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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Chapter 2: The MARCKS protein amount is differently regulated by calpain during toxic effects of methylmercury between SH-SY5Y and EA.hy926 cells

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,