Effects of inorganic mercury and methylmercury on osteoclasts and osteoblasts in the scales of the marine teleost as a model system of bone

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Effects of Inorganic Mercury and Methylmercury on Osteoclasts and Osteoblasts in the Scales of the Marine Teleost as a Model System of Bone

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To evaluate the effects of inorganic mercury (InHg) and methylmercury (MeHg) on bone metabolism in a marine teleost, the activity of tartrate-resistant acid phosphatase (TRAP) and alkaline phosphatase (ALP) as indicators of such activity in osteoclasts and osteoblasts, respectively, were examined in scales of nibbler fish (*Girella punctata*). We found several lines of scales with nearly the same TRAP and ALP activity levels. Using these scales, we evaluated the influence of InHg and MeHg. TRAP activity in the scales treated with InHg (10^{-5} and 10^{-4} M) and MeHg (10^{-6} to 10^{-4} M) during 6 hrs of incubation decreased significantly. In contrast, ALP activity decreased after exposure to InHg (10^{-5} and 10^{-4} M) and MeHg (10^{-6} to 10^{-4} M) for 18 and 36 hrs, although its activity did not change after 6 hrs of incubation. As in enzyme activity 6 hrs after incubation, mRNA expression of TRAP (osteoclastic marker) decreased significantly with InHg and MeHg treatment, while that of collagen (osteoblastic marker) did not change significantly. At 6 hrs after incubation, the mRNA expression of metallothionein, which is a metal-binding protein in osteoblasts, was significantly increased following treatment with InHg or MeHg, suggesting that it may be involved in the protection of osteoblasts against mercury exposure up to 6 hrs after incubation. To our knowledge, this is the first report of the effects of mercury on osteoclasts and osteoblasts using marine teleost scale as a model system of bone.

Key words: inorganic mercury, methylmercury, osteoblasts, osteoclasts, scales, nibbler fish

INTRODUCTION

Mercury is widely distributed in aquatic environments. Elemental mercury (Hg^0) appears to be emitted from fumaroles due to volcanic activity in the bottom of the sea (Sakamoto et al., 1995; Selin, 2009). Hg^0 has been shown to be oxidized to inorganic mercury (InHg) in aquatic environments, and particularly easily in seawater (Yamamoto, 1996). InHg is convertible to methylmercury (MeHg) in natural environments, and most of the mercury in fish tissue has been reported to be MeHg (WHO, 2008; WHO, 2010; Depew et al., 2012). The toxicokinetics (absorption, distribu-

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tion, metabolism, and excretion) of mercury are highly dependent on the form of mercury (WHO, 2008; WHO, 2010). MeHg is a highly toxic compound that bioaccumulates significantly in fish. In long-finned eels, MeHg was approximately 1,000,000 times higher than in environmental water (Redmayne et al., 2000). Humans are mainly exposed to MeHg through consumption of fish with bioaccumulations of mercury (WHO, 2008). In humans, the main target organs of MeHg are brain, kidney, and liver (WHO, 2008). Many studies have thus focused on these tissues. Little attention, however, has been paid to bone metabolism in this area of research, as mercury bioaccumulation in bone was shown to be extremely low compared to that in neural tissues (Boyer et al., 1978; Doyle, 1979; Berglund et al., 2000).

As mentioned above, muscle in fish is the main edible part, and it accumulates MeHg (Depew et al., 2012). In largemouth bass, the total mercury concentration in muscle was highly correlated with that of scales (r = 0.89) (Lake et al., 2006), indicating the importance of examining the interaction between mercury exposure and bone metabolism in fish. Additionally, Lake et al. suggested that assessment of mercury in fish scales may be suitable for prediction of mercury levels in muscle tissue. Mercury accumulated in the scales of fish (herring and sockeye salmon) was also detected by a combination of laser ablation with relatively low-cost cold vapor atomic fluorescence spectrophotometry (Beaudin et al., 2010). Fish scales may thus be another mercury-sensitive organ.

Considering the above, we developed the assay system with nibbler fish to examine the effects of mercury on bone metabolism in marine teleosts. First, we morphologically examined the colocalization of osteoclasts and osteoblasts in the scales of nibbler fish. We then determined the location of scales with similar cell activities to enhance accuracy and reproducibility, and developed an assay system, which we used to examine the effects of InHg and MeHg on scale osteoclasts and osteoblasts. To confirm the effects of InHg and MeHg on osteoclasts and osteoblasts, the mRNA expression of an osteoclast marker (tartrate-resistant acid phosphatase: TRAP) and an osteoblastic marker (collagen type I, α1: COL1A1) were partially sequenced and investigated using a quantitative PCR method. In addition, the mRNA expression of metallothionein (MT), which is a metal-binding protein that protects the organism from heavy metal (Hamer, 1986; Klaassen et al., 1999; Jin et al., 2002), was also examined.

In marine teleosts, this is the first study to indicate that both InHg and MeHg suppress marker enzyme activities of osteoclasts and osteoblasts in scales as a model system of bone.

MATERIALS AND METHODS

Animals

Nibbler fish (*Girella punctata*) (both sexes, n = 80, 64.3 ± 1.3 g) were captured by fishing in Tsukumo Bay of the Noto Peninsula (Ishikawa Prefecture). After acclimation for around two weeks, these fish were used in the present experiments. All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of Kanazawa University.

Morphological study of osteoclasts and osteoblasts in the scales of nibbler fish

Scales were collected from nibbler fish anesthetized with 0.04% of a 2-phenoxyethanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan) solution and fixed using a 4% paraformaldehyde solution neutralized with a phosphate buffer solution (pH 7.2; Wako) for 5 min. Subsequently, osteoclasts were observed by TRAP staining using the methods of Cole and Walters (1987). Osteoblasts were detected by alkaline phosphatase (ALP) staining using a kit (NBT/BCIP Stock Solution, Roche Applied Science, Mannheim, Germany). After staining, the osteoclasts and osteoblasts were observed under a microscope.

Development of an in vitro assay system with the scales of nibbler fish

Nibbler fish were anesthetized with a 0.04% 2-phenoxyethanol (Wako) solution, and body scales were then removed. The location of the scales used in the present study is indicated in Fig. 1. In nibbler fish, we chose a line of scales of approximately the same size. In each line, the removed scales were put into a 96-well microplate in turn, and divided into a control and an experimental group (See Fig. 1). To examine the variance in osteoclastic and osteoblastic activity in the two groups of each line, we measured the activity of osteoclasts and osteoblasts with TRAP and ALP as the respective markers, as these markers have been shown to be affected by a number of hormones and other factors in osteoclasts and osteoblasts in mammals (Vaes, 1988; Dimai et al., 1998; Suda et al., 1999).

The methods for measuring TRAP and ALP activities were as follows. An aliquot of 100 μl of an acid buffer (0.1 M sodium acetate, including 20 mM tartrate, pH 5.3) or an alkaline buffer (100 mM Tris-HCl, pH 9.5; 1 mM $MgCl_2$) was added to each well. Then, the scales were put into their own well in a 96-well microplate. This microplate was frozen at –80°C immediately and then kept at –20°C until analysis. An aliquot of 100 μl of 20 mM para-nitrophenyl phosphate in an acid or alkaline buffer was then added to each well of a melted solution in the microplate. This plate was incubated at 23°C for 60 min while being shaken. After incubation, the reaction was stopped by adding 50 μl of 3 N NaOH. One-hundred-fifty μl of a colored solution was transferred to a new plate, and the absorbance was measured at 405 nm. The absorbance was converted into the amount of produced para-nitrophenol (pNP) using a standard curve for pNP.

After measuring the activity of both TRAP and ALP, the size of the scales was measured with Image J. Afterward, TRAP and ALP activities were normalized to the surface area (mm^2) of each scale (Suzuki et al., 2009).

Effects of InHg and MeHg on TRAP and ALP activities in the cultured scales of nibbler fish

Scales collected from nibbler fish under anesthesia with a

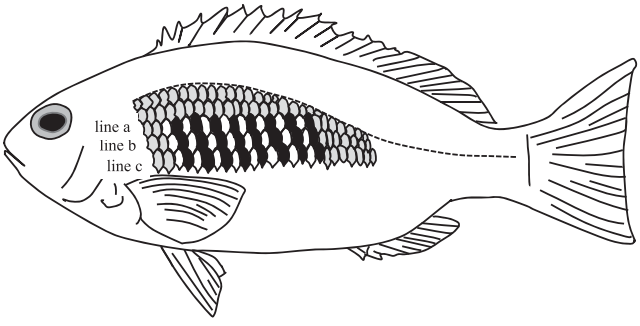

Fig. 1. Location of the scales used in the present study. In nibbler fish, we chose the lines (a, b, and c) of scales of approximately the same size. In each line, the removed scales were separated into two groups, a control group and an experimental group. The scales of group 1 are shown in white; the other scales (group 2) are indicated in black.

0.04% 2-phenoxyethanol (Wako) solution were incubated for 6 hrs in L-15 medium (Life Technologies Corporation, Carlsbad, CA, USA) with added InHg ($HgCl_2$) and MeHg (CH_3HgCl) (each 10^{-6} to 10^{-4} M; Wako) and compared with a mercury-free medium as a control. After incubation, TRAP and ALP activities were measured using the same method described above. Furthermore, to evaluate the influence of InHg and MeHg on osteoblasts with longer exposure times, scales were incubated in mercury for 18 and 36 hrs.

Sequencing of TRAP, COL1A1, MT, and elongation factor 1α

Scales were collected from nibbler fish under anesthesia with a 0.04% 2-phenoxyethanol (Wako) solution. Total RNAs were prepared from scales of nibbler fish using a total RNA isolation kit (NucleoSpin RNA II, Takara Bio Inc., Otsu, Japan). Complementary DNA was synthesized using the PrimeScriptTM RT reagent kit (Takara Bio Inc.). Using cDNA from the scales of nibbler fish, partial fragments of TRAP and COL1A1 were amplified by degenerate PCR method. The degenerated primer sets for TRAP (sense: GCNGGNAAYCAYGAYCA; antisense 1: TCIADRAARTTICCNGCNC; antisense 2: ARRTTRTGRTCRTGICCRCA) and for COL1A1 (sense: TGYCAYCCNGAYTGGAA; antisense: YTCDATYTCRTTISWNCC) were designed from multiple amino acid sequence alignments of each vertebrate family member. To amplify the TRAP cDNA fragment, particularly, nested PCR was done.

The sequence of the open reading frame in MT was determined by use of the 3' RACE System for Rapid Amplification of cDNA Ends (Life Technologies Corporation). The gene-specific primer for MT (ATGGATCCNTGCGAA) was also designed by the multiple amino acid sequence alignment of MT vertebrate family members. These sequences were analyzed by a direct sequence method using an ABI PRISM 3130xl Genetic Analyzer.

To normalize each mRNA expression, elongation factor 1α (EF-1α) was also cloned partially. The primers (sense: CCATGTCGACTCTGGGAAAT; antisense: TTCAGGAACTTGGGGTTGTC) were designed from the sequence of lamprey EF1α (AB183717). Then, we also determined the partial sequence of EF-1α by direct sequencing.

Changes in TRAP, COL1A1, and MT mRNA expression of the scales by InHg and MeHg treatment

Scales were collected from nibbler fish under anesthesia with a 0.04% 2-phenoxyethanol (Wako) solution. To examine changes in TRAP, COL1A1, and MT mRNAs that responded to InHg or MeHg, these scales were incubated for 6 hrs in L-15 medium (Life Technologies Corporation) with added InHg (10^{-5} M) or MeHg (10^{-5} M) and compared with the control (without mercury). After incubation, the scales were frozen at –80°C for mRNA analysis.

Total RNAs were prepared from scales of nibbler fish, and cDNA was synthesized using a kit as described above. The PCR primer sequences—sense: TGGATGAGAAGCCCAGAGG; antisense: CCGCAGAGGTAAGCAGTGG—were designed from TRAP (AB874604). The primer sets for COL1A1 (AB874603) (sense: 5′-GTGAGGTCGCCAAGAAGAAC-3′; antisense: 5′-ATGAGACGCAGGAAGGTCAG-3′) and for MT (AB874602) (sense: 5′-TCAAAGAGTGGAAAATGCAACTG-3′; antisense: 5′-GATGGGCAGCATGAGCAG-3′) were used. EF-1α (AB874605) was amplified using the following primers: sense: 5′- GTATGGTCGTCACCTTTGCTC-3′; antisense: 5′- GTGGGTCGTTCTTGCTGTC-3′. The PCR amplification was performed using the real-time Mx3000p PCR apparatus (Agilent Technologies, Santa Clara, CA, US) (Suzuki et al., 2011a). The annealing temperature of TRAP, COL1A1, MT, and EF-1α was 60°C. The initial reaction condition was 10 s at 95°C, followed by

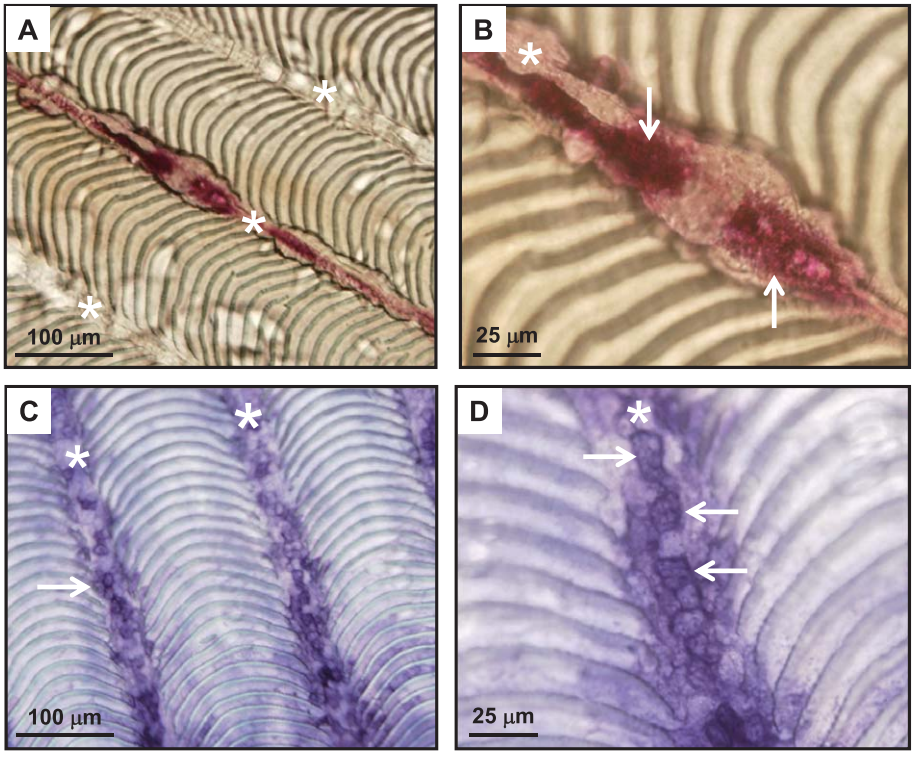

Fig. 2. Microscopic views of nibbler fish scales stained for osteoclasts **(A, B)** and osteoblasts **(C, D)**. Panels **(B)** and **(D)** are enlarged views of **(A)** and **(C)**, respectively. Arrows indicate multinucleated osteoclasts **(B)** and osteoblasts **(D)**. Asterisks show grooves of the scales in nibbler fish.

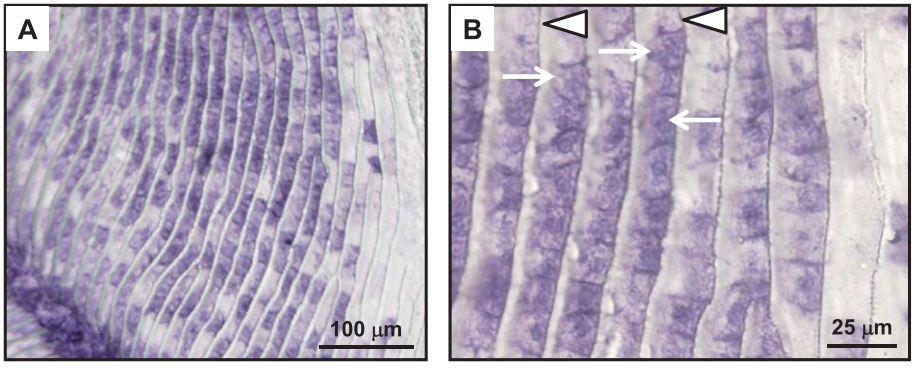

Fig. 3. Microscopic views of nibbler fish scales stained for osteoblasts. Panel **(B)** is an enlarged view of **(A)**. Arrows indicate osteoblasts. Arrowheads show ridges of the scales in nibbler fish.

40 cycles of denaturation at 95°C for 10 s and annealing/extension at 60°C for 40 s. The TRAP, COL1A1, and MT mRNA levels were normalized to the EF-1α mRNA level.

Statistical analysis

Comparison between group 1 and 2 (each 8 scales) (See Fig. 1) was assessed by Student's *t*-test. The mean value from eight individual scales of one nibbler fish in the control group was compared with that in the experimental group. The data were assessed using the paired *t*-test, and the significance level chosen was $P < 0.05$. All results are expressed as means ± SEM ($n = 6$ or 8).

RESULTS

Osteoclasts and osteoblasts in the scales of nibbler fish

We detected osteoclasts in the scales of nibbler fish. Arrows indicate multinucleated osteoclasts (Fig. 2B) in the grooves (asterisks in Fig. 2A, B) of the scales. ALP-stained osteoblasts (arrows in Fig. 2D) were detected around the grooves (asterisks in Fig. 2C, D). Osteoclastic and osteoblastic cells were colocalized in the grooves of the scales. In addition, ALP-stained osteoblasts (arrows in Fig. 3B) were detected around the ridges (arrowheads in Fig. 3B).

Comparison of TRAP and ALP activities among the alternate scales in each line

In each line, TRAP activity was not changed significantly among alternative scales (Fig. 4A). In the case of ALP activity as well, there was no significant difference between the respective value of group 1 and group 2 (Fig. 4B).

Effects of InHg and MeHg on TRAP activity in the cultured scales of nibbler fish

The results are shown in Fig. 5. TRAP activity significantly decreased by InHg (10^{-5} and 10^{-4} M, $P < 0.05$) treatment at 6 hrs (Fig. 5A). Also, MeHg was significantly decreased in the TRAP activities of the scales at 6 hrs (10^{-6} and 10^{-5} M: $P < 0.05$, 10^{-4} M: $P < 0.01$) (Fig. 5B).

Effects of InHg and MeHg on ALP activity in the cultured scales of nibbler fish

During 6 hrs of incubation, ALP activity did not change from the values in the control group (Fig. 6A). However, ALP activity in the InHg-treated scales decreased significantly at 18 (10^{-5} and 10^{-4} M: $P < 0.05$) and 36 (10^{-5} M: $P < 0.05$; 10^{-4} M: $P < 0.01$) hrs of incubation from the value of the control scales (Fig. 6B, C).

Similar changes were induced by MeHg. ALP activity did not change at 6 hrs (Fig. 7A). In MeHg as well as InHg, ALP activity in the treated scales decreased significantly at 18 hrs (10^{-6} M, $P < 0.05$; 10^{-5} and 10^{-4} M, $P < 0.01$) and 36 hrs (10^{-6} M, $P < 0.05$; 10^{-5} M, $P < 0.01$; 10^{-4} M, $P < 0.001$) of incubation from the values of the control scales (Fig. 7B, C).

Sequencing of TRAP, COL1A1, MT, and EF-1α

The sequences of TRAP, COL1A1, and MT are shown in Fig. 8. We determined the sequences of TRAP, COL1A1, and MT from the scales of nibbler fish. Deduced amino acid sequences of TRAP and MT showed high identity to salmon (84.6%) in TRAP and to goldfish (75.0%) in MT. In TRAP and MT, there was a relatively high sequence identity to other vertebrate counterparts (TRAP: 57.7% to human and 60.0% to mouse; MT: 65.6% to human and 69.9% to rat). However, the sequence of COL1A1 was well-conserved among vertebrates, at least in the obtained sequence. Sequence identity of nibbler fish COA1A1 to goldfish, human, and mouse COL1A1 was 88.7, 82.7, and 84.2%, respectively.

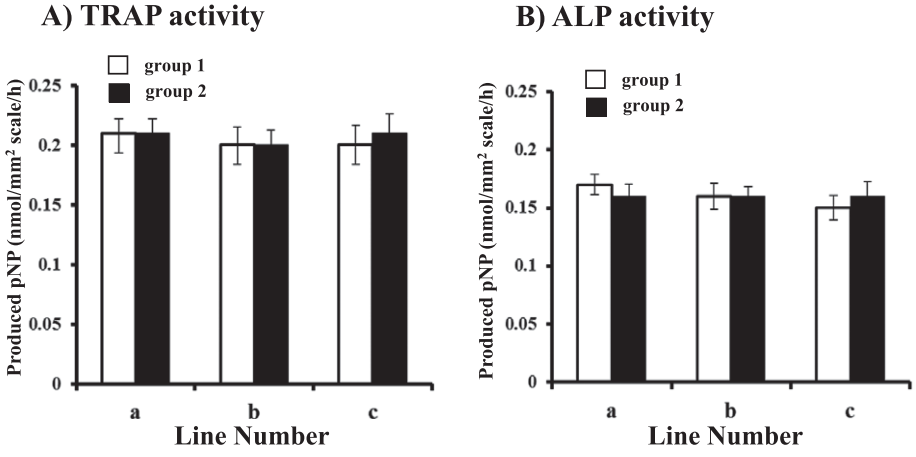

Fig. 4. TRAP **(A)** and ALP **(B)** activities of the scales in two groups in each line. There was no significant difference in TRAP and ALP activity values in scales between group 1 and group 2 (n = 8 scales).

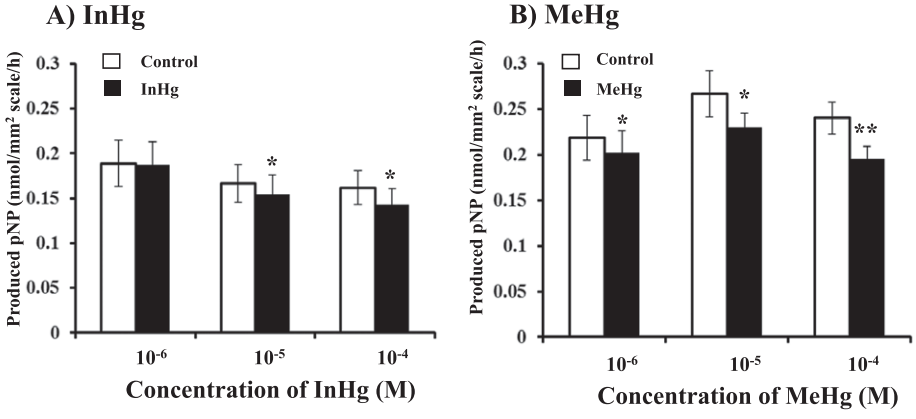

Fig. 5. Effect of InHg (10^{-6} to 10^{-4} M) (A) and MeHg (10^{-6} to 10^{-4} M) (B) on TRAP activity in cultured scales at 6 hrs. * and ** indicate statistically significant differences at $P < 0.05$ and $P < 0.01$, respectively, from the values of the control scales (n = 6 samples; one sample from one fish).

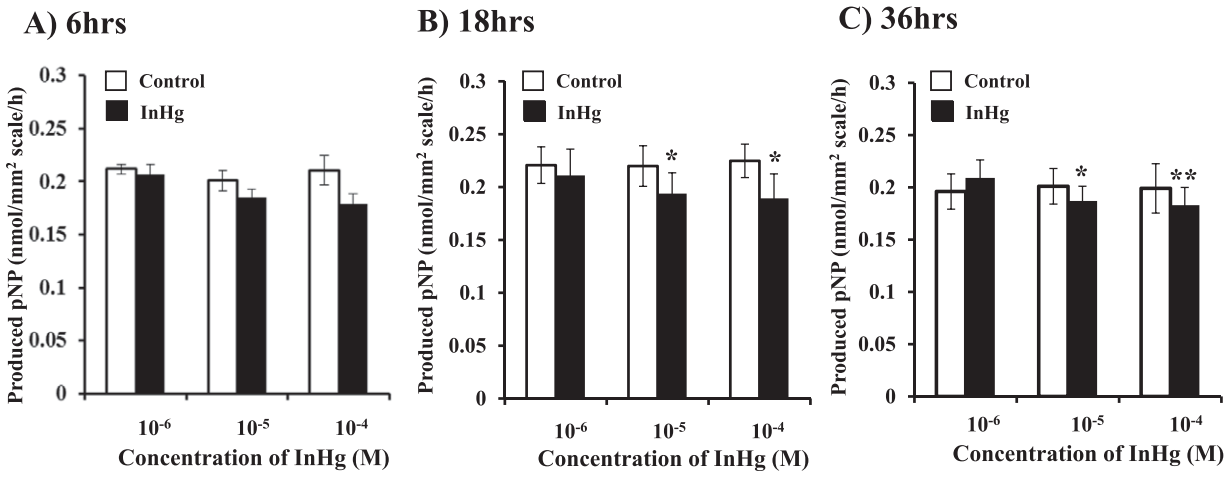

Fig. 6. Effect of InHg (10^{-6} to 10^{-4} M) on ALP activity in the cultured scales at 6 **(A)**, 18 **(B)**, and 36 hrs **(C)**. * and ** indicate statistically significant differences at $P < 0.05$ and $P < 0.01$, respectively, from the values of the control scales ($n = 6$ samples; one sample from one fish).

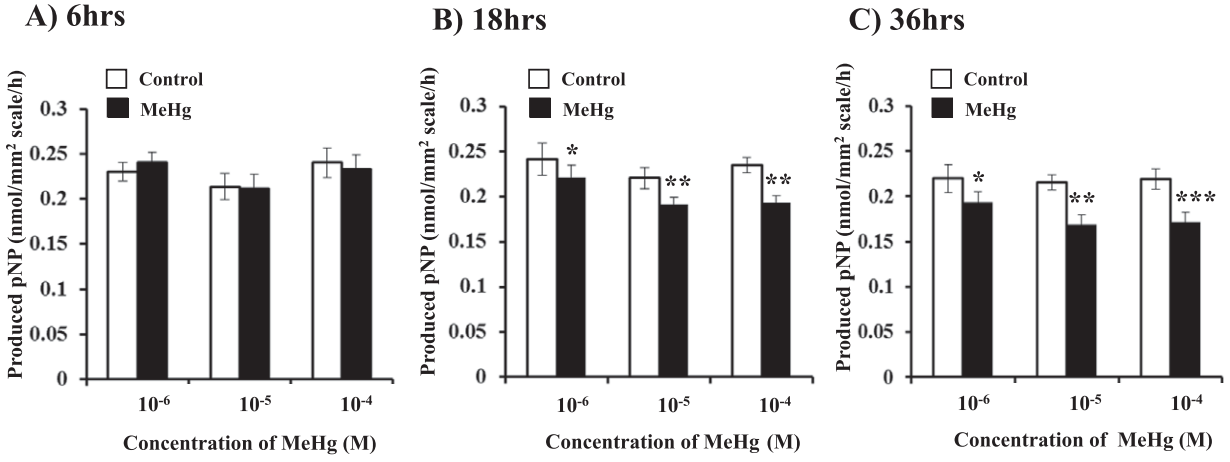

Fig. 7. Effect of MeHg (10^{-6} to 10^{-4} M) on ALP activity in the cultured scales at 6 (A), 18 (B), and 36 hrs (C). *, **, and *** indicate statistically significant differences at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, from the values of the control scales ($n = 6$ samples; one sample from one fish).

Changes in TRAP, COL1A1, and MT mRNA expression of the scales by InHg and MeHg treatment

The relative ratio of amplified TRAP, COL1A1, and MT cDNAs to EF-1α cDNA is shown in Fig. 9. After 6 hrs of incubation, the expression of TRAP mRNA in both InHg- and MeHg-treated scales was lower than that in the control scales (InHg: $P < 0.05$; MeHg: $P < 0.01$), although COL1A1 mRNA did not change significantly. Conversely, MT mRNA expression was increased by both InHg ($P < 0.05$) and MeHg treatment ($P < 0.01$).

DISCUSSION

In this study, we found that the enzyme activity of TRAP (osteoclastic marker) was decreased at 6 hrs following treatment by both InHg and MeHg. In osteoblasts, also, both InHg and MeHg inhibited the ALP enzyme activity at 18 and 36 hrs after incubation. It is known that the scales are calcified tissue that contains osteoclasts and osteoblasts (Bereiter-Hahn and Zylberberg, 1993; Yoshikubo et al., 2005; Suzuki et al., 2007; Suzuki et al., 2008; de Vrieze et al., 2010; Suzuki et al., 2011a; Thamamongood et al., 2012, Yano et al., 2013). Additionally, it has been reported that scales are a better potential internal calcium reservoir than body skeletons, jaws, and otoliths examined by the ^{45}Ca-labeling study of calcified tissues of goldfish and killifish (Mugiya and Watabe, 1977). In fish, thus, scales accumulate mercury and sensitively respond to mercury exposure in a short time.

In nibbler fish (a marine teleost), we have shown that both InHg and MeHg directly affect osteoclasts by both analyses of TRAP enzyme activity and TRAP mRNA expression in the present study. In goldfish (freshwater teleost), we previously reported that InHg (10^{-5} to 10^{-3} M) (Suzuki et al., 2011b) and MeHg (10^{-8} to 10^{-6} M) (Suzuki et al., 2004) significantly suppressed TRAP enzyme activity. Particularly, the response of MeHg in nibbler fish is lower than that in goldfish. It is known that the exchange of calcium in the scales of freshwater teleost is faster than that in marine teleost because freshwater teleosts live in a low-calcium environment. In fact, the response of the calcemic hormone

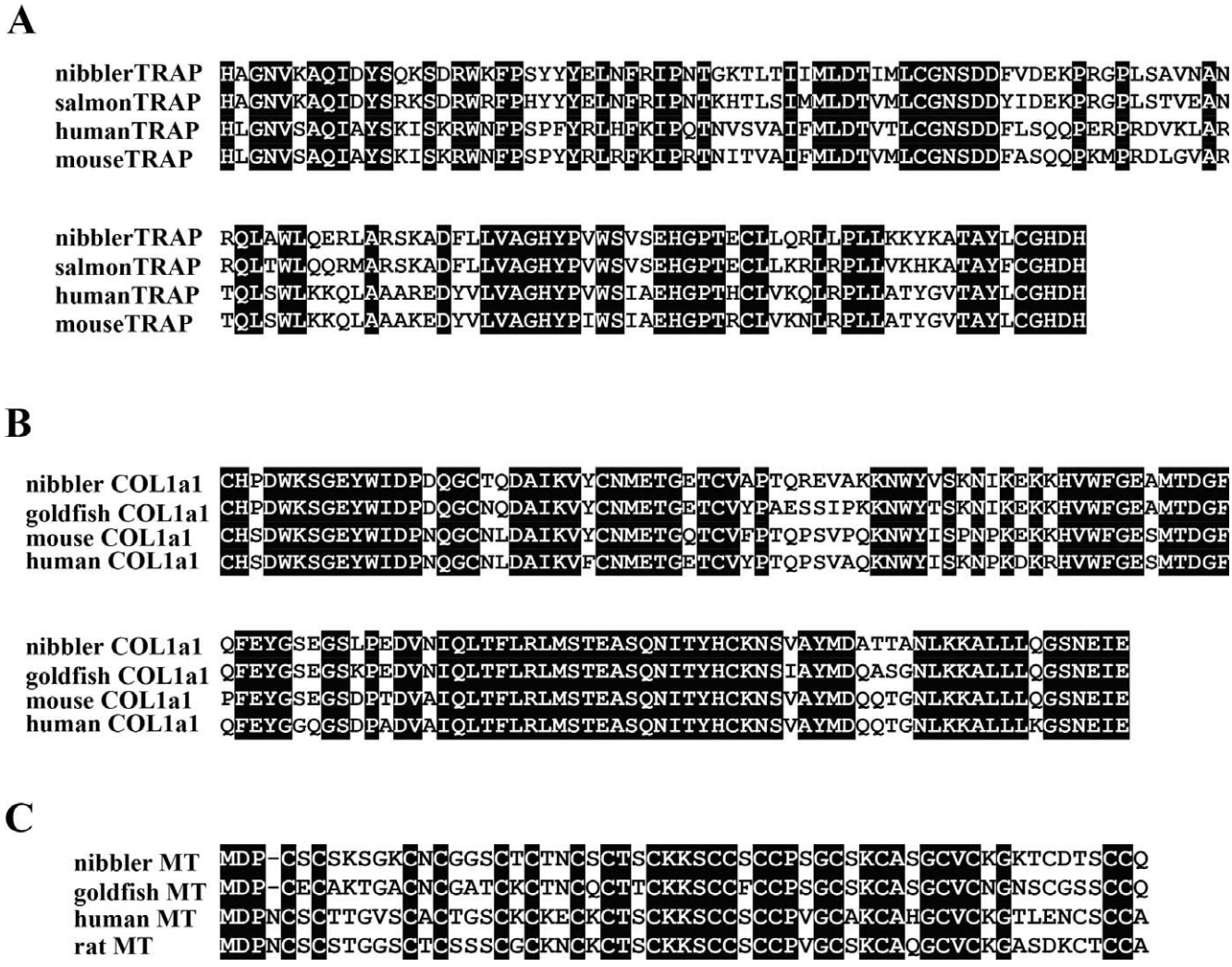

Fig. 8. Alignment of amino acid sequences of TRAP **(A)**, COL1A1 **(B)**, and MT **(C)**. Conserved amino acid sequences are indicated by black boxes. The accession numbers are as follows: nibbler TRAP, AB874604; salmon TRAP, NP_001134890.1; human TRAP, NP_001602.1; mouse TRAP, NP_031414.1; nibbler COL1A1, AB874603; goldfish COL1A1, BAG72200.1; human COL1A1, CAA98968.1; mouse COL1A1, AAA88912.1; nibbler MT, AB874602; goldfish MT, AAB32777.1; human MT, NP_789846.1; rat MT, NP_620181.1.

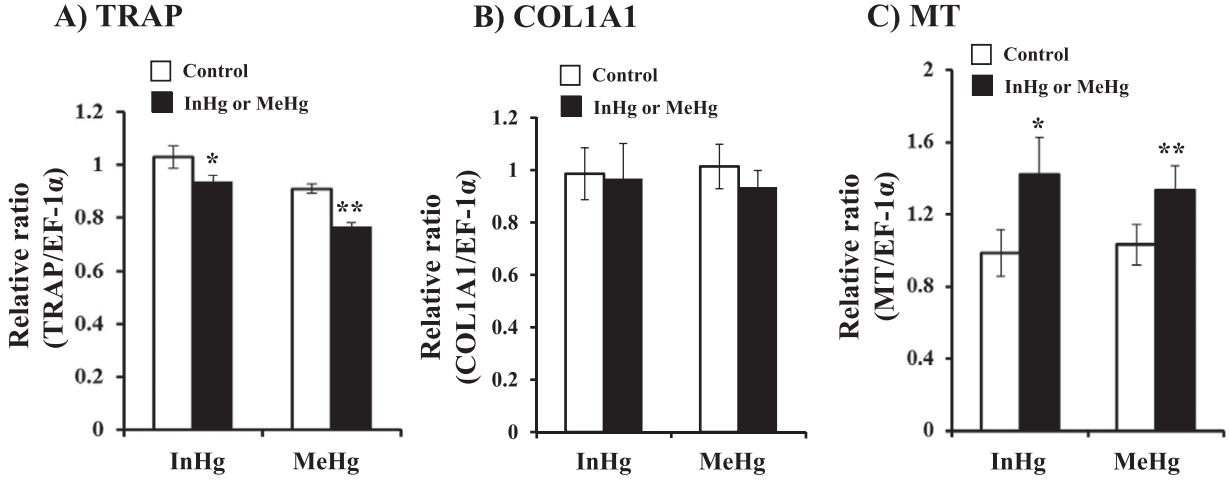

Fig. 9. Expression of TRAP **(A)**, COL1A1 **(B)**, and MT **(C)** mRNAs in the InHg- and MeHg-treated scales of nibbler fish at 6 hrs of incubation. TRAP, COL1A1, and MT mRNA levels were normalized to the EF-1α mRNA level. * and ** indicate statistically significant differences at $P < 0.05$ and $P < 0.01$, respectively, from the values of the control scales ($n = 8$ samples; one sample from one fish).

estrogen in the scale osteoclasts of freshwater teleosts was higher than that in those of marine teleosts (Persson et al., 1994; Persson et al., 1995; Guerreiro et al., 2002). We believe that osteoclasts in freshwater teleosts have some important roles in calcium metabolism as compared with those in marine teleosts.

In the case of osteoblasts, the ALP enzyme activity did not change from the values in the control group during 6 hrs of incubation with either InHg or MeHg. However, ALP activity in the InHg (10^{-5} and 10^{-4} M)- and MeHg (10^{-6} to 10^{-4} M)-treated scales decreased significantly at 18 and 36 hrs of incubation from the value of the control scales. From each marker mRNA expression analysis, at 6 hrs of incubation, the expression of TRAP mRNA in InHg- and MeHg-treated scales was significantly lower than that in the control scales, although the mRNA expression of COL1A1 (osteoblastic marker) did not change significantly. Conversely, MT mRNA expression significantly increased with both InHg and MeHg treatment. This result is similar to those from studies in mammals, as it has been demonstrated that MT plays a protective role in mercury-induced toxicity in bone (Jin et al., 2002). As it has been reported that osteoblasts express MT and protect from heavy metal (Angle et al., 1990; Nagata and Lönnerdal, 2011), the activation of MT in osteoblasts may be involved in resistance to mercury.

In mammals, the influence of mercury on bone metabolism has been studied mainly by in vivo experiments and investigated in bone formation or osteoblastic activity (Yonaga et al., 1985; Jin et al., 2002). Both MeHg and InHg inhibited the growth of tibia in rats (Yonaga et al., 1985), and InHg decreased the serum levels of osteoblastic markers (ALP and osteocalcin) in rats (Jin et al., 2002). Mammalian bone has resorptive cells (osteoclasts) and formative cells (osteoblasts). These cells act on the regulation of calcium homeostasis in mammals as well as in fish. Simultaneous incubation of osteoclasts and osteoblasts is therefore necessary for the evaluation of the effects of mercury on bone metabolism. However, a co-culture of osteoclasts and osteoblasts is not particularly easy to obtain because of the difficulty in handling osteoclasts. Pre-osteoclasts are triggered by the receptor activator NF-κB—the receptor activator of the NF-κB ligand binding of osteoclasts and osteoblasts followed by differentiation, activation, and multinucleation (Suda et al., 1999; Teitelbaum, 2000; Hofbauer et al., 2004). In the present study, therefore, we used scales from a marine teleost to develop an in vitro assay system. This system can be used to simultaneously detect both osteoclastic and osteoblastic activities with TRAP and ALP enzyme activities. Furthermore, we determined TRAP, COL1A1, and MT cDNAs from the scales of nibbler fish. Thus, we conclude that the in vitro assay system we developed may be a useful means for analyzing heavy metal in bone metabolism. In the future, we will further examine different toxic mechanisms between MeHg and InHg using fish scales as a model of bone.

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