Effects of Inorganic Mercury on Osteoclasts and Osteoblasts of the Goldfish Scales In Vitro

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INTRODUCTION
Heavy metals such as mercury, cadmium and copper are known to be extremely toxic to organisms. Mercury has been recognized as an environmental contaminant since the Minamata disaster in the late 1950s. Minamata disease which was caused by the consumption of marine fishes severely polluted with mercury from local industrial discharge due to this Minamata disaster (Takeuchi et al., 1978; Takeuchi, 1982). This extremely adverse situation occurred because of mercury, a highly toxic compound, was severely bio-accumulated (in case of long-finned eels: approx. 1,000,000 times higher than environmental water) by fish (Redmayne et al., 2000).

The effect of mercury on the central nervous system has widely studied and revealed that mercury is a neuro-toxic material, and its poisoning effect is characterized by the damage in discrete portions of the brain, such as the visual cortex and the granule layer of the cerebellum (Castoldi et al., 2001). As bio-accumulation of mercury in bone is lower than that in neural tissues (Boyer et al., 1978; Doyle, 1979; Berglundi et al., 2000), much attention has not been given to bone in this area of research.

Recently, Lake et al. (2006) reported that the corre-
lation between the total mercury concentration of the scales and that of the muscles was high (r=0.89), and suggested the suitability for prediction of muscle tissue by the assessment of available mercury in the fish scales. It is known that the scales are calcified tissue which contains osteoclasts and osteoblasts (Bereiter-Hahn and Zylberberg, 1993; Suzuki et al., 2000; Yoshikubo et al., 2005; Suzuki et al., 2007) and is reported that the scales are a better potential internal calcium reservoir than the body skeletons, jaws and otolithes, examined by the multiple–labelling study for the calcified tissues of goldfish and killifish (Mugiya and Watabe, 1977). In fishes, thus, the scale accumulates mercury and seems to be a sensitive tissue for mercury.

Recently, we have developed a novel in vitro assay system using goldfish scale (Suzuki et al., 2000; Suzuki and Hattori, 2002) because the scale is a very active tissue of calcium regulation in fish described above. In the present study, therefore, we examined the effect of inorganic mercury (HgCl₂) on the scale osteoclasts and osteoblasts. To confirm the effects of HgCl₂ on osteoclasts and osteoblasts, the mRNA expressions of osteoclastic markers (tartrate–resistant acid phosphatase: TRAP and cathepsin K) and osteoblastic marker (insulin–like growth factor–I: IGF–I) were investigated using reverse–transcription (RT)–PCR. Furthermore, 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), was also examined using RT–PCR.

MATERIALS AND METHODS

Animals

Our previous study (Suzuki et al., 2000) indicated that the sensitivity for calcemic hormone such as estrogen and calcitonin was higher in mature female than immature female goldfish (Carassius auratus). Therefore, mature female goldfish (n=12, 35.50 ± 1.30 g) were purchased from commercial source (Higashikawa Fish Farm, Yamatokoriyama, Japan) and used in the scale incubation for 6 hrs in MEM supplemented with 10–8–10–3 M ethyl 3–aminobenzoate, methanesulfonic acid salt (MS–222, Sigma–Aldrich, Inc., MO, USA) and antibiotic and 20 mM HEPES (Research Organics Inc., OH, USA) (20 mM) was added to MEM and adjusted to pH 7.0. After filtration, MEM was used in this experiment for analyzing the effect of HgCl₂ on TRAP and ALP activities in the cultured goldfish scales. Scales collected from goldfish under anesthesia with MS–222. To examine changes in TRAP, cathepsin K, IGF–I, and MT mRNAs that responded to HgCl₂, these scales were incubated for 18 hrs in MEM (containing antibiotic and 20 mM HEPES) supplemented with HgCl₂ (10–4 M) and compared with the control (without metals).

Effect of HgCl₂ on TRAP and ALP activities in the cultured scales of goldfish

A 1% penicillin–streptomycin mixture (ICN Biomedicals Inc., OH, USA) was added to Eagle’s minimum essential medium (MEM; ICN Biomedicals Inc.). HEPES (Research Organics Inc., OH, USA) (20 mM) was added into MEM and adjusted to pH 7.0. After filtration, MEM was used in this experiment for analyzing the effect of HgCl₂ on TRAP and ALP activities in the cultured goldfish scales. Scales collected from goldfish under anesthesia with ethyl 3–aminobenzoate, methanesulfonic acid salt (MS–222, Sigma–Aldrich, Inc., MO, USA) and incubated for 6 hrs in MEM supplemented with 10–4–10–1 M HgCl₂ (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and compared with control (HgCl₂–free medium). To evaluate the effect of HgCl₂ on osteoclasts and osteoblasts, furthermore, scales were incubated with HgCl₂ (10–2, 10–1, 10–0, and 10+ M) for comparatively longer exposure times, namely 18, 36, and 64 hrs. After incubation, scales were fixed in 10% formalin in a 0.05 M cacodylate buffer (pH 7.4) followed by a storage in a 0.05 M cacodylate buffer at 4 °C until analysis.

The measurement methods of TRAP and ALP activities have been described by Suzuki and Hattori (2002). We detected the respective enzyme activity from one scale by transferring each scale into a 96–well–microplate and directly incubating it with the substrate in each well. The procedure of TRAP measurement was as follows. Each scale was transferred to its own well in a 96–well microplate after measurement of the scale weight. An aliquot of 200 μl of 10 mM para–nitrophenyl–phosphate and 20 mM tartrate in a 0.1 M sodium acetate buffer (pH 5.3) was added to each well. This plate was then incubated at 20 °C for 30 min while being shaken. After incubation, the reaction was stopped by adding 50 μl of 2 N NaOH. One hundred and 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. The results are shown as means ± SEM of eight scales.

ALP activities were measured using an alkaline buffer (100 mM Tris–HCl, pH 9.5; 1 mM MgCl₂; 0.1 mM ZnCl₂). Other conditions were the same as those for the measurement of TRAP activity.

Changes of TRAP, cathepsin K, IGF–I, and MT mRNA expression in HgCl₂–treated scales for 18 hrs of culture

Scales were collected from goldfish under anesthesia with MS–222. To examine changes in TRAP, cathepsin K, IGF–I, and MT mRNAs that responded to HgCl₂, these scales were incubated for 18 hrs in MEM (containing antibiotic and 20 mM HEPES) supplemented with HgCl₂ (10+ M) and compared with the control (without metals). We previously reported that IGF–I mRNA expression decreased at 18 hrs of incubation (Suzuki and Hattori, 2003). Therefore, this incubation period was adopted. After incubation, the scales were frozen at −80 °C for mRNA analysis.

Total RNAs were prepared from the goldfish scales using a total RNA isolation kit (Nippon Gene, Tokyo, Japan). RT–PCR was performed using Oligotex–dT 30 Super (Takara Bio Inc., Otsu, Japan) as an oligo dT primer to prevent genomic DNA contamination (Suzuki et al., 1997). The gene–specific primers (TRAP 5': AACTTC–CGATTCCCTGAAACG; TRAP 3': GCCCAGCCACCAGGATATA; cathepsin K 5': GCTATGGATCCGTGCGAATGC; MT 3': GGAGACGCTGTGCGGG; IGF–I 3': GGAGCCACACCAAAAGG; MT 5': CTGGCGTCTACGCTACAT) reported by Azuma et al. (2007) were used. IGF–I and MT cDNAs were also amplified using gene specific primers (IGF–I 5': GGAGACGCTGTGCGGG; IGF–I 3': GCCCAGCCACCAGGATATA; cathepsin K 5': GCTATGGATCCGTGCGAATGC; MT 3': GGAGACGCTGTGCGGG; MT 5': GCCCAGCCACCAGGATATA).
HgCl$_2$ Inhibits the Scale Osteoclastic and Osteoblastic Activities

CTCCTCATTGACAGCAGCT). These were designed from the nucleotide sequences of respective cDNA (IGF–I: Kermouni et al., 1998; MT: Chan, 1994). β–actin cDNA using a primer set (5’-CAGCTGTCGACACGACGAGT; 3’-CCATCTCGCTGCTCAAAGTC) (Chan et al., 1998) were also amplified. The conditions for PCR amplification were denaturation for 0.5 min at 96 °C, annealing for 1 min at 55 °C, and extension for 2 min at 72 °C, followed by a single cycle at 72 °C for 30 min. The cycle numbers for the amplification in TRAP, cathepsin K, IGF–I, MT, and β–actin cDNAs were determined by ensuring that PCR amplification was at submaximum and the intensity of the band corresponded exactly to the amount of starting material. The PCR products were analyzed on a 2.5% NuSive GTG agarose gel (FMC BioProducts, ME, USA) and stained with ethidium bromide. The band densities were estimated using a computer program (NIH Image J). The mRNA levels of TRAP, cathepsin K, IGF–I and MT were normalized to the mRNA level of β–actin.

Statistical analysis

The statistical significance was assessed by one–way ANOVA followed by Dunnett test. The significance level chosen was as $P<0.05$.

RESULTS

Effect of HgCl$_2$ on TRAP activity in the cultured scales of goldfish

HgCl$_2$ was significantly decreased the TRAP activities of the scales by 6 hrs of incubation ($P<0.01$ for $10^{-5}$ M; $P<0.001$ for $10^{-4}$ and $10^{-3}$M) (Fig. 1). Thus, increased doses of HgCl$_2$ resulted in greater effects on decreasing TRAP activities dose–dependently.

By the long incubation time period (18 to 64 hrs), only at $10^{-4}$M, significant difference ($P<0.01$) between HgCl$_2$–treated scales and control scales was obtained by

Fig. 1. Effect of HgCl$_2$ ($10^{-8}$ to $10^{-3}$ M) on TRAP activity in the cultured scales incubated for 6 hrs. **, *** indicate statistically significant differences at $P<0.01$ and $P<0.001$, respectively, from the value in the control scales.

Fig. 2. Effect of HgCl$_2$ ($10^{-7}$ to $10^{-4}$ M) on TRAP activity in the cultured scales incubated for 18, 36, and 64 hrs. ** indicates statistically significant difference at $P<0.01$ from the values in the control scales.

Fig. 3. Effect of HgCl$_2$ ($10^{-7}$ to $10^{-3}$ M) on ALP activity in the cultured scales incubated for 6 hrs. There was no significant difference between HgCl$_2$–treated scales and control scales.

Fig. 4. Effect of HgCl$_2$ ($10^{-7}$ to $10^{-3}$ M) on ALP activity in the cultured scales incubated for 18, 36, and 64 hrs. *, **, *** indicate statistically significant differences at $P<0.05$, $P<0.01$ and $P<0.001$, respectively, from the values in the control scales.
18 hrs of incubation (Fig. 2).

**Effect of HgCl₂ on ALP activity in the cultured scales of goldfish**

The ALP activity of the control scales by 6 hrs of incubation was 5.33 ± 0.41 (nmol produced pNP/mg scale/hr) which did not show any difference with HgCl₂–treated groups (10⁻⁸ to 10⁻³M) (Fig. 3). Thus, the ALP activity did not change during 6 hrs of incubation with HgCl₂ compared to the control.

However, the ALP activity in the HgCl₂–treated scales decreased significantly by 36 hrs (P<0.05 for 10⁻³M, 10⁻²M or 10⁻¹M) and 64 hrs (P<0.05 for 10⁻²M; P<0.01 for 10⁻¹M and P<0.001 for 10⁻²M) of incubation from the values of the control scales although it did not change at 18 hrs of incubation (Fig. 4).

**Changes of TRAP, cathepsin K, IGF–I, and MT mRNA expression in HgCl₂–treated scales**

After 18 hrs of incubation, the mRNA expressions of TRAP, cathepsin K and IGF–I in HgCl₂–treated scales were significantly (P<0.001 for TRAP; P<0.001 for cathepsin K and P<0.001 for IGF–I) lower than those in the control scales (Fig. 5). Conversely, the mRNA expression was significantly (P<0.001) increased for MT when treated with HgCl₂ (Fig. 5).

**DISCUSSION**

The present study demonstrated that fish scale sensitively responded to HgCl₂. A high co–relation of mercury between scales and muscles was reported in largemouth bass (Lake et al., 2006). This indicates that accumulation of mercury is occurred in the fish scale although mercury did not accumulate in the vertebral bone of fish (Camusso et al., 1995). It is also well–known that the scale is a more active tissue in fish calcium regulation than vertebral bone (Mugiyama and Watabe, 1977; Yamada, 1961; Berg, 1968; Bereiter–Hahn and Zylberberg, 1993). Therefore, we strongly believe that the fish scale is capable to accumulate mercury and respond to mercury similarly like calcium.

In mammals, the influence of mercury on bone metabolism has been studied only by *in vivo* experiments and investigated in bone formation or osteoblastic activity (Yonaga et al., 1985; Jin et al., 2002). Mercury inhibited the growth of tibia in rats (Yonaga et al., 1985) and decreased serum levels of osteoblastic markers (ALP and osteocalcin) (Jin et al., 2002). In our knowledge, our study is the first to indicate direct effect of inorganic mercury on osteoclasts. The inhibitory action of HgCl₂ on osteoclasts after 6 hrs incubation was stronger than that of 18 to 64 hrs incubation. As for organic mercury, similar results were obtained in our scale assay system (Suzuki et al., 2004). Furthermore, we recently succeed to clone osteoclastic markers: TRAP and cathepsin K in fish for the first time (Azuma et al., 2007) and examined mRNA expressions of these markers in the HgCl₂–treated scales. In the present study, we confirmed that the both mRNA expressions of TRAP and cathepsin K decreased as TRAP enzyme activity did.

It was found that the mRNA expression of MT in HgCl₂–treated scales increased in the present study. This result is similar to that in mammals because it has been demonstrated that MT plays a protective role in mercury–induced toxicity in bone (Jin et al., 2002). Fish are aquatic animals with scales that are always exposed to environmental water. In an *in vitro* experiment for 6 and 18 hrs of incubation, therefore, osteoblasts may be resistant to mercury as a result of MT production. On the other hand, IGF–I mRNA expression decreased compared to the control. As IGF–I participates in osteoblastic growth and differentiation, we speculated that mercury has toxic effect on osteoblasts under long–term exposure.

We previously demonstrated that the osteogenesis of regenerating scale is very similar to that of mammalian membrane bone and a good model of osteogenesis (Yoshikubo et al., 2005). Using this system, furthermore, we first demonstrated that calcitonin, a hypocalcemic hormone, suppressed osteoclastic activity in teleosts as well as in mammals (Suzuki et al., 2000) and that melatonin, a major hormone secreted from the pineal gland, suppressed the functions in both osteoclasts and osteoblasts (Suzuki and Hattori, 2002). Osteoblasts in the scale responded to estrogen as they do in mammals (Yoshikubo et al., 2005). In addition, the effects of endocrine disrupters, such as bisphenol–A (Suzuki and Hattori, 2003) and tributyltin (Suzuki et al., 2006), and heavy metals, i.e., cadmium and organic mercury (Suzuki et al., 2004), on osteoblasts and osteoclasts have been examined. Moreover, we indicated that cadmium (even at 10⁻¹⁰M) responded to TRAP activity in the scale (Suzuki et al., 2004). Considering these results together with present data, our scale assay system will be useful for analysis of environmental contaminant on bone metabolism.
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