tips. These cells were treated with MeHg for 24 hr, after which the images of the wound areas were obtained by using an inverted microscope IX70 (Olympus, Tokyo, Japan). The percentage of area covered by the migrated cells was measured using ImageJ software (NIH, Bethesda, MD, U.S.A.).

3.4. Tube formation assay

Tube formation assay was performed as previously reported [44, 45], with slight modifications. In brief, the surface of 24-well plates was coated with 100 µl of Corning Matrigel basement Membrane Matrix (bD biosciences), which was allowed to polymerize at 37°C for 30 min. EA.hy926 cells were seeded on to the Matrigel-coated wells (3×10^4 cells/cm^2) with or without MeHg. The images were taken at 12 hr after seeding. The length of the tube was measured by using ImageJ software (NIH, Bethesda, MD, U.S.A.).

3.5. Measurement of NO production

NO production was measured as previously described [35, 56]. Two days before the experiments, cells were seeded at a density of 8.8×10^4 cells/cm^2 in a

100-mm dish. After changing the medium to DMEM without phenol red, the medium was collected from the dish at 24 hr after addition of MeHg. Accumulated NO_2 in the medium was measured using the NO_2/NO_3Assay KitFX (Dojindo) in accordance with the manufacturer's instructions. The fluorescence intensity of the sample was measured using an Infinite M200 FA plate reader (TECAN, Männedorf, Switzerland).

3.6. Transfection of siRNA and plasmid DNA

Screen*FectA* (Wako, Osaka, Japan) was used for both siRNA and plasmid DNA transfections. MARCKS siRNA (HSS180966) and negative control siRNA were purchased from Invitrogen(Carlsbad, CA, U.S.A.). EA.hy926 cells were mixed with siRNA and then seeded on 35-mm dishes (1×10^4 cells/cm^2) at 48 hr before the experiments, according to the manufacturer's instructions. For plasmid DNA transfection, cells were seeded on 35-mm dishes at a density of 2.5×10^4 cells/cm^2. After 24 hr incubation, GFP-fused wild-type MARCKS-expression plasmids [77] or control pEGFP-N1 (Clontech, Palo Alto, CA, U.S.A.) was transfected to the cells for 24 hr.

3.7. Western blotting

Western blotting was performed as previously described [76, 77]. In brief,

two days before the experiments, cells were seeded at a density of 1×10^4 cells/cm^2. Cells were treated with MeHg after 4 hr of starvation. The primary antibodies used were anti-MARCKS, anti-NOS3 (Santa Cruz biotechnology, Santa Cruz, CA,U.S.A.), anti-pS159/163 MARCKS (Cell Signaling Technology, Danvers, MA, U.S.A.) and anti-β-actin antibody (Sigma-Aldrich). Immunoreactive proteins were detected using Luminata Forte Western HRP substrate (Millipore, Billerica, MA, U.S.A.) and quantified by densitometric analysis using Image J software (NIH, Bethesda, MD, U.S.A.). The MARCKS and eNOS expression or MARCKS phosphorylation was normalized to the amount of β-actin or pan-MARCKS, respectively.

3.8. Statistical analysis

All values are expressed as the means ± SEM of the number of independent experiments. Statistical differences between two means were evaluated by the Student's *t*-test. Multiple comparisons were performed using one-way analysis of variance followed by Dunnett's test. Differences were considered significant at $P<0.05$.

4. RESULTS

4.1. Effect of MeHg on endothelial cell viability

To determine the effect of MeHg on cell viability, EA.hy926 cells were treated with 0.1–10 μM MeHg for 24 hr. MeHg elicited a decrease in cell viability in a dose-dependent manner (Fig. 1A). At MeHg concentration higher than 1 μM, significant decrease in cell viability was observed. We assessed the involvement of MARCKS in MeHg-induced decrease in cell viability by using EA.hy926 cells with MARCKS knockdown or MARCKS overexpression. Transfection of siRNA for MARCKS or MARCKS-expression plasmid caused decrease in MARCKS expression to 36.0 ± 8.4% (Fig. 2A) or increase in MARCKS expression to 148.0 ± 7.9% (Fig. 2B), in comparison with control mock-transfected cells. In cells with MARCKS knockdown, cell viability was decreased in comparison with control siRNA-transfected cells (Fig. 1B), suggesting the involvement of MARCKS in endothelial cell proliferation. In addition, decrease in cell viability induced by 3 μM MeHg for 24 hr was significantly augmented in cells with MARCKS knockdown (Fig. 1C). Although cells with MARCKS overexpression showed similar cell viability as control cells (GFP) (Fig. 1D), MeHg-induced decrease in cell viability was significantly suppressed in cells with MARCKS overexpression (Fig. 1E). Flow cytometric analysis of the cell cycle of the cells treated with MeHg (0.1–3 μM) showed that there was no alteration in the distribution of cells in the G1, S or G2/M phase (Fig. 3).

4.2.Effect of MeHg on cell migration

To determine the effect of MeHg on cell functions, we first observed the effect of MeHg on cell migration by a wound healing assay. Incubation of cells with 0.1–3 μM MeHg for 24 hr showed dose-dependent inhibition of cell migration of EA.hy926 cells (Fig. 4A). Significant inhibition by MeHg was observed at concentrations higher than 0.3 μM. In cells with MARCKS knockdown or overexpression, the cell migration was significantly suppressed or augmented, respectively (Fig.4B and D), suggesting the role of MARCKS in the migration of endothelial cells as reported previously [40, 96]. However, 0.3 μM MeHg-induced inhibition of cell migrations was not altered in both cells with MARCKS knockdown or overexpression (Fig. 4C and E).

4.3. Effect of MeHg on tube formation

EA.hy926 cells were seeded onto Matrigel-coated plates, and then, the tube formation of EA.hy926 cells was analyzed by measurement of the tube length. In the presence of 0.1–1 μM MeHg, tube length was significantly decreased in a dose-dependent manner (Fig. 5A). Although MARCKS knockdown or overexpression in EA.hy926 cells significantly decreased or increased the tube length on Matrigel (Fig.

5B and D), respectively, the modification of MARCKS expression did not alter the tube length in the presence of 1 μM MeHg (Fig. 5C and E).

4.4. Effect of MeHg on NO production

Next, we examined the effect of MeHg on NO production by EA.hy926 cells, because NO has been shown to play an important role inthe regulation of vascular tones [52, 89]. In the presence of 0.1–1 μM MeHg, spontaneous NO production by EA.hy926 cells for 24 hr was significantly inhibited in a dose-dependent manner (Fig. 6A). MARCKS knockdown or overexpression did not change the spontaneous NO production of EA.hy926 cells during the 24 hr observation (Fig. 6B and D). In contrast, in cells with MARCKS knockdown, 0.3 μM MeHg-induced inhibition of spontaneous NO production was significantly augmented (Fig. 6C). Furthermore, MARCKS overexpression in EA.hy926 cells significantly suppressed the inhibition of NO production by MeHg (Fig. 6E).

4.5. *Effect of MeHg on expression of MARCKS, eNOS and phosphorylation of MARCKS*

Finally, we observed the effect of MeHg on MARCKS expression or phosphorylation, since alteration of MARCKS expression/phosphorylation has been reported in MeHg-treated neuroblastoma cells [77]. Western blotting using

specificantibodies (Fig. 7A) showed a decrease in MARCKS expression (Fig. 7B) and biphasic increase in MARCKS phosphorylation by MeHg in a dose-dependent manner (Fig. 7C). At 24 hr after exposure to MeHg, significant differences were observed in the MARCKS expression in cells exposed to 3 μM MeHg and in the MARCKS phosphorylation in cells exposed to concentrations higher than 0.3 μM MeHg. In contrast, there was no alteration in the expression of eNOS by treatment of MeHg (Fig. 7D and 7E).

5. DISCUSSION

EA.hy926 cells exposed to MeHg for 24 hr showed a dose-dependent decrease in cell viability. Significant decrease in cell viability was observed at concentrations higher than 1 μM MeHg. The concentration of MeHg that caused significant decrease in cell viability was in accordance with that reported previously in neuroblastoma SH-SY5Y cells and primary human endothelial cells, such as brain microvascular endothelial cells and umbilical vein endothelial cells [32, 44, 77]. MeHg has been reported to elicit cell growth inhibition by interfering with the cell cycle process [43]. However, in this study, flow cytometric analysis of the cell cycle showed that there were no significant differences between control and MeHg-treated cells, suggesting that the decrease in the cell viability cannot be attributed to the toxic effect of MeHg on the cell cycle process. Our group has previously reported

that MARCKS knockdown accelerates MeHg-induced decrease in cell viability in neuroblastoma SH-SY5Y cells [77]. Thus, in this study, we studied the effect of MeHg on cell viability by using MARCKS knockdown/overexpression experiments in EA.hy926 cells. Although MARCKS overexpression did not alter the cell viability of EA.hy926 cells, MARCKS knockdown caused significant decrease in the cell viability in comparison with control siRNA-transfected cells. The observed decrease in the cell viability may be due to the suppression of cell proliferation, which is regulated by MARCKS [70, 71, 96]. MARCKS knockdown, as previously reported in neuroblastoma cells, significantly accelerated MeHg-induced decrease in cell viability in EA.hy926 cells. In addition, in cells with MARCKS overexpression, suppression of the MeHg toxicity was observed. These results support the fact that MARCKS is involved in MeHg toxicity not only in neuronal cells but also in endothelial cells.

The migration of endothelial cells is one of the key processes in angiogenesis, which is involved in a wide range of physiological and pathophysiological events, such as wound healing, cancer and cardiovascular diseases. Treatment of cells with MeHg significantly and dose-dependently inhibited EA.hy926 cell migration in the wound healing assay and tube formation on the Matrigel. These observations are in agreement with a previous report using primary human endothelial cells [32, 33, 44, 45]. In the wound healing assay, we observed significant inhibition of migration at

0.3 μM MeHg, which is a lower concentration than that which induced significant decrease in the cell viability assay, suggesting that the inhibition of migration may be one of the principal toxic actions of MeHg on EA.hy926 cells. Since the involvement of MARCKS in cell migration has been reported in many types of cells, including endothelial cells [27, 40, 63, 97], we observed the effects of MARCKS knockdown/overexpression on EA.hy926 cell migration and the effects of MeHg exposure on the cell migration. In cells with MARCKS knockdown by siRNA, cell migration was significantly suppressed in comparison with control cells, whereas overexpression of MARCKS accelerated cell migration in the wound healing assay. These results indicated the role of MARCKS in cell migration of EA.hy926 cells. However, the effects of MARCKS knockdown/overexpression on MeHg-induced inhibition of migration were not observed. Furthermore, we observed similar results for the tube formation of EA.hy926 cells on Matrigel. Therefore, it seems likely that MARCKS is not involved in the MeHg toxic effect on cell migration and tube formation of EA.hy926 cells under our experimental conditions.

Next, we examined the effect of MeHg on spontaneous NO production by EA.hy926 cells, because NO has been shown to play an important role in the regulation of vascular tones [52, 89]. We have previously reported that vasodilation induced by acetylcholine, which is dependent on NO production from endothelial cells, was decreased in a basilar artery isolated from MeHg-exposed mice [35, 37].

In this study, we showed that treatment of 0.3 μM MeHg significantly inhibited NO production, but not expression of eNOS, in a dose-dependent manner. Taken together, these results indicate that the inhibition of NO production in endothelial cells is one of the principal toxic actions of MeHg. Although MARCKS knockdown/overexpression did not change spontaneous NO production, MeHg-induced decrease in NO production in EA.hy926 cells was significantly accelerated or inhibited by MARCKS knockdown or overexpression, respectively, suggesting the involvement of MARCKS in MeHg-induced toxicity on NO production in EA.hy926 cells. Although the role of MARCKS in the transport of extracellular l-arginine, which is the immediate substrate for NO synthesis in bovine aortic endothelial cells, has been reported [88], further studies are needed to determine whether MARCKS directly functions as a regulator of NO production in endothelial cells.

Finally, we examined the effects of MeHg on MARCKS expression and phosphorylation in EA.hy926 cells, since we reported that alteration in MARCKS expression or phosphorylation has consequences on the MeHg-induced neurotoxicity in neuroblastoma cells [77]. EA.hy926 cells exposed to MeHg showed a dose-dependent decrease in MARCKS expression, although a significant difference was only found at higher (3 μM) concentrations of MeHg. However, MeHg exposure elicited a biphasic increase in MARCKS phosphorylation, and

significant differences were observed at concentrations higher than 0.3 μM at 24 hr after the treatment. Since the interactions between MARCKS and its target molecules, such as actin and phosphatidylinositol 4,5-bisphosphate, are regulated by phosphorylation at the effector domain of MARCKS [8, 40], it is likely that the phosphorylation of MARCKS induced by MeHg is directly involved in the MeHg toxicity on EA.hy926 cells. MeHg is known to induce reactive oxygen species (ROS) production, including hydrogen peroxide (H_2O_2). Since the distinct role of MARCKS accompanying its phosphorylation in H_2O_2-mediated signaling pathway in bovine aortic endothelial cells has been reported [38, 41], MARCKS is possibly phosphorylated through mechanisms associated with MeHg-induced H_2O_2production in EA.hy926 cells. Although we previously reported that, in neuroblastoma cells, the MARCKS phosphorylation by MeHg exposure was mediated by protein kinase C activation and occurred in a Ca^{2+}-dependent manner, the phosphorylation mechanisms in EA.hy926 cells are still not clear and remain to be elucidated. MeHg has been reported to elicit calpain activation accompanying intracellular Ca^{2+}elevation, and calpain inhibitor suppresses MeHginduced decrease in cell viability in neuroblastoma cells and rat cerebellar neurons [64, 73]. Since the regulation of MARCKS functions by calpain proteolytic cleavage has also been reported, it is possible that calpain activation induced by MeHg exposure causes

alteration in the MARCKS functions in a phosphorylation-independent manner [17, 46].

6. CONCLUSION

In summary, we showed that MeHg exposure induced a dose-dependent decrease in cell viability, migration, tube formation on Matrigel and NO production. MeHg exposure also elicited a decrease in MARCKS expression and an increase in MARCKS phosphorylation in EA.hy926 cells. Furthermore, alteration of MeHg-induced decrease in cell viability and NO production was observed in cells with MARCKS knockdown or overexpression. The findings of our study suggest the broad role of MARCKS in endothelial cell functions and show that MARCKS is involved in MeHg-induced toxicity in endothelial cells. It has been reported that MARCKS plays roles in cell proliferation, migration and tube formation of endothelial cells through the regulation of actin polymerization and sequestering phospholipid phosphatidylinositol 4,5-bisphosphate [40, 100]. Future studies are needed to determine the precise roles of MARCKS on the toxicity of MeHg on endothelial cells.

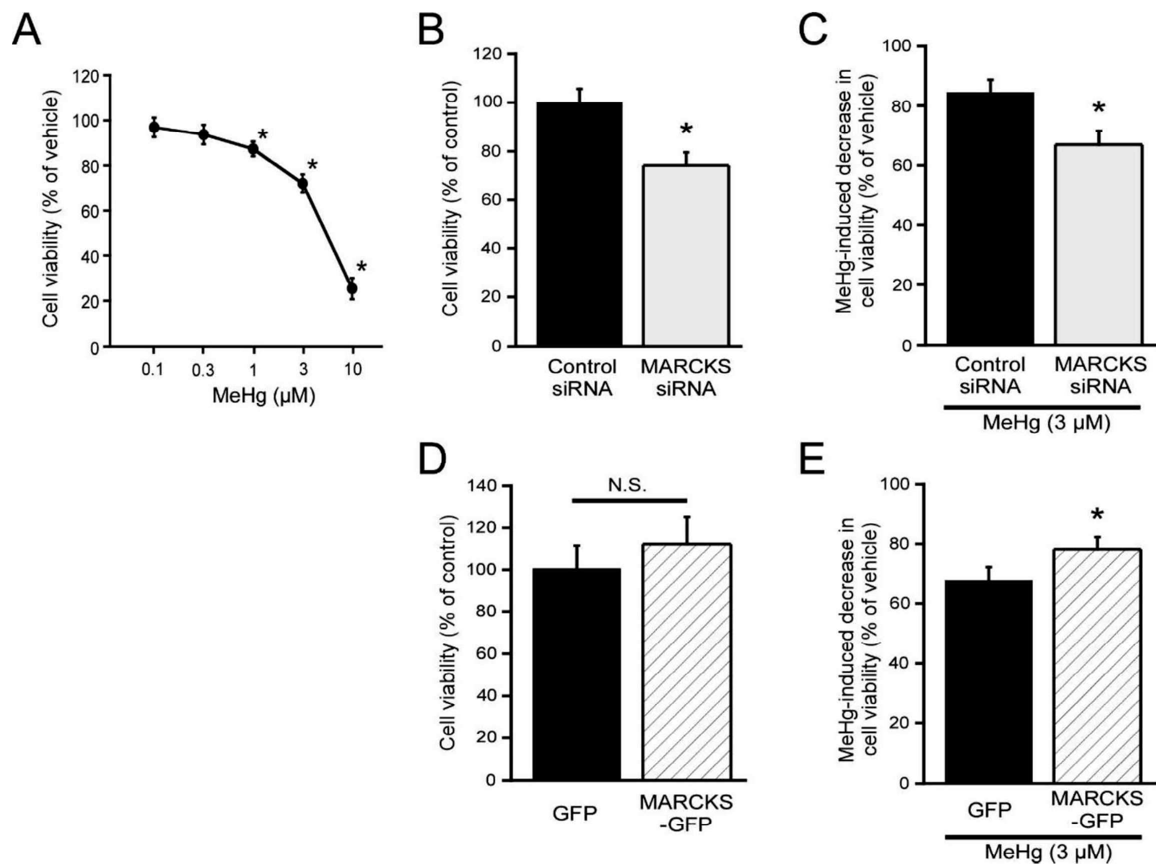

Fig. 1. Effect of MeHg on cell viability and involvement of MARCKS. Effect of MeHg on cell viability (A, n=9), effect of MARCKS knockdown on cell viability (B, n=9) or MeHg-induced decrease in cell viability (C, n=9), and effect of MARCKS overexpression on cell viability (D, n=8) or MeHg-induced decrease in cell viability (E, n=8) were examined 24 hr after addition of MeHg by cell viability assay in EA.hy926 cells. Data are expressed as a percentage of vehicle-treated or mock-transfected cells (control). Results shown are the mean ± SEM. *$P<0.05$, as compared with vehicle-treated or mock-transfected cells. N.S.; not significant.

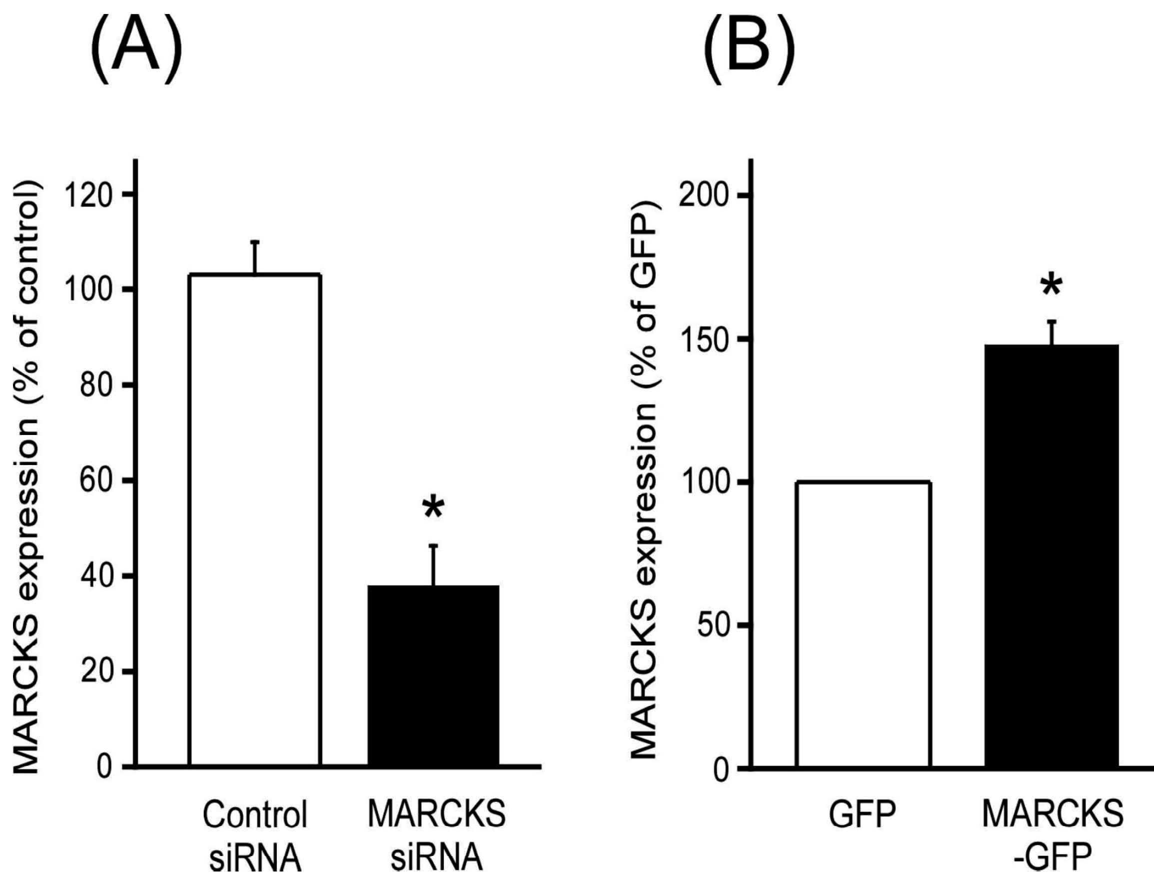

Fig. 2. Effect of MARCKS siRNA or MARCKS plasmid on MARCKS expression. Changes in MARCKS expressioninduced by transfection of siRNA (A, n=4) or plasmid DNA (B, n=4) were determined by densitometric analysis. Data are expressed as a percentage of mock-transfected cells (control). Results shown are the mean ± SEM. *$P<0.05$, as compared with control mock-transfected cells.

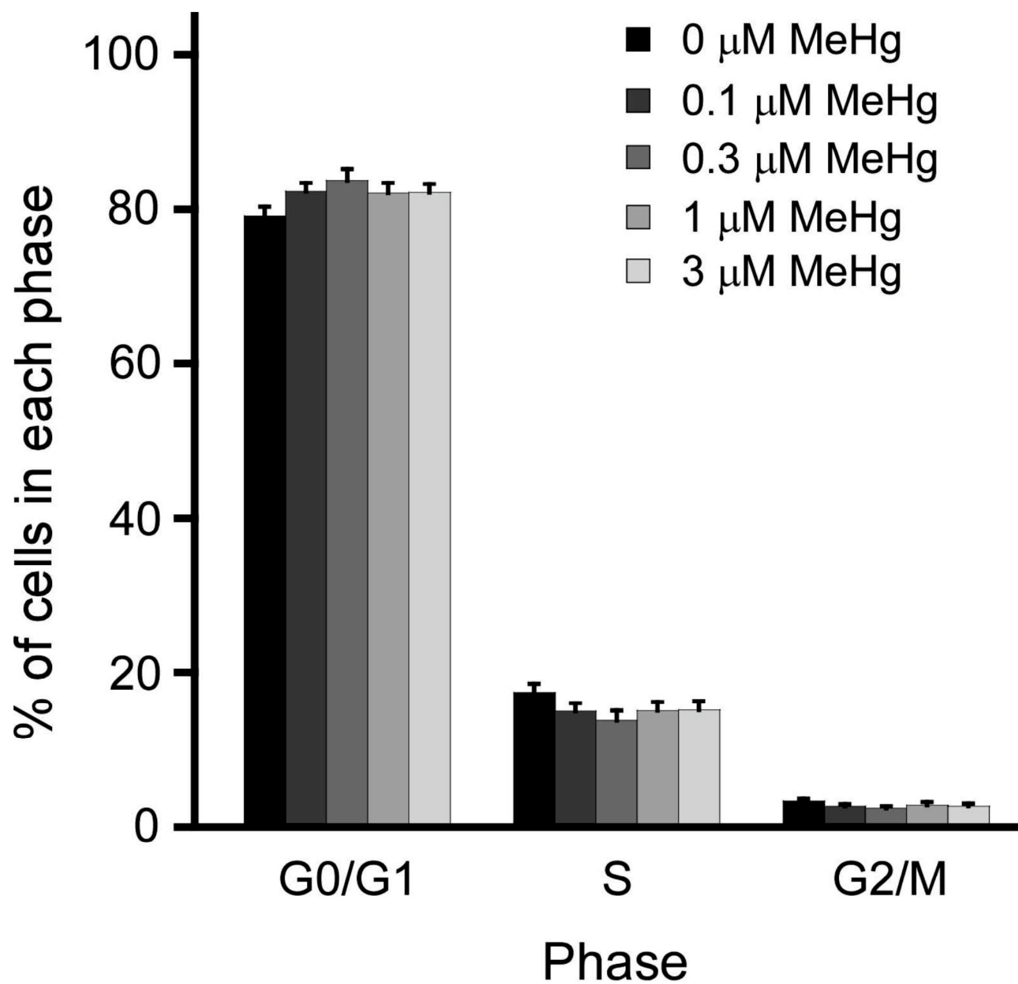

Fig. 3. Effect of MeHg on the cell cycle. Change in the G0/G1-, S- and G2/M-phase distribution following MeHg exposure were determined based on the DNA amountby flow cytometry (BD FSCSCalibur HG ™, BD Biosciences Cell Analysis group). Data are expressed as a percentage of cells in each phase divide total of cells. Results shown are the mean ± SEM (n=6).

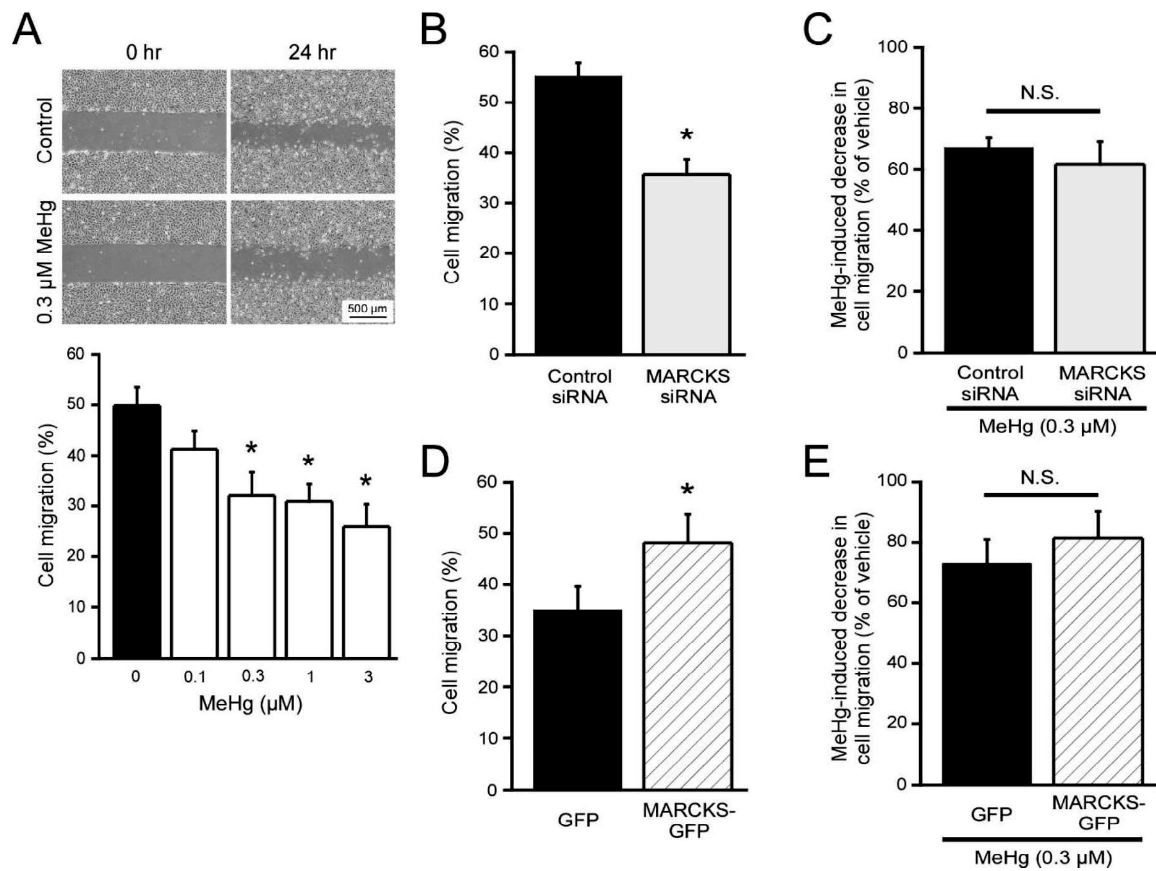

Fig. 4. Effect of MeHg on cell migration and involvement of MARCKS. Effect of MeHg on cell migration (A, n=5), effect of MARCKS knockdown on cell migration (B, n=10) or MeHg-induced decrease in cell migration (C, n=10), and effect of MARCKS overexpression on cell migration (D, n=8) or MeHg-induced decrease in cell migration (E, n=8) were examined 24 hr after addition of MeHg by wound healing assay in EA.hy926 cells. Results shown are the mean ± SEM. *$P<0.05$, as compared with vehicle-treated or mock-transfected cells. N.S.; not significant.

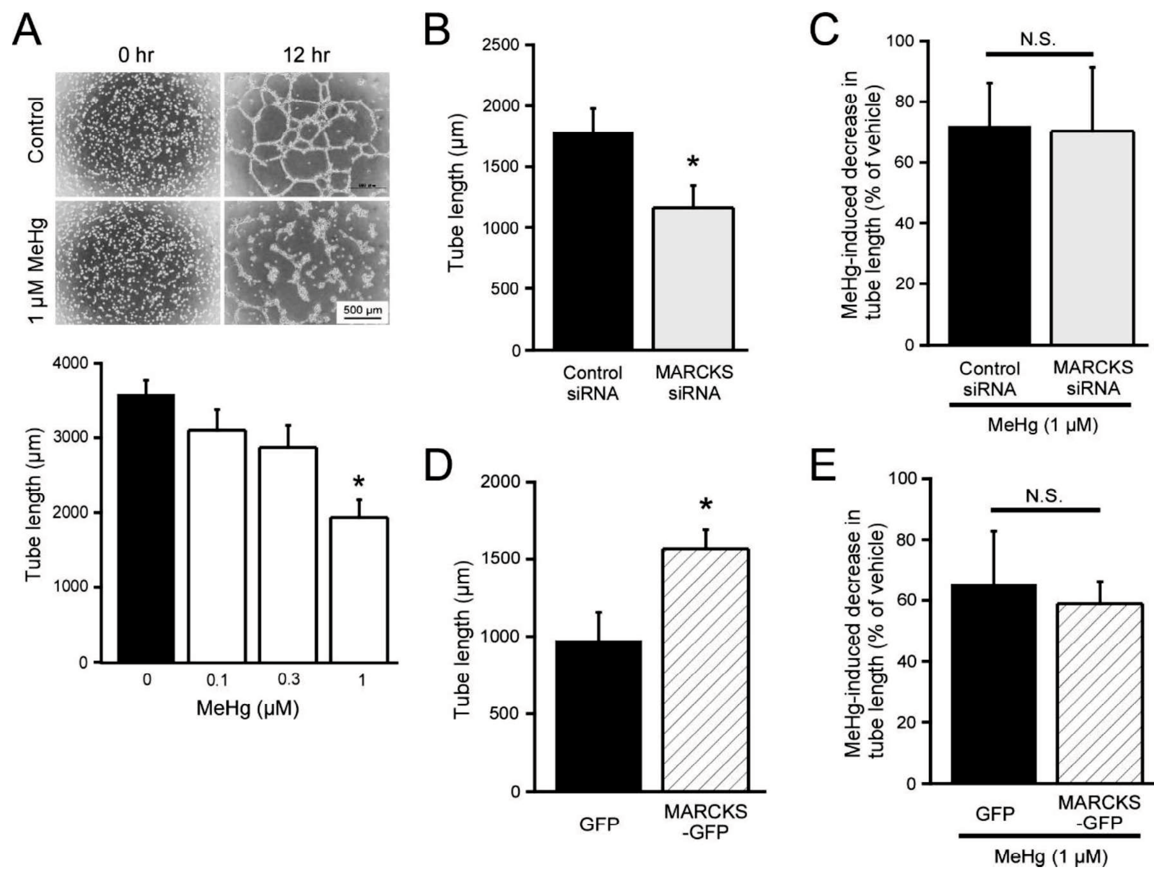

Fig. 5. Effect of MeHg on tube formation and involvement of MARCKS. Effect of MeHg on tube formation (A, n=9), effect of MARCKS knockdown on tube formation (B, n=5) or MeHg-induced decrease in tube formation (C, n=5), and effect of MARCKS overexpression on tube formation (D, n=4) or MeHg-induced decrease in tube formation (E, n=4) were examined 12 hr after seeding of cells with or without MeHg by measurement of tube formation of EA.hy926 cells. Results shown are the mean ± SEM. **P<0.05*, as compared with vehicle-treated or mock-transfected cells. N.S.; not significant.

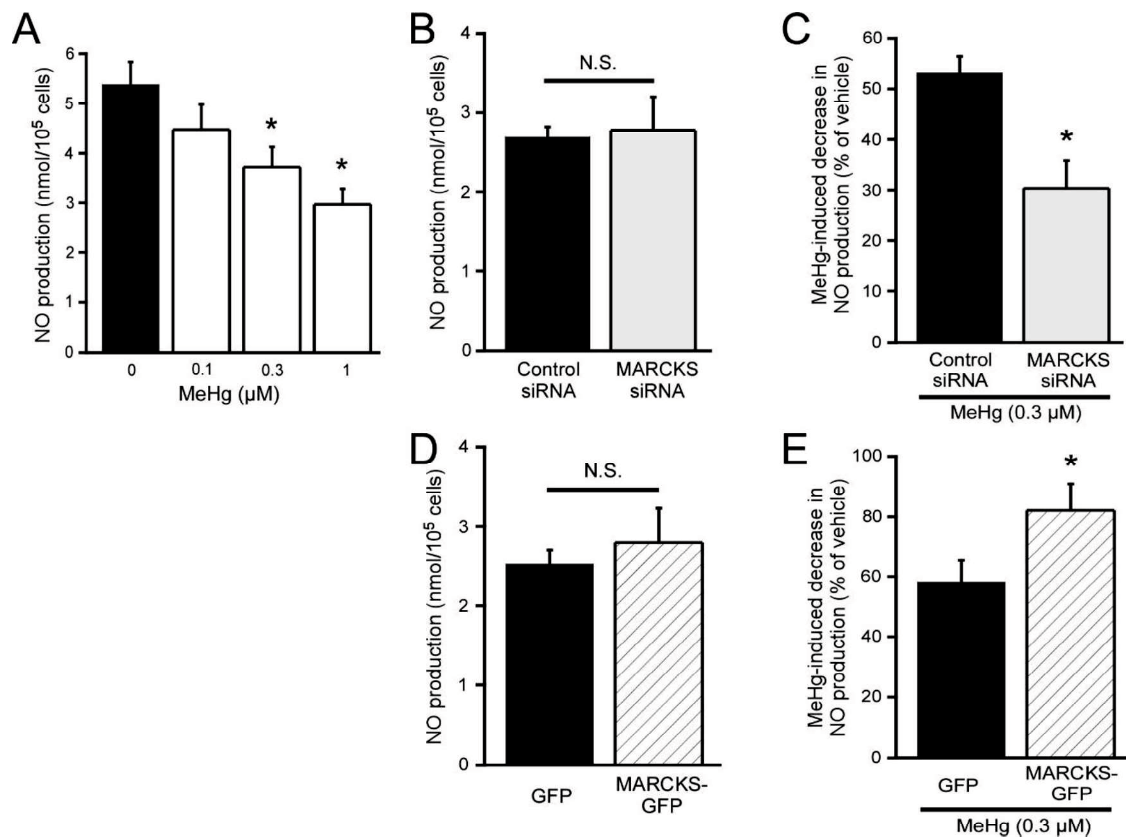

Fig. 6. Effect of MeHg on NO production and involvement of MARCKS. Effect of MeHg on NO production (A), effect of MARCKS knockdown on NO production (B) or MeHg-induced decrease in NO production (C), and effect of MARCKS overexpression on NO production (D) or MeHg-induced decrease in NO production (E) were examined 24 hr after addition of MeHg by measurement of spontaneous NO production. Results shown are the mean ± SEM (n=6). *$P<0.05$, as compared with vehicle-treated or mock-transfected cells. N.S.; not significant.

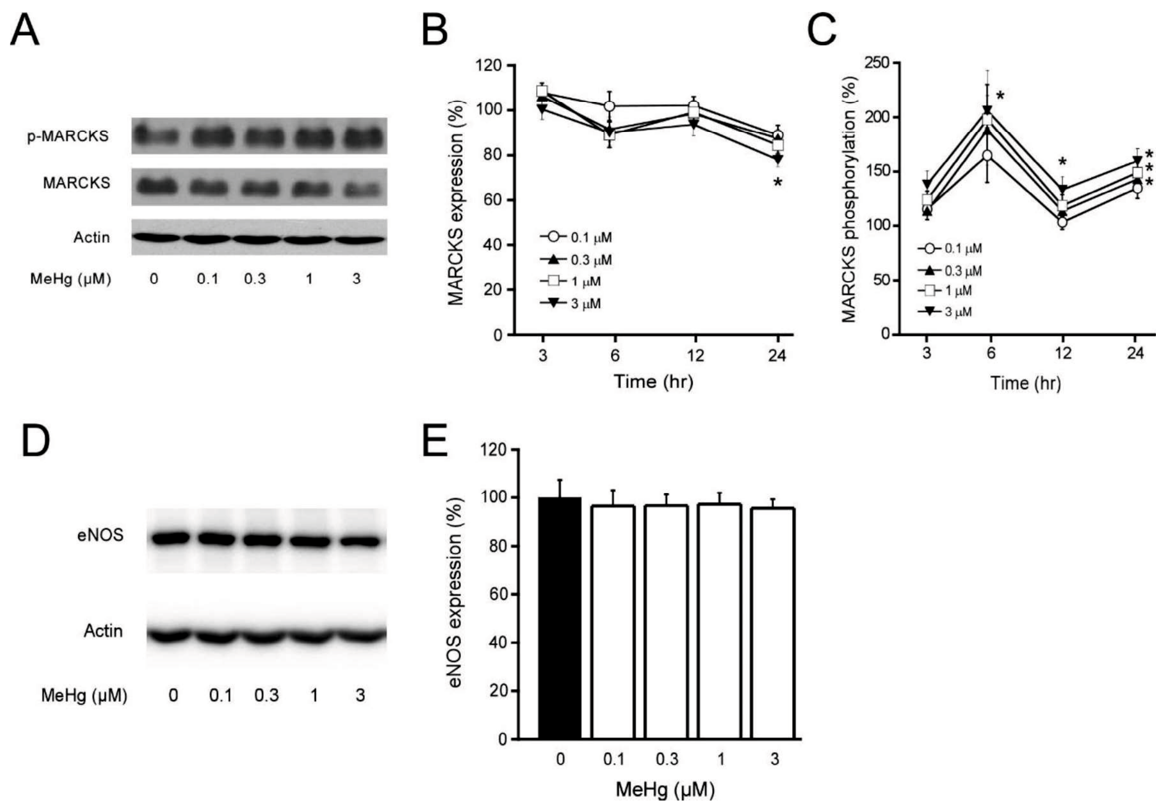

Fig. 7. Effect of MeHg on expression of MARCKS, eNOS and phosphorylation of MARCKS. Representative immunoblots of MARCKS, phosphorylated-MARCKS (P-MARCKS) (A) and eNOS (D) by specific antibodies. Changes in MARCKS expression (B, n=9), MARCKS phosphorylation (C, n=9) and eNOS expression (E, n=4) induced by MeHg were determined by densitometric analysis. Data are expressed as a percentage of vehicle-treated cells (control). Results shown are the mean ± SEM. $*P<0.05$, as compared with control.

Chapter 2

Study 2

The MARCKS protein amount is differently regulated by calpain during toxic effects of methylmercury between SH-SY5Y and EA.hy926 cells

1. ABSTRACT.

Methylmercury (MeHg) is an environmental contaminant which shows severe toxicity on human and animals. However, the molecular mechanisms mediating MeHg toxicity are not completely understood. Our group has previously reported that the Myristoylated alanine-rich C kinase substrate(MARCKS) protein is involved in the MeHg toxicity on SH-SY5Y neuroblastoma and EA.hy926 vascular endothelial cell lines. On the other hand, calpain, a Ca^{2+}-dependent protease, is suggested to be associated with the MeHg toxicity. Since MARCKS is known as a substrate of calpain, we investigated relationship between calpain activation and cleavage of MARCKS, and its role in MeHg toxicity. In SH-SY5Y cells, MeHg induced a decrease in cell viability accompanying calcium mobilization, calpain activation, and a decrease in MARKCS expression. However, pretreatment with calpain inhibitors attenuated the decrease in cell viability and MARCKS expression only induced by 1 μM but not by 3 μM MeHg. In cells with MARCKS-knockdown, calpain inhibitors failed to attenuate the decrease in cell viability by MeHg. In EA.hy926 cells, although MeHg caused calcium mobilization and a decrease in MARCKS expression, calpain activation was not observed. These results indicated that involvement of calpain in the regulation of MARCKS was dependent on the cell type and concentration of MeHg. In SH-SY5Y cells, calpain-mediated proteolysis of MARCKS was involved in cytotoxicity induced by low concentration of MeHg.

Keywords: Calpain, MARCKS, Methylmercury

2. INTRODUCTION

Methylmercury (MeHg) is a potent environmental toxic pollutant that is generated by bacterial methylation of inorganic mercury in an aquatic environment [85]. The central nervous system is the main target of MeHg toxicity [19, 20, 91] in humans and experimental animal models [10]. For example, prenatal MeHg intoxication has been implicated in neurodevelopmental disorders such as mental retardation and motor and cognitive dysfunction [39]. The cardiovascular system has also been reported as a target of MeHg [11, 69]. We recently showed that mice exposed to MeHg *in vivo*develop high blood pressure and impaired endotheliumdependent vasodilation [37].

Myristoylated alanine-rich C kinase substrate (MARCKS) is a major substrate of protein kinase C and is expressed in many tissues [2], including the brain and endothelial cells [40, 53, 80]. In neurons, the functions of MARCKS in dendrite branching, dendritic-spine morphology, growth cone guidance, neurite outgrowth, and higher brain functions, such as learning and memory, have been reported [9, 24, 48, 54, 76]. In endothelial cells, MARCKS has been shown to regulate proliferation, cell migration, cell permeability, and nitric oxide production [38, 40, 57, 87, 96]. Our group has previously reported that in human neuroblastoma and endothelial cell lines, MeHg induces a significant decrease in MARCKS amount, and that the decrease in cell viability induced by MeHg is accelerated in MARCKS

knockdown cells [77, 87], suggesting that MARCKS plays an important role in MeHg cytotoxicity. However, the precise mechanisms underlying the regulation of MARCKS content by MeHg exposure remain unclear.

Calpain is a cytosolic, Ca^{2+}-activated, neutral cysteine protease. The well-studied calpain isoforms, calpain 1 (μ-calpain) and calpain 2 (m-calpain), are ubiquitously expressed and regulate important functions of neuronal [6] and endothelial cells [23]. MeHg induces calpain activation, which is involved in MeHg cytotoxicity *in vitro* [14, 49, 73, 86] and *in vivo* [7, 94, 99]. Furthermore, regulation of MARCKS function by calpain proteolytic cleavage has been suggested [17, 46, 84].

In the present study, we determined the contribution of MeHg-induced calpain activation to the regulation of full-length MARCKS content in a human neuroblastoma cell line, SH-SY5Y, and in a human endothelial cell line, EA.hy926, by means of different concentrations of MeHg, potent cell-permeating calpain I and II inhibitors, or MARCKS small interfering RNA (siRNA) knockdown cells. Our results indicated that the participation of calpain in the regulation of MARCKS protein content was dependent on the cell type and concentration of MeHg. In SH-SY5Y cells, MARCKS proteolysis by calpain was found to be involved in cytotoxicity induced by a low concentration of MeHg. These findings add to our

understanding of the distinct molecular mechanisms of MeHg-induced cytotoxicity toward different types of cells.

3. MATERIALS AND METHODS

3.1. Cell culture

SH-SY5Y or EA.hy926 cells (ATCC, Manassas, VA, U.S.A.) were grown in Dulbecco's modified Eagle's medium mixed 1:1 with Ham's F-12 (Wako, Osaka, Japan) or *Dulbecco's modified Eagle's medium*(Sigma-Aldrich, St. Louis, MO, U.S.A.) supplemented with 10% fetal bovine serum, respectively. Both cell lines were grown at 37°C in a humidified atmosphere with 5% CO_2. Two days before experiments, cells were seeded in 96-well plates or 35-mm dished at a density of 7×10^4 (for SH-SY5Y cells) or 1×10^4 cells/cm^2 (for EA.hy926 cells). For all experiments, cells were treated with methylmercury (MeHg) chloride (Kanto Chemical, Tokyo, Japan) dissolved in distilled water for 24 hr after 4 hr serum-starvation.

3.2. Cell viability assay

To evaluate MeHg cytotoxicity, cell viability was measured by using the WST-8 assay Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). SH-SY5Y or

EA.hy926 cells in a 96-well plate were treated with 0.3 μM calpain inhibitors, MDL-28170 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) or calpeptin (Cayman Chemical, Michigan, U.S.A.), dissolved inDimethyl Sulfoxide(DMSO) for 2 hr before addition of MeHg. DMSO (0.1%) was used as vehicle of calpain inhibitors. The absorbance of formazan dye solution in the WST-8 assay was measured using an Infinite M200 FA plate reader (TECAN, Männedorf, Switzerland).

3.3.Measurement of intracellular Ca^{2+} mobilization

Intracellular Ca^{2+} mobilization induced by MeHg was monitored by using Calcium kit II - Fluo 4 (Dojindo) as previously described [60, 62]. Cells in a 96-well plate were incubated with 2.5 μM fluo-4 AM for 1 hr at 37°C. Fluo-4 fluorescence at 518 nm emission after excitation at 495 nm were measured using an Infinite M200 FA plate reader(TECAN, Männedorf, Switzerland) at 37°C.

3.4.Western blotting

Western blotting was performed as previously described [76, 77]. Cells in 35-mm dishes were treated withcalpain inhibitors and MeHg as described above. The primary antibodies used were anti-MARCKS (JK-8) (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), anti-α-Fodrin (αII-Spectrin) (*Enzo*Life Sciences, New

York, U.S.A.) and anti-β-actin antibody (Sigma-Aldrich). Immunoreactive proteins were detected using Luminata Forte Western HRP substrate (Millipore, Billerica, MA, U.S.A.) and quantified by densitometric analysis using Image J software (NIH, Bethesda, MD, U.S.A.). Expression of MARCKS and α-Fodrin were standardized by the amount of β-actin.

3.5.Knock-down of MARCKS expression

Screen*FectA* Plus (Wako, Osaka, Japan) was used for siRNA transfections for both cell lines. MARCKS siRNA (HSS180966) and negative control siRNA were purchased from Invitrogen (Carlsbad, CA, U.S.A.). SH-SY5Y or EA.hy926 cells were mixed with 20 nM or 3.5 nM siRNA, respectively, and then seeded on a 96-well plate or 35-mm dishes at 2 days before experiments.

3.6. Statistical analysis

All values are expressed as the means ± SEM. Statistical differences between two means were evaluated by the Student's *t*-test. Multiple comparisons were performed using one-way analysis of variance followed by Dunnett's or Tukey's test. Differences were considered significant at $P < 0.05$.

4. RESULTS

4.1. Suppression of MeHg-induced decrease in cell viability by calpain inhibitors

Treatment of MeHg for 24 hr caused a decrease in cell viability of SH-SY5Y or EA.hy926 cells in a dose-dependent manner (Fig. 8A). Lower concentration of MeHg, 1 μM for SH-SY5Y or 3 μM for EA.hy926 cells, elicited approximately 30% decrease in cell viability, while higher concentration of MeHg, 3 μM for SH-SY5Y or 10 μM for EA.hy926 cells, elicited approximately 60% decrease in cell viability (Fig. 8A). In SH-SY5Y cell, pretreatment of 0.3 μM calpain inhibitors, MDL-28170 or capeptin, for 2 hr before addition of MeHg suppressed a decrease in cell viability induced by low concentration (1 μM) but not by high concentration (3 μM) of MeHg (Fig. 8B and 8C). In contrast, a decrease in cell viability induced by low (3 μM) or high (10 μM) concentration of MeHg in EA.hy926 cells were not altered by the pretreatment of calpain inhibitors (Fig. 8D and 8E).

4.2. Calcium mobilization and calpain activation induced by MeHg

Calpain inhibitors showed different effects on a decrease in cell viability induced by MeHg, which was depend on the concentration of MeHg and cell types. Therefore, we confirmed the effect of MeHg on intracellular Ca^{2+} concentration, since calpain activation is mediated through intracellular Ca^{2+} mobilization. Low and high concentration of MeHg elicited significant increase in Ca^{2+} concentration in both type of cells (Fig. 9A and 9B).

Moreover, we determined MeHg-induced calpain activation in SH-SY5Y and EA.hy926 cells by monitoring of cleavage of alpha-spectrin into 150/145 kDa calpain-dependent spectrin breakdown products (SBDP) by Western blotting. In SH-SY5Y cells, low and high concentration of MeHg significantly increased the expression of 150/145 kDa SBDP (Fig. 10A-C), suggesting that calpain was activated by MeHg in SH-SY5Y cells. The calpain activation by low and high concentration of MeHg was suppressed by pretreatment of calpain inhibitors, although the inhibitors suppressed a decrease in cell viability induced by only low concentration of MeHg (Fig. 8). On the other hand, in EA.hy926 cells, MeHg did not cause an increase in 150/145 kDa SBDP (Fig. 10D-F) in accordance with the results in the effect of calpain inhibitors on the MeHg-induced decrease in cell viability (Fig. 8).

4.3. Suppression of MeHg-induced decrease in MARCKS expression by calpain inhibitors

We previously reported that a decrease in MARCKS expression plays important role in the MeHg cytotoxicity in both SH-SY5Y and EA.hy926 cells. Furthermore, it has been reported that MARCKS is cleaved by activated calpain *in vitro* [14, 49, 73, 86]. Therefore, we determined the effect of calpain inhibitors on a decrease in MARCKS expression induced by MeHg. In SH-SY5Y and EA.hy926

cells, treatment of low or high concentration of MeHg caused significant decrease in full-length MARCKS expression as previously reported (Fig. 11). In SH-SY5Y cells, although calpain activation was elicited by both low or high concentration of MeHg, calpain inhibitors suppressed only a decrease in MARCKS expression induced by low concentration of MeHg which is in accordance with the effect of calpain inhibitors on MeHg-induced decrease in cell viability (Fig. 11A-C). On the other hand, in EA.hy926 cells, MeHg-induced decrease in MARCKS expression was not altered by the pretreatment of calpain inhibitors (Fig. 11D-F).

4.4. Effect of calpain inhibitors on MeHg-induced decrease in cell viability and MARCKS expression in SH-SY5Y cells with MARCKS-knockdown.

To clarify the relationship between the calpain-mediated decrease in MARCKS expression and the decrease in cell viability induced by MeHg, we observed the effect of calpain inhibitors on a decrease in MARCKS expression and cell viability induced by MeHg in MARCKS-knockdown cells (Fig. 12). Expression of MARCKS in SH-SY5Y cells treated with MARCKS siRNA was decreased to approximately 40% of control SH-SY5Y cells. In MARCKS-knockdown cells, a decrease in MARCKS expression by low concentration of MeHg and the effect of calpain inhibitors on the MARCKS expression was not observed (Fig. 12A and 12B).

In accordance with the observation, a decrease in cell viability by low concentration of MeHg was not altered by pretreatment of calpain inhibitors (Fig. 12C).

5. DISCUSSION

The aim of this study was to investigate the relation between calpain activation and proteolysis of MARCKS in MeHg toxicity to the SH-SY5Y neuroblastoma cell line and EA.hy926 vascular endothelial cell line. In our previous studies, amount of the full-length MARCKS protein was significantly decreased by treatment with MeHg, and this protein seems to play a key role in the MeHg toxicity [77, 87]. In addition, the involvement of calpain in the MeHg toxicity had been suggested [7, 14, 49, 73, 86, 94, 99]. Hence, it is important to identify the precise mechanisms behind the regulation of MARCKS levels by MeHg exposure.

We first determined the involvement of calpain in the MeHg-induced decrease in viability of SH-SY5Y and EA.hy926 cells by means of potent cell-permeating calpain I and II inhibitors: MDL-28170 and calpeptin (Fig. 8). In SH-SY5Y cells, the decrease in cell viability induced by the low concentration of MeHg, which causes approximately a 30% decrease, was significantly attenuated by the pretreatment with calpain inhibitors (Fig. 8B) as previously reported in rat cerebellar granules [73]. However, the pretreatment effects of calpain inhibitors were not

observed at the high concentration of MeHg (Fig. 8C). We confirmed that the low and high concentrations of MeHg caused not only a significant increase in the fluo-4 fluorescence ratio in comparison with vehicle-treated cells (Fig. 9A) but also a significant increase in the amount of calpaingenerated 150/145 kDa SBDP. Furthermore, the increase in 150/145 kDa SBDP amounts was almost abrogated by the pretreatment with calpain inhibitors (Fig. 10B and 10C). These results suggested that the participation of calpain in MeHg toxicity was different at different concentrations of MeHg in SH-SY5Y cells, even though calpain was activated by both the low and high concentration of MeHg. In contrast, in EA.hy926 cells, although MeHg significantly decreased cell viability, the pretreatment effect of calpain inhibitors was not observed regardless of the concentration of MeHg (Fig. 8D and 8E). MeHg significantly increased the fluo-4 fluorescence ratio (Fig. 9B), while calpain activation by MeHg was not detected (Fig. 10E and 10F). Since expression of calpain in EA.hy926 cells has been reported [66], it seems that the zero effect of calpain inhibitors on the MeHg-induced decrease in viability of EA.hy926 cells was due to the absence of calpain activation by MeHg. It has been reported that calpain activation was regulated not only by calcium mobilization but also by phospholipids or ERK/MAP kinase signaling pathway [75, 98], suggesting that MeHg-induced signaling events in EA.hy926 cells may not be sufficient to induce calpain activation.

Next, we focused on the contribution of calpain to the MeHg-induced downregulation of MARCKS because we have previously reported the participation of MARCKS in MeHg toxicity to SH-SY5Y and EA.hy926 cells [77, 87]. It has been shown that calpain regulates MARCKS function by proteolytic cleavage [17] during myoblast fusion, adhesion, and migration [15, 17] and airway mucin secretion [46]. In accordance with the results on cell viability, the MeHg-induced decrease in full-length MARCKS amount was significantly suppressed by the pretreatment with calpain inhibitors at the low concentration of MeHg (Fig. 11B), but not at the high concentration of MeHg in SH-SY5Y cells (Fig. 11C). Besides, calpain inhibitors had no effect on the decrease in MARCKS amounts by MeHg in EA.hy926 cells (Fig. 11E and 11F). These results suggest that the low concentration of MeHg downregulates MARCKS through calpain activation in SH-SY5Y cells.

Finally, we examined the effect of calpain inhibitors on the MeHg-induced decrease in viability and downregulation of full length MARCKS in SH-SY5Y cells with the MARCKS knockdown (Fig. 12). In the MARCKS knockdown cells, the decrease in MARCKS amount by the low concentration of MeHg was not detected (Fig. 12B). In addition, pretreatment with calpain inhibitors had no effect on the amount of full-length MARCKS. On the other hand, a significant decrease in cell viability caused by the low concentration of MeHg in control cells was augmented in cells with the MARCKS knockdown (Fig. 12C) as previously reported [77],

suggesting that MARCKS plays a key role in MeHg cytotoxicity. In line with the result on MARCKS content, pretreatment with calpain inhibitors did not alter the decrease in cell viability induced by MeHg. These results suggest that the calpain-mediated decrease in MARCKS amount mediates MeHg toxicity to SH-SY5Y cells at the low concentration of this chemical. Recently, Pierozan et al.proposed that the cytoskeleton is an end point of MeHg cytotoxicity [65]. Because it has been demonstrated that in vitrocleavage of MARCKS by calpain increases its actin-binding activity [94], MARCKS cleaved by calpain may exert its effect via actin reorganization, in addition to the increase in MARCKS phosphorylation [77] in MeHg toxicity to SH-SY5Y cells.

Here, we demonstrated that the function of calpain in the regulation of MARCKS protein amounts is dependent on the cell type and concentration of MeHg. In SH-SY5Y cells, proteolysis of MARCKS by calpain mediates the cytotoxicity caused by the low concentration of MeHg. The mechanisms of the MeHg-driven decrease in cell viability and in MARCKS amount remain unknown in EA.hy926 cells and for the high concentration MeHg in SH-SY5Y cells. Interestingly, cleavage of MARCKS by calpain is dependent of its phosphorylation and localization [17]. In addition, it has been reported that cathepsin B, a lysosomal cysteine protease, and ubiquitin-proteasome proteolytic pathway were involved in degradation of MARCKS in neurons [26, 55]. However, it has not been reported whether the

cathepsin B or ubiquitin-proteasome system participates in mechanisms of MeHg toxicity on SH-SY5Y and EA.hy926 cells. Involvement of these mechanisms should be clarified for a better understanding of the role of MARCKS proteins in MeHg cytotoxicity. A schematic representation of the regulation of full-length MARCKS amounts by MeHg in SH-SY5Y and EA.hy926 cells is provided in Fig. 13.These findings should elucidate the distinct molecular mechanisms of MeHg toxicity to various cell types.

6. CONCLUSIONS

In this study, we demonstrated that involvement of calpain in the regulation of MARCKS protein was dependent on the cell type and concentration of MeHg. In SH-SY5Y cells, proteolysis of MARCKS by calpain was involved in cytotoxicity induced by low concentration of MeHg. The mechanisms how MeHg cause decrease in cell viability and MARCKS expression still remains unknow in EA.hy926 cells, and at high concentration MeHg in SH-SY5Y. It should be clarified to further understand the role MARCKS proteins in MeHg cytotoxicity. A schematic representation of the regulation of full-length MARCKS expression by MeHg in SH-SY5Y and EA.hy926 cells is provided in Fig. 13. These findings add to our understanding of the distinct molecular mechanisms of MeHg-induced cytotoxicity on different type of cells.

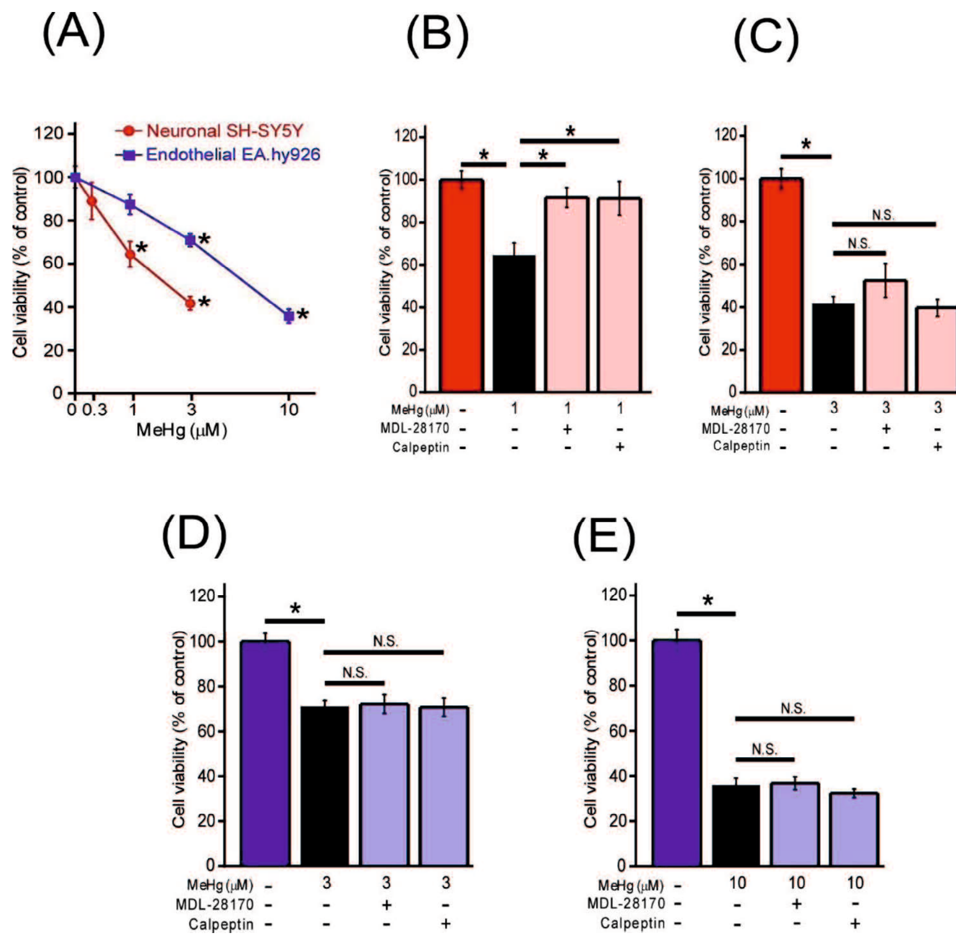

Fig. 8. Suppression of MeHg-induced decrease in cell viability by calpain inhibitors. MeHg induced dose-dependent decrease in cell viability of SH-SY5Y and EA.hy926 cells (A, n=9-10). Effect of calpain inhibitors (0.3 μM MDL-28170 or 0.3 μM calpeptin) on a decrease in cell viability induced by 1 μM (B, n=9) or 3 μM (C, n=9) MeHg in SH-SY5Y cells. Effect of calpain inhibitors (0.3 μM MDL-28170 or 0.3 μM calpeptin) on a decrease in cell viability induced by 3 μM (D, n=5) or 10 μM (E, n=5) MeHg in EA.hy926 cells. Data are expressed as a percentage of vehicle-treated cells (control). Results shown are the mean ± SEM. *P< 0.05, as compared with control (A) or as indicated (B-E). N.S.; not significant.

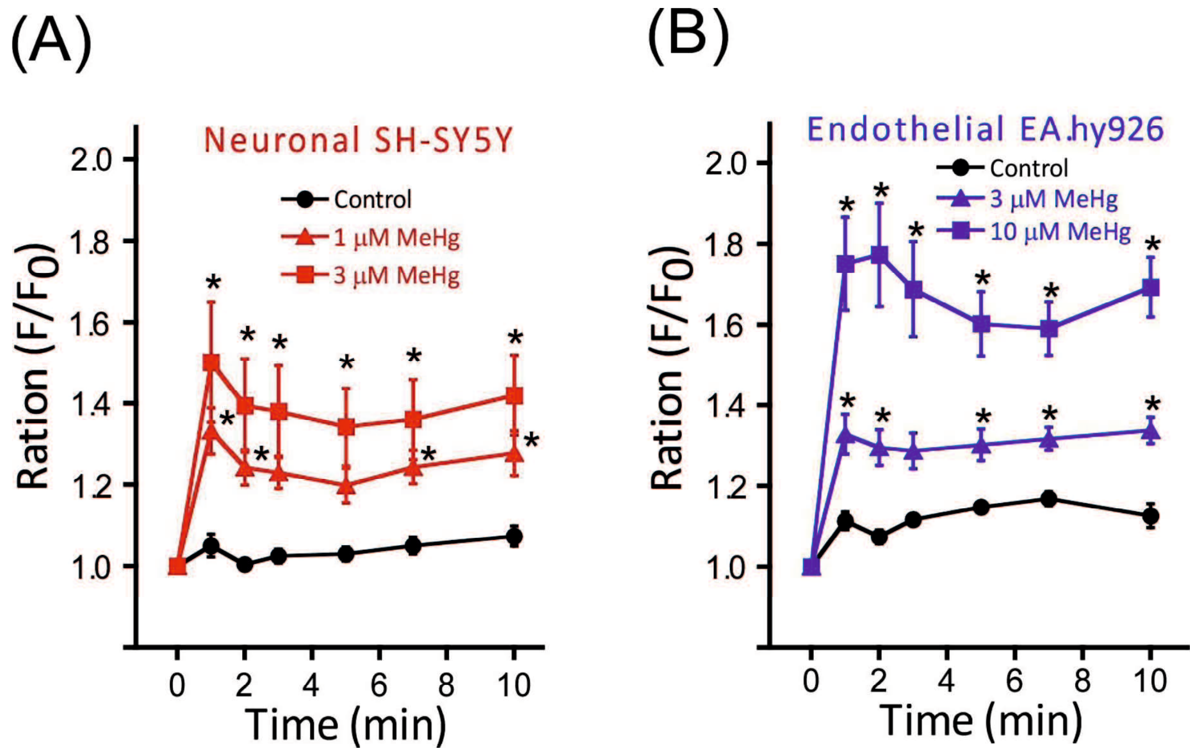

Fig. 9. Calcium mobilization induced by MeHg. MeHg induced dose-dependent increase in fluo-4 F/F_0 ratio changes in SH-SY5Y (A, n=7) and EA.hy926 cells (B, n=6). Data are expressed as a percentage of vehicle-treated cells (control). Results shown are the mean ± SEM. *$P < 0.05$, as compared with control.

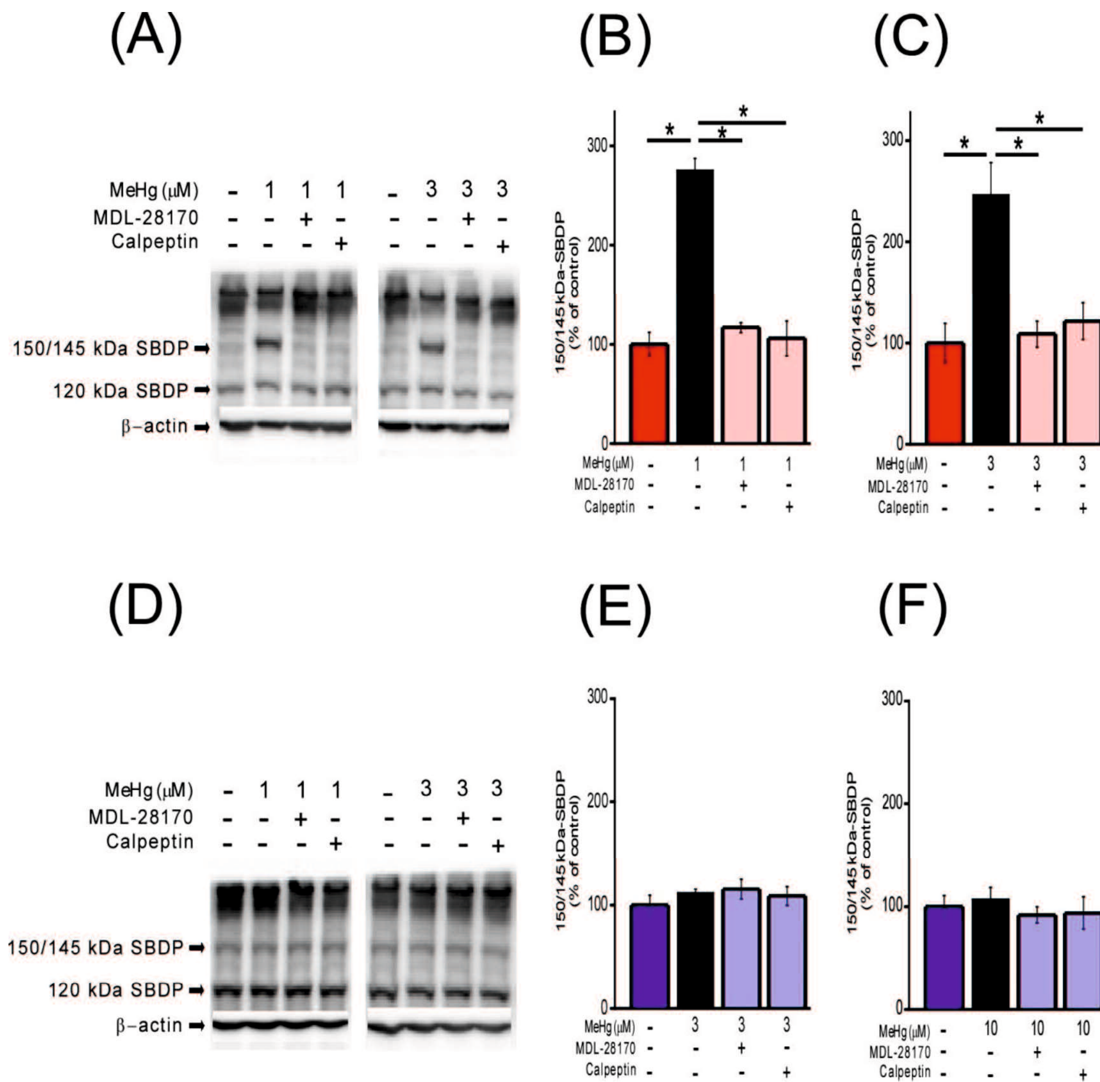

Fig. 10. Calpain activation induced by MeHg and effect of calpain inhibitors. MeHg induced change in 150/145 kDa SBDP was investigated in SH-SY5Y (A-C, n=5) and EA.hy926 cells (D-F, n=6). Representative immunoblots of 150/145 and 120 kDa SBDP and β-actin by specific antibodies are shown (A and D). Changes in 150/145 kDa SBDP were determined by densitometric analysis (B-C and E-F). Data are expressed as a percentage of vehicle-treated cells (control). Results shown are the mean ± SEM. $^{*}P < 0.05$.

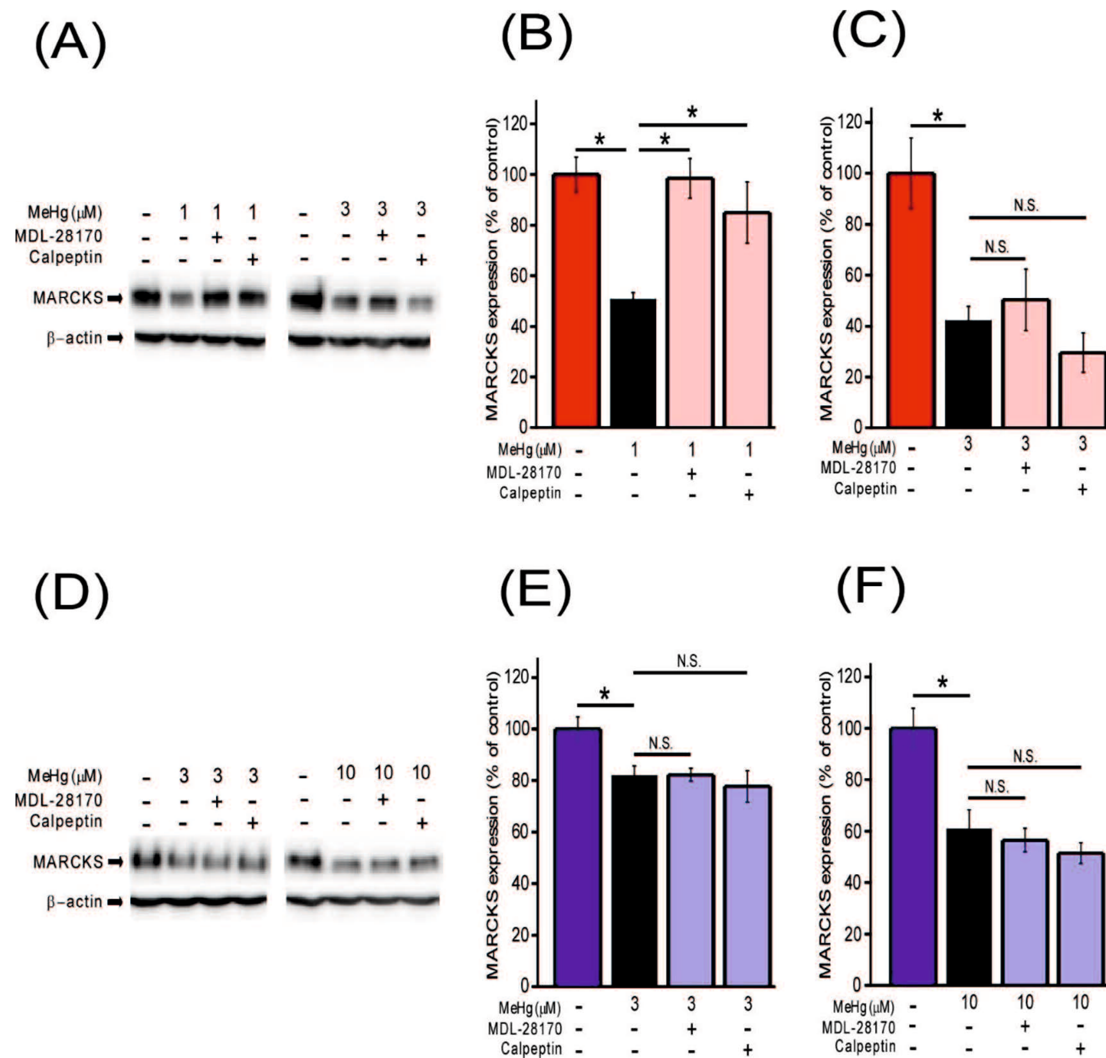

Fig. 11. Suppression of MeHg-induced decrease in MARCKS expression by calpain inhibitors. MeHg-induced decrease in full-length MARCKS expression and effect of calpain inhibitors were investigated in SH-SY5Y (A-C, n=5) and EA.hy926 cells (D-F, n=6). Representative immunoblots of MARCKS and *β*-actin by specific antibodies are shown (A and D). Changes in MARCKS expression was determined by densitometric analysis (B-C and E-F). Data are expressed as a percentage of vehicle-treated cells (control). Results shown are the mean ± SEM. $^{*}P < 0.05$.

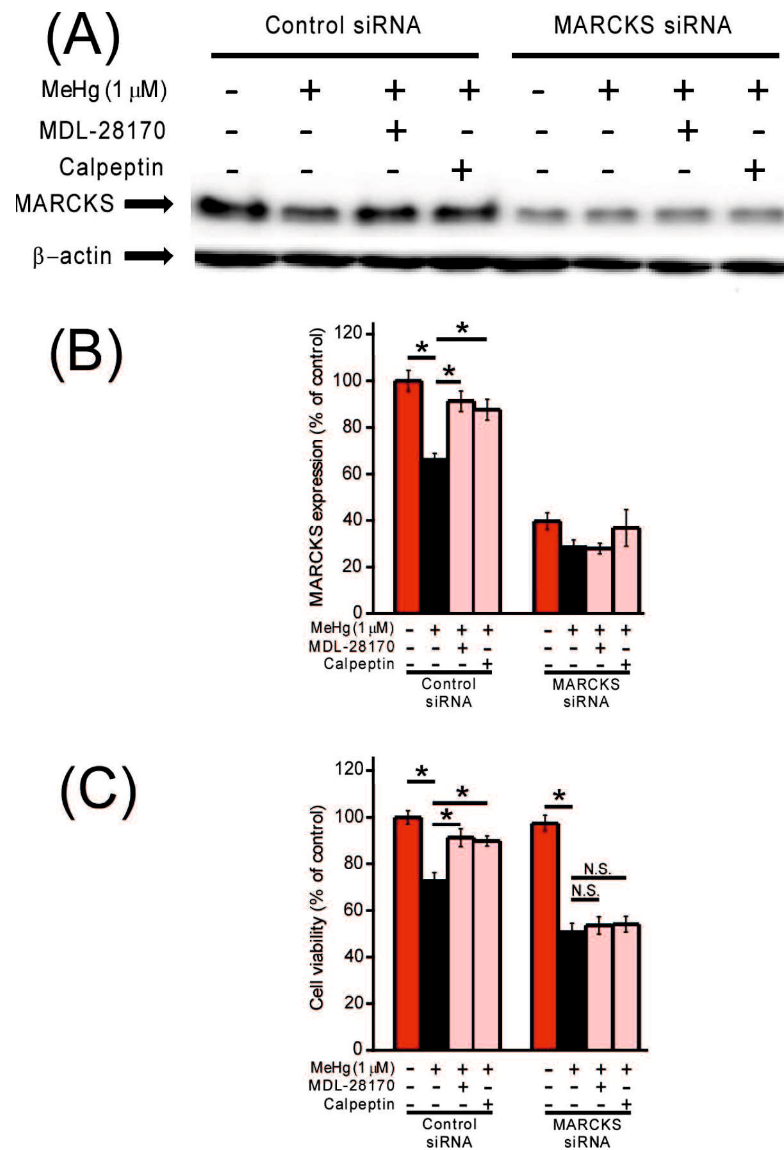

Fig. 12. Effect of calpain inhibitors on MeHg-induced decrease in cell viability and MARCKS expression in SH-SY5Y cells with MARCKS-knockdown. Representative immunoblots of MARCKS and *β*-actin by specific antibodies are shown (A). Effect of MeHg and calpain inhibitors on full-length MARCKS expression (B, n=5) and cell viability (C, n=8) in control and MARCKS-knockdown cells. Data are expressed as a percentage of vehicle-treated cells. Results shown are the mean ± SEM. *P< 0.05. N.S.; not significant.

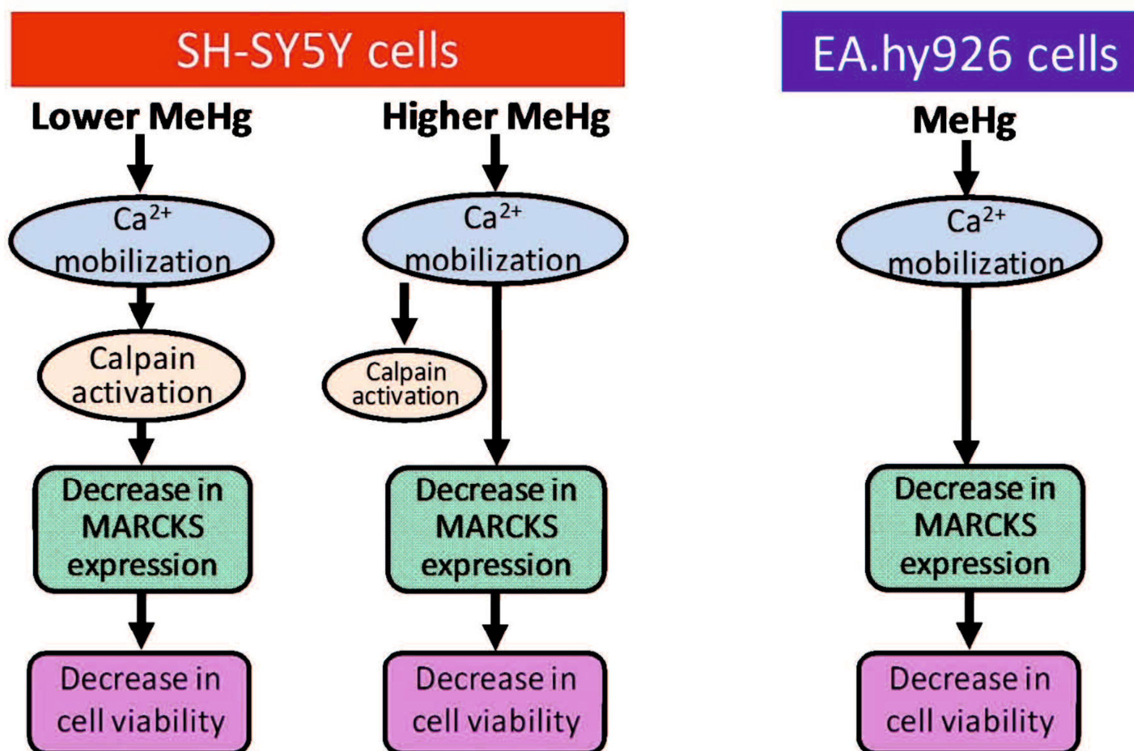

Fig. 13. Schematic representation of the regulation of full-length MARCKS expression by MeHg in SH-SY5Y and EA.hy926 cells. Involvement of calpain in the regulation of MARCKS protein is dependent on cell types and the concentration of MeHg. In SH-SY5Y cells, MARCKS proteolysis by calpain is involved in cytotoxicity of low concentration of MeHg.

GENERAL DISCUSSION

EA.hy926 cells exposed to MeHg for 24 hr showed a dose-dependent decrease in cell viability. Significant decrease in cell viability was observed at concentrations higher than 1 μM MeHg. The concentration of MeHg that caused significant decrease in cell viability was in accordance with that reported previously in neuroblastoma SH-SY5Y cells and primary human endothelial cells, such as brain microvascular endothelial cells and umbilical vein endothelial cells [32, 44, 77]. MeHg has been reported to elicit cell growth inhibition by interfering with the cell cycle process [43]. However, in this study, flow cytometric analysis of the cell cycle showed that there were no significant differences between control and MeHg-treated cells, suggesting that the decrease in the cell viability cannot be attributed to the toxic effect of MeHg on the cell cycle process. Our group has previously reported that MARCKS knockdown accelerates MeHg-induced decrease in cell viability in neuroblastoma SH-SY5Y cells [77]. Thus, in this study, we studied the effect of MeHg on cell viability by using MARCKS knockdown/overexpression experiments in EA.hy926 cells. Although MARCKS overexpression did not alter the cell viability of EA.hy926 cells, MARCKS knockdown caused significant decrease in the cell viability in comparison with control siRNA-transfected cells. The observed decrease in the cell viability may be due to the suppression of cell proliferation, which is regulated by MARCKS [70, 71, 96]. MARCKS knockdown, as previously

reported in neuroblastoma cells, significantly accelerated MeHg-induced decrease in cell viability in EA.hy926 cells. In addition, in cells with MARCKS overexpression, suppression of the MeHg toxicity was observed. These results support the fact that MARCKS is involved in MeHg toxicity not only in neuronal cells but also in endothelial cells.

The migration of endothelial cells is one of the key processes in angiogenesis, which is involved in a wide range of physiological and pathophysiological events, such as wound healing, cancer and cardiovascular diseases. Treatment of cells with MeHg significantly and dose-dependently inhibited EA.hy926 cell migration in the wound healing assay and tube formation on the Matrigel. These observations are in agreement with a previous report using primary human endothelial cells [32, 33, 44, 45]. In the wound healing assay, we observed significant inhibition of migration at 0.3 μM MeHg, which is a lower concentration than that which induced significant decrease in the cell viability assay, suggesting that the inhibition of migration may be one of the principal toxic actions of MeHg on EA.hy926 cells. Since the involvement of MARCKS in cell migration has been reported in many types of cells, including endothelial cells [27, 40, 63, 97], we observed the effects of MARCKS knockdown/overexpression on EA.hy926 cell migration and the effects of MeHg exposure on the cell migration. In cells with MARCKS knockdown by siRNA, cell migration was significantly suppressed in comparison with control cells, whereas

overexpression of MARCKS accelerated cell migration in the wound healing assay. These results indicated the role of MARCKS in cell migration of EA.hy926 cells. However, the effects of MARCKS knockdown/overexpression on MeHg-induced inhibition of migration were not observed. Furthermore, we observed similar results for the tube formation of EA.hy926 cells on Matrigel. Therefore, it seems likely that MARCKS is not involved in the MeHg toxic effect on cell migration and tube formation of EA.hy926 cells under our experimental conditions.

Next, we examined the effect of MeHg on spontaneous NO production by EA.hy926 cells, because NO has been shown to play an important role in the regulation of vascular tones [52, 89]. We have previously reported that vasodilation induced by acetylcholine, which is dependent on NO production from endothelial cells, was decreased in a basilar artery isolated from MeHg-exposed mice [35, 37]. In this study, we showed that treatment of 0.3 μM MeHg significantly inhibited NO production, but not expression of eNOS, in a dosedependent manner. Taken together, these results indicate that the inhibition of NO production in endothelial cells is one of the principal toxic actions of MeHg. Although MARCKS knockdown/overexpression did not change spontaneous NO production, MeHg-induced decrease in NO production in EA.hy926 cells was significantly accelerated or inhibited by MARCKS knockdown or overexpression, respectively, suggesting the involvement of MARCKS in MeHg-induced toxicity on NO production in

EA.hy926 cells. Although the role of MARCKS in the transport of extracellular l-arginine, which is the immediate substrate for NO synthesis in bovine aortic endothelial cells, has been reported [88], further studies are needed to determine whether MARCKS directly functions as a regulator of NO production in endothelial cells.

Finally, we examined the effects of MeHg on MARCKS expression and phosphorylation in EA.hy926 cells, since we reported that alteration in MARCKS expression or phosphorylation has consequences on the MeHg-induced neurotoxicity in neuroblastoma cells [77]. EA.hy926 cells exposed to MeHg showed a dose-dependent decrease in MARCKS expression, although a significant difference was only found at higher (3 μM) concentrations of MeHg. However, MeHg exposure elicited a biphasic increase in MARCKS phosphorylation, and significant differences were observed at concentrations higher than 0.3 μM at 24 hr after the treatment. Since the interactions between MARCKS and its target molecules, such as actin and phosphatidylinositol 4,5-bisphosphate, are regulated by phosphorylation at the effector domain of MARCKS [8, 40], it is likely that the phosphorylation of MARCKS induced by MeHg is directly involved in the MeHg toxicity on EA.hy926 cells. MeHg is known to induce reactive oxygen species (ROS) production, including hydrogen peroxide (H_2O_2). Since the distinct role of MARCKS accompanying its phosphorylation in H_2O_2-mediated signaling pathway

in bovine aortic endothelial cells has been reported [38, 41], MARCKS is possibly phosphorylated through mechanisms associated with MeHg-induced H_2O_2 production in EA.hy926 cells. Although we previously reported that, in neuroblastoma cells, the MARCKS phosphorylation by MeHg exposure was mediated by protein kinase C activation and occurred in a Ca^{2+}-dependent manner, the phosphorylation mechanisms in EA.hy926 cells are still not clear and remain to be elucidated. MeHg has been reported to elicit calpain activation accompanying intracellular Ca^{2+} elevation, and calpain inhibitor suppresses MeHg-induced decrease in cell viability in neuroblastoma cells and rat cerebellar neurons [64, 73]. Since the regulation of MARCKS functions by calpain proteolytic cleavage has also been reported, it is possible that calpain activation induced by MeHg exposure causes alteration in the MARCKS functions in a phosphorylation-independent manner [17, 46].

The aim of second study was to investigate the relation between calpain activation and proteolysis of MARCKS in MeHg toxicity to the SH-SY5Y neuroblastoma cell line and EA.hy926 vascular endothelial cell line. In our previous studies, amount of the full-length MARCKS protein was significantly decreased by treatment with MeHg, and this protein seems to play a key role in the MeHg toxicity [77, 87]. In addition, the involvement of calpain in the MeHg toxicity had been

suggested [7, 14, 49, 73, 86, 94, 99]. Hence, it is important to identify the precise mechanisms behind the regulation of MARCKS levels by MeHg exposure.

We first determined the involvement of calpain in the MeHg-induced decrease in viability of SH-SY5Y and EA.hy926 cells by means of potent cell-permeating calpain I and II inhibitors: MDL-28170 and calpeptin (Fig. 8). In SH-SY5Y cells, the decrease in cell viability induced by the low concentration of MeHg, which causes approximately a 30% decrease, was significantly attenuated by the pretreatment with calpain inhibitors (Fig. 8B) as previously reported in rat cerebellar granules [73]. However, the pretreatment effects of calpain inhibitors were not observed at the high concentration of MeHg (Fig. 8C). We confirmed that the low and high concentrations of MeHg caused not only a significant increase in the fluo-4 fluorescence ratio in comparison with vehicle-treated cells (Fig. 9A) but also a significant increase in the amount of calpaingenerated 150/145 kDa SBDP. Furthermore, the increase in 150/145 kDa SBDP amounts was almost abrogated by the pretreatment with calpain inhibitors (Fig. 10B and 10C). These results suggested that the participation of calpain in MeHg toxicity was different at different concentrations of MeHg in SH-SY5Y cells, even though calpain was activated by both the low and high concentration of MeHg. In contrast, in EA.hy926 cells, although MeHg significantly decreased cell viability, the pretreatment effect of calpain inhibitors was not observed regardless of the concentration of MeHg (Fig.

8D and 8E). MeHg significantly increased the fluo-4 fluorescence ratio (Fig. 9B), while calpain activation by MeHg was not detected (Fig. 10E and 10F). Since expression of calpain in EA.hy926 cells has been reported [66], it seems that the zero effect of calpain inhibitors on the MeHg-induced decrease in viability of EA.hy926 cells was due to the absence of calpain activation by MeHg. It has been reported that calpain activation was regulated not only by calcium mobilization but also by phospholipids or ERK/MAP kinase signaling pathway [75, 98], suggesting that MeHg-induced signaling events in EA.hy926 cells may not be sufficient to induce calpain activation.

Next, we focused on the contribution of calpain to the MeHg-induced downregulation of MARCKS because we have previously reported the participation of MARCKS in MeHg toxicity to SH-SY5Y and EA.hy926 cells [77, 87]. It has been shown that calpain regulates MARCKS function by proteolytic cleavage [17] during myoblast fusion, adhesion, and migration [15, 17] and airway mucin secretion [46]. In accordance with the results on cell viability, the MeHg-induced decrease in full-length MARCKS amount was significantly suppressed by the pretreatment with calpain inhibitors at the low concentration of MeHg (Fig. 11B), but not at the high concentration of MeHg in SH-SY5Y cells (Fig. 11C). Besides, calpain inhibitors had no effect on the decrease in MARCKS amounts by MeHg in

EA.hy926 cells (Fig. 11E and 11F). These results suggest that the lowconcentration of MeHg downregulates MARCKS through calpain activation in SH-SY5Y cells.

Finally, we examined the effect of calpain inhibitors on the MeHg-induced decrease in viability and downregulation of full length MARCKS in SH-SY5Y cells with the MARCKS knockdown (Fig. 12). In the MARCKS knockdown cells, the decrease in MARCKS amount by the low concentration of MeHg was not detected (Fig. 12B). In addition, pretreatment with calpain inhibitors had no effect on the amount of full-length MARCKS. On the other hand, a significant decrease in cell viability caused by the low concentration of MeHg in control cells was augmented in cells with the MARCKS knockdown (Fig. 12C) as previously reported [77], suggesting that MARCKS plays a key role in MeHg cytotoxicity. In line with the result on MARCKS content, pretreatment with calpain inhibitors did not alter the decrease in cell viability induced by MeHg. These results suggest that the calpain-mediated decrease in MARCKS amount mediates MeHg toxicity to SH-SY5Y cells at the low concentration of this chemical. Recently, Pierozan et al. (2017) proposed that the cytoskeleton is an end point of MeHg cytotoxicity [65]. Because it has been demonstrated that in vitro cleavage of MARCKS by calpain increases its actin-binding activity [94], MARCKS cleaved by calpain may exert its effect via actin reorganization, in addition to the increase in MARCKS phosphorylation [77] in MeHg toxicity to SH-SY5Y cells.

Here, we demonstrated that the function of calpain in the regulation of MARCKS protein amounts is dependent on the cell type and concentration of MeHg. In SH-SY5Y cells, proteolysis of MARCKS by calpain mediates the cytotoxicity caused by the low concentration of MeHg. The mechanisms of the MeHg-driven decrease in cell viability and in MARCKS amount remain unknown in EA.hy926 cells and for the high concentration MeHg in SH-SY5Y cells. Interestingly, cleavage of MARCKS by calpain is dependent of its phosphorylation and localization [17]. In addition, it has been reported that cathepsin B, a lysosomal cysteine protease, and ubiquitin-proteasome proteolytic pathway were involved in degradation of MARCKS in neurons [26, 55]. However, it has not been reported whether the cathepsin B or ubiquitin-proteasome system participates in mechanisms of MeHg toxicity on SH-SY5Y and EA.hy926 cells. Involvement of these mechanisms should be clarified for a better understanding of the role of MARCKS proteins in MeHg cytotoxicity. A schematic representation of the regulation of full-length MARCKS amounts by MeHg in SH-SY5Y and EA.hy926 cells is provided in Fig. 13. These findings should elucidate the distinct molecular mechanisms of MeHg toxicity to various cell types.

GENERAL CONCLUSION

The present thesis revealed that 1) characteristics of MeHg toxicity on endothelial cells, 2) involvement of MARCKS on its toxicity, and 3) different toxic mechanism of MeHg between neuronal and endothelial cells.In the first study, the author showed that MeHg exposure induced a dose-dependent decrease in cell viability, migration, tube formation on Matrigel and NO production in EA.hy926 cells. MeHg exposure also elicited a decrease in MARCKS expression and an increase in MARCKS phosphorylation in EA.hy926 cells. Furthermore, alteration of MeHg-induced decrease in cell viability and NO production was observed in cells with MARCKS knockdown or overexpression. The findings suggest the broad role of MARCKS in endothelial cell functions and show that MARCKS is involved in MeHg-induced toxicity in endothelial cells. In the second study, in SH-SY5Y cells, MeHg induced a decrease in cell viability accompanying calcium mobilization, calpain activation, and a decrease in MARKCS expression. However, pretreatment with calpain inhibitors attenuated the decrease in cell viability and MARCKS expression only induced by 1 μM but not by 3 μM MeHg. In cells with MARCKS-knockdown, calpain inhibitors failed to attenuate the decrease in cell viability by MeHg; in EA.hy926 cells, although MeHg caused calcium mobilization and a decrease in MARCKS expression, calpain activation was not observed.These results suggest that participation of calpain in the regulation of MARCKS amounts is

dependent on the cell type and concentration of MeHg. In SH-SY5Y cells, calpain-mediated proteolysis of MARCKS is involved in cytotoxicity induced by a low concentration of MeHg. These findings from this thesis will stimulate and support further progress in research on toxic mechanisms of MeHg in central nervous system and cardiovascular system.

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