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Benz[*a*]anthracene Decreases Plasma Calcium Levels Resulting from Influence of Scale Osteoclastic and Osteoblastic Activities in Goldfish

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Abstract: In the present study, the effects of polycyclic aromatic hydrocarbons (PAHs) on plasma calcium, osteoblasts and osteoclasts were investigated. Goldfish were intraperitoneally injected with benz[*a*]anthracene (BaA) (5μg/g body weight) (around 10^{-5} M). BaA induced hypocalcemia at 24 and 48 h and thereafter the level recovered to control levels at 72 h. Goldfish scales possess both osteoclasts (bone resorption cells) and osteoblasts (bone forming cells). The marker enzyme activity (tartrate-resistant acid phosphatase: TRAP) of osteoclasts in goldfish scales decreased at 12 and 24 h after BaA injection. In BaA injected goldfish scales, the marker enzyme (alkaline phosphatase, ALP) gradually decreased at 48 and 72 h. In addition, 4-hydroxybenz[*a*]anthracene (4-OHBaA) that is one of the metabolites of BaA by conversion enzyme (P4501A1) was detected in the bile of goldfish at 12, 24, 48, and 72 h after administration of BaA to goldfish. We have recently found the toxicity of monohydroxylated polycyclic aromatic hydrocarbons (OHPAHs), metabolites of PAHs, in bone metabolism. We reported that 4-OHBaA suppressed both TRAP and ALP activities in the cultured scales of goldfish. Therefore, it was probable that 4-OHBaA metabolized from BaA has toxicity for osteoclasts and osteoblasts in goldfish. These phenomena are a cause of the disruption of the bone metabolism and the induction of spinal deformities.

Keywords: Benz[*a*]anthracene, 4-Hydroxybenz[*a*]anthracene, Teleost Scale, Osteoblasts, Osteoclasts, Calcium. Goldfish

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants derived from petroleum and produced by combustion of fossil fuel, wood and other organic materials (Lima *et al*., 2003), as well as in cigarette smoke (Lee *et al*., 2002). It has been reported that PAHs affects bone metabolism in mammals. For example, PAHs (benzo[*a*]pyrene and 7,12-dimethylbenz[*a*]-anthracene) present in cigarette smoke induced bone loss in an ovariectomized rat (Lee *et al*., 2002). In addition, PAHs inhibited osteoclastogenesis in rabbit osteoclasts and the RAW264.7 cells (mouse monocyte macrophage cell line) (Voronov *et al*., 2005).

In the aquatic environment as well as atmospheric environment, storm water runoff and atmospheric deposition are one of sources for aquatic PAHs contamination (Lima *et al*., 2003; Li and Daler, 2004). In fact, spinal bone deformity was reported in pacific herring and pink salmon by PAHs (Barron *et al*., 2004; Billiard *et al*., 2006). Much attention should be given to bone metabolism in fish.

It is known that the osteoclasts and osteoblasts in teleost scale are similar morphological bone-like features which is found in avian and mammalian membrane bone (Yamada, 1961; Bereiter-Hahn and Zylberberg, 1993; Suzuki *et al*., 2000, 2007, 2008). The scales of some teleosts contain as much as 20% of the total body calcium and are thus a better potential internal calcium reservoir rather than vertebral bone during periods of increased calcium demand, such as sexual maturation and starvation (Yamada, 1961; Berg, 1968; Mugiya and Watabe, 1977; Bereiter-Hahn and Zylberberg, 1993). A co-relation between mercury levels in the scales and in the muscles was reported in largemouth bass (Lake *et al*., 2006) although mercury did not accumulate in the vertebral bone of fish (Camusso *et al*., 1995). This strongly indicates that the scale is a more active organ in bone metabolism than vertebral bone. Considering these results, we developed an original *in vitro* assay using goldfish scales (Suzuki et al., 2000, 2007; Suzuki and Hattori, 2002). This system can simultaneously detect the activities of both scale osteoclasts and osteoblasts with tartrate-resistant acid phosphatase (TRAP) and alkaline phosphatase (ALP) as markers, as shown by the fact that, in mammals, the effects of hormones and some bioactive substances on osteoclasts and osteoblasts have been investigated using TRAP and ALP as respective markers (Vaes, 1988; Dimai *et al*., 1998; Suda *et al*., 1999). 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.

In the present study, we examined plasma calcium levels and both scale osteoclastic and osteoblastic activities after intraperitoneal injection of benz[*a*]anthracene (BaA)(5μg/g body weight) (around 10^{-5} M) to goldfish. Furthermore, the concentration of 4-hydroxybenz[*a*]anthracene (4-OHBaA) that is one of the metabolites of BaA by conversion enzyme (P4501A1) was measured in the bile of BaA-injected goldfish because we previously reported that 4-OHBaA inhibited osteoclastic and osteoblastic activities in the cultured scales of goldfish (fresh water

teleost) and wrasse (marine teleost)(Suzuki *et al*., 2009a).

The present study is the first to demonstrate that BaA decreases plasma calcium levels in goldfish resulting from influence of osteoclasts and osteoblasts activities in the scales of goldfish.

Materials and Methods

Animals:

Suzuki *et al.* (2000) reported that the sensitivity for calcemic hormones was higher in mature female than in mature male teleosts. Therefore, we have used female goldfish (*Carassius auratus*) in the present study which were purchased from commercial source (Higashikawa Fish Farm, Yamatokoriyama, Japan). Fish were fed a commercial pellet diet every morning and were kept in tap water at 26 C before the start of experiment.

All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of Kanazawa University.

Effects of BaA on plasma calcium levels in the goldfish:

In the experimental group, goldfish (n = 25) were anesthetized with 0.03% ethyl 3-aminobenzoate, methanesulfonic acid salt (Sigma-Aldrich, Inc., MO, USA) and then BaA (Nakarai Chemicals Ltd., Kyoto, Japan) (5μg/g body weight) (around 10^{-5} M) was injected intraperitoneally. BaA was firstly dissolved in ethanol and then 0.9% NaCl was added to make the desired stock solution. The goldfish in the control group (n = 25) were injected intraperitoneally with saline (0.9% NaCl containing 0.1% ethanol) in the same manner as experimental goldfish. These goldfish were kept in the aquarium for 72 h (3 days). This experimental period was adopted because hormonal and toxicological effects were influenced in goldfish during 3 days (Suzuki *et al*., 2004a, 2011; Omori et al., 2012; Yachiguchi *et al*., 2014). During the experimental periods, these goldfish were fasted to exclude intestinal calcium uptake from diets. After 12, 24, 48, and 72 h of the injection, blood samples were collected from the caudal vessel of anesthetized goldfish by using heparinized syringes from both control and experimental groups at each interval from each group (n = 5). The collected blood was put into a 1.5 ml tube and centrifuged at 15,000 rpm for 3 min. Then, the separated plasma was immediately frozen and kept at – 80 C until use. The plasma total calcium levels (mg/100 ml) were determined using an assay kit (Calcium E, Wako Pure Chemical Industries).

Effects of BaA on osteoclastic and osteoblastic activities in the scales of goldfish:

Prior to BaA or saline (0.9% NaCl containing 0.1% ethanol) injection, the scales (TRAP for 8 scales; ALP for 8 scales) were removed under anesthesia with 0.03% ethyl 3-aminobenzoate, methanesulfonic acid salt (Sigma-Aldrich). At 12, 24, 48, and 72 h after BaA or saline (0.9% NaCl containing 0.1% ethanol) injection, scales (TRAP for 8 scales; ALP for 8 scales) were extracted from anesthetized goldfish to examine the influences of BaA on the osteoclasts and osteoblasts with TRAP and ALP as respective marker.

For measuring TRAP and ALP activities, 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 in a 96-well microplate. Then, each scale was put into its separate well. This microplate was immediately frozen at -80 C and then kept at -20 C until analysis. To analyze the TRAP and ALP activities, an aliquot of 100 µl of 20 mM para-nitrophenyl-phosphate in 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 microplate well with a melted acid buffer or alkaline buffer solution. This plate was incubated at 20 C for 30 min while being shaken. After incubation, the reaction was stopped by adding 50 µl of a 3 N NaOH-20 mM EDTA solution. A colored solution of 150 µl was transferred to a new plate, and the absorbance was measured at 405 nm. The absorbance was converted into the amount of para-nitrophenol (pNP) produced by using a standard curve for pNP. Detailed methods described by Suzuki *et al*. (2009b).

The mean for TRAP or ALP activity (8 scales from one fish) in experimental group was compared with that in control group. Considering the variation among individuals, the values indicates the ratio of the value of each time course for the initials value in respective goldfish.

Measurement of 4-OHBaA in the bile of BaA-injected goldfish:

At each time course, goldfish (n=5) were anesthetized with ethyl 3-aminobenzoate, methanesulfonic acid salt (Sigma-Aldrich), and dissected. Then, bile was collected with a syringe from each gallbladder. The collected bile was incubated with β-glucuronidase and arylsulfatase to hydrolyze BaA-glucuronide and -sulphate, respectively. An aliquot of the solution was injected into a high-performance liquid chromatograph equipped with a fluorescence detector. The analyte was separated on a column (Discovery RP Amide C16, 250 x 4.6 mm i.d., 5 µm; Sigma-Aldrich). Isocratic elution was employed using 10 mM phosphate buffer solution (pH 7.0)/ acetonitrile (55/45, v/v). The flow rate was kept at 1.0 ml/min and the column temperature was maintained at 40C. The concentration of 4-OHBaA was quantified by using deuterated 1-hydroxypyrene as an internal standard. The detailed procedure is described by Suzuki *et al.* (2015).

Statistical analysis:

The statistical significance between the control and experimental groups was assessed by Student's t-test. The selected significance level was $P < 0.05$.

Results

Effects of BaA on plasma calcium levels in the goldfish:

Figure 1 indicates plasma calcium levels after BaA administration. BaA induced a significant hypocalcemia at 24 ($P < 0.01$) and 48 h ($P < 0.05$). Thereafter, plasma calcium levels recovered at 72 h after injection of BaA.

Effects of BaA on osteoclastic and osteoblastic activities in the scales of goldfish:

The results of osteoclastic activity are shown in Figure 2. BaA decreased osteoclastic activity temporally. A significant decrease between control group and experimental group was obtained at 12 ($P < 0.05$) and 24 h ($P < 0.05$). Thereafter, osteoclastic activity was recovered towards control levels.

The response to BaA in osteoblasts was slower than that in osteoclasts (Fig. 3). The inhibitory effects (Fig. 3) were observed at 48 ($P < 0.01$) and 72 h ($P < 0.001$).

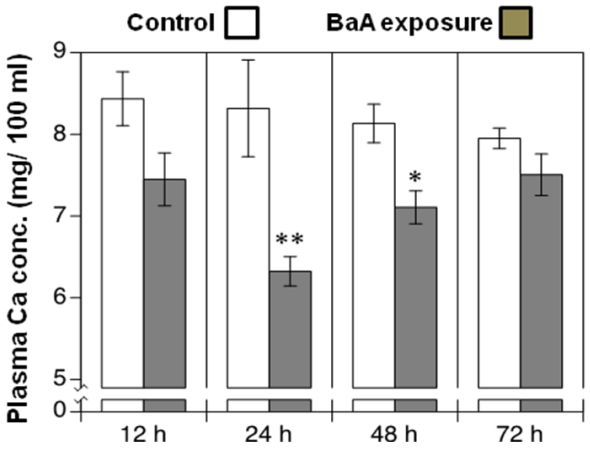

Fig. 1. Effects of benz[*a*]anthracene (BaA) on plasma calcium (Ca) levels in goldfish. Asterisk and double asterisk indicate statistically significant differences at $P < 0.05$ and $P < 0.01$, respectively, from the values in the control goldfish.

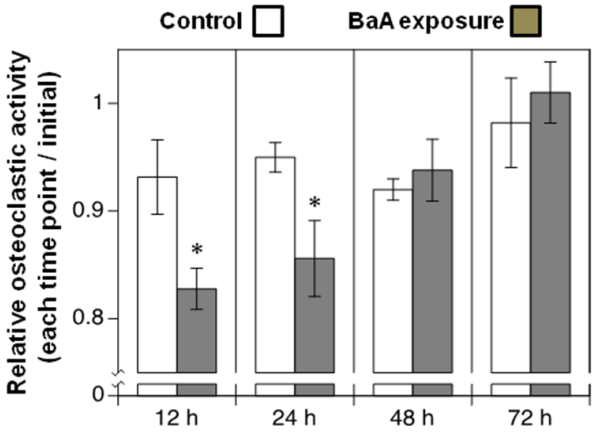

Fig. 2. Effects of benz[*a*]anthracene (BaA) on osteoclastic activity in goldfish. The mean for tartrate-resistant acid phosphatase (TRAP) (8 scales from one fish) in experimental group was compared with that in control group. The values indicate the ratio of the value of each time course for the initial value in respective goldfish (5 individuals for each time course). Asterisk indicates statistically significant differences at $P < 0.05$ from the values in the control goldfish.

Changes in 4-OHBaA in the bile of BaA-injected goldfish:

4-OHBaA was detected in the bile of goldfish after administration of BaA. At 48 h after BaA injection, 4-OHBaA levels was highest (Fig. 4). In the bile of control goldfish, 4-OHBaA could not be detected.

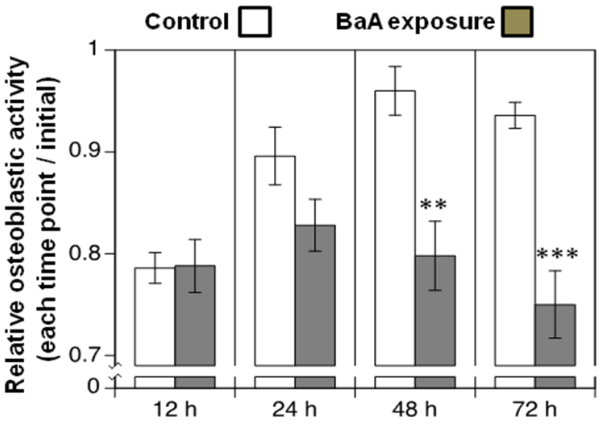

Fig. 3. Effects of benz[*a*]anthracene (BaA) on osteoblastic activity in goldfish. The mean for alkaline phosphatase (ALP) (8 scales from one fish) in experimental group was compared with that in control group. The values indicate the ratio of the value of each time course for the initials value in respective goldfish (5 individuals for each time course). Double asterisk and triple asterisk indicate statistically significant differences at $P < 0.01$ and $P < 0.001$, respectively, from the values in the control goldfish.

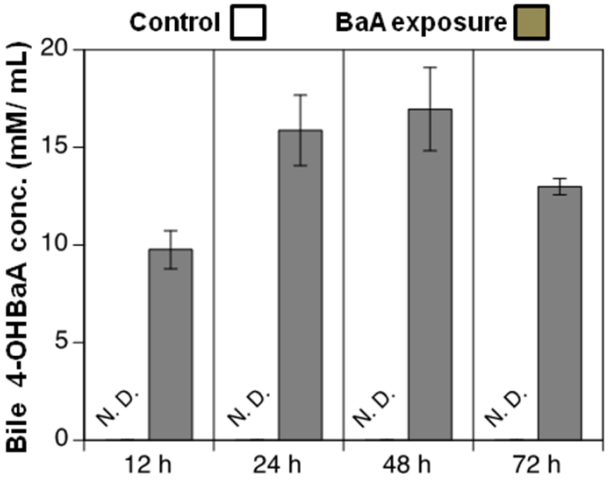

Fig. 4. Changes in 4-hydroxybenz[*a*]anthracene (4-OHBaA) of the bile of goldfish after benz[*a*]anthracene (BaA) injection. 4-OHBaA did not detect in the control goldfish.

Discussion

We indicated that BaA (5μg/g body weight) (around 10^{-5} M) suppressed osteoclastic activity at 12 and 24 h after injection. Resulting from inhibition of osteoclasts in the scales of goldfish, plasma calcium levels decreased. Therefore, we are the first to

demonstrate that BaA directly influence plasma calcium concentration. In our previous study, cadmium induced a hypocalcemia in goldfish (Suzuki *et al*., 2004b). Goldfish were kept in water containing cadmium (10^{-7} M) for 2, 4, and 8 days and after 4 days of exposure, there was noticed a significant hypocalcemia (Suzuki *et al*., 2004b). Thereafter, plasma calcium levels further decreased at 8 days after exposure to cadmium (10^{-7} M) (Suzuki *et al*., 2004b). In the present study, plasma calcium levels recovered to control levels at 72 h due to the increased osteoclast activity. In the case of cadmium, however, plasma calcium levels could not recover to control levels. Therefore, toxicity of BaA seems to be less than that of cadmium.

We detected 4-OHBaA in the bile of goldfish after administration of BaA. It is possible that that metabolized 4-OHBaA suppresses osteoclastic and osteoblastic activities in the scales of goldfish. Suzuki *et al*. (2009a) reported that 4-OHBaA inhibited osteoclastic and osteoblastic activities in the cultured scales of goldfish and wrasse. After short-term incubation (6 h), the sensitivity of osteoclasts in the scales of both goldfish and wrasse was higher than that of osteoblasts in the scales of both fish (Suzuki *et al*., 2009a). Therefore, OHBaAs may firstly inhibit osteoclastic activity and then suppress osteoblastic activity. These phenomena are a cause of the disruption of the bone metabolism and the induction of spinal deformities.

PAHs, such as benzo[*a*]pyrene, were shown to inhibit osteogenesis in rat bone marrow cells (Andreou *et al*., 2004). Benzo[*a*]pyrene and 7,12-dimethylbenz[*a*]-anthracene have also been shown to inhibit osteoclastogenesis in rabbit (Voronov *et al*., 2005). The present data are consistent with these results. PAHs have been reported to be converted into OHPAHs in the presence of cytochrome P450 in human bone marrow cells (McCord *et al*., 1996). In mammals as well as fish, therefore, we strongly suggest that metabolized OHPAHs cause toxicity to bone tissue.

Teleost scales, like the mammalian endoskeleton, are known to work as a potential internal calcium reservoir (Suzuki *et al*., 2008). We recently detected cathepsin K and TRAP mRNA expression in scale osteoclasts (Azuma *et al*., 2007). In osteoblasts, we detected osteoblast-specific markers, such as runt-related transcription factor 2, osterix, type 1 collagen, ALP, and osteocalcin (Thamamongood *et al*., 2012). It has also been demonstrated that the osteogenesis of regenerating scale is quite similar to that of mammalian membrane bone (Yoshikubo *et al*., 2005). Therefore, the features of osteoclasts and osteoblasts in scales are similar to those found in mammals. In addition, calcitonin, a hypocalcemic hormone, directly suppressed osteoclastic activity in normal scales of teleosts as well as in mammalian bone (Suzuki *et al*., 2000). In our experiments, osteoclasts were activated by parathyroid hormone (a calcium-regulating hormone) in goldfish scale as well as in mammalian bone (Suzuki *et al*., 2011). Also, 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*., 2004b), on osteoblasts and osteoclasts have been examined using the cultured goldfish scales. We indicated

that cadmium (even at 10^{-13} M) had an effect on the osteoclastic activity in the scales of goldfish (Suzuki *et al*., 2004b). Moreover, we indicated that seawater polluted with highly concentrated PAHs inhibited osteoblastic activity in the scales of goldfish even if polluted seawater was directly added into culture medium at dilution rates of 500 times (Suzuki *et al*., 2016). Thus, fish scale assay seems to be very useful to evaluate the effect of environmental pollutants on the bone metabolism.

Storm water runoff and atmospheric deposition are now the largest sources of aquatic PAHs contamination (Lima *et al*., 2003; Li and Daler, 2004). In addition to pollution from the atmosphere to the water, accidental oil spills, such as those from the Deepwater Horizon, the Exxon Valdez, and the Nakhodka, directly caused PAH pollution in a marine environment (Bue *et al*., 1998; Heintz *et al*., 2000; Hayakawa *et al*., 2006; de Soysa *et al*., 2012). In the Nakhodka C-heavy oil, 210 μg/g of benz[*a*]anthracene having four aromatic rings was detected (Hayakawa *et al*., 2006). This concentration is similar to that used in the present experiment. Immediately after an oil spill, the high level of PAHs influenced marine animals, including fish. For a long time (more than 14 years), furthermore, the toxicity of PAHs originating from an oil spill affected many marine animals (for a review, see Peterson *et al*., 2003). In teleosts, PAHs have reproductive (Hoffmann and Oris, 2006) and developmental toxicity (Barron *et al*., 2004; Billiard *et al*., 2006). Also, immune toxicity has been reported (for a review see, Reynaud and Deschaux, 2006). Taking these facts together with our study, much attention should be given to aquatic PAHs contamination.

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