Comparative Biochemistry and Physiology, Part C 189 (2016) 54–60

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

Sodium fluoride induces hypercalcemia resulting from the upregulation of both osteoblastic and osteoclastic activities in goldfish, *Carassius auratus*

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ARTICLE INFO

Article history:
Received 31 May 2016
Received in revised form 20 July 2016
Accepted 22 July 2016
Available online 27 July 2016

Keywords:
Sodium fluoride
Osteoblasts
Osteoclasts
Scales
Calcium metabolism
RANKL
OPG
Goldfish

ABSTRACT

The influence of sodium fluoride (NaF) on calcium metabolism was examined in goldfish (fresh water teleost). At 2 days after administration of NaF (500 ng/g body weight; 5 μg/g body weight) (around 10^{-5} to 10^{-4} M in goldfish), we indicated that plasma calcium levels upregulated in both doses of NaF-treated goldfish. To examine the mechanism of hypercalcemia by NaF treatments, therefore, direct effects of NaF on osteoblasts and osteoclasts in goldfish were investigated by an original assay system using teleost scale which has osteoblasts, osteoclasts and bone matrix. Alkaline phosphatase activity in the scales increased with the treatment of NaF (10^{-6} and 10^{-5} M) during 6 h of incubation. Also, tartrate-resistant acid phosphatase activity increased after exposure to NaF (10^{-5} M) at the 6 h of incubation. To investigate the osteoclastic activation, the mRNA expression of osteoclastogenesis related factors were examined. The receptor activator of the nuclear factor-κB ligand (RANKL) which is known as a factor for osteoclastogenesis, increased in the NaF-treated scales after 6 h of incubation. The ratio of RANKL/osteoprotegerin (osteoclastogenesis inhibitory factor) significantly increased after 6 h of incubation. Resulting from the increase of RANKL mRNA level, the expression of transcription-regulating factors was significantly increased. Furthermore, the expression of functional genes, cathepsin K and matrix metalloproteinase-9 mRNA, was significantly increased. In our knowledge, this is the first report concerning the effects of NaF on osteoblasts and osteoclasts in teleosts. We concluded that NaF influences calcium metabolism *via* osteoclastic activation in goldfish.

1. Introduction

Fluoride is abundant in environmental water. A major source of fluoride is drinking water, including fluoridated community drinking water and underground water contaminated by geological sources. An appropriate range of fluoride is thought to be safe and effective for caries reduction (Fordyce et al., 2007). In the case of aquatic animals, however, fluoride tends to accumulate in the exoskeleton of invertebrates and the bone tissue of fish (see a review, Camargo, 2003). These results indicated that fluoride affects calcium metabolism in fish. However, the direct effects of fluoride on osteoblasts and osteoclasts have not yet been elucidated in any fish, although there are several studies regarding the toxicity of fluoride in fish. Superior bioassay is strongly desired to analyze the effect of this chemical on both osteoclasts and osteoblasts in teleosts.

In all vertebrates, blood calcium levels are strictly kept at a constant concentration (around 2.5 mM) in spite of changing the internal milieu

Abbreviations: ALP, alkaline phosphatase; CTSK, cathepsin K; EF1α, elongation factor 1α; MMP-9, matrix metalloproteinase-9; NaF, sodium fluoride; NFATc1, nuclear factor of activated T-cells and cytoplasmic 1; TRAF6, TNF receptor-associated factor 6; TRAP, tartrate-resistant acid phosphatase; OPG, osteoprotegerin; RANK, receptor activator of the nuclear factor-κB; RANKL, receptor activator of the nuclear factor-κB ligand.

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http://dx.doi.org/10.1016/j.cbpc.2016.07.006
1532-0456/

or external environment (Dacke, 1979). The scales of teleosts have an important role in regulating blood calcium because teleost scales, having both osteoblasts and osteoclasts, are known to function as potential internal calcium reservoirs similar to those in the endoskeletons of mammals, especially during increased calcium demand, such as sexual maturation or starvation (Yamada, 1961; Berg, 1968; Mugiya and Watabe, 1977; Bereiter-Hahn and Zylberberg, 1993; Suzuki et al., 2000; Yoshikubo et al., 2005; Suzuki et al., 2007; Ohira et al., 2007). We detected both cathepsin K and tartrate-resistant acid phosphatase (TRAP) mRNA expression in the osteoclasts of goldfish scales (Azuma et al., 2007). Several osteoblastic markers (such as osteocalcin, type 1 collagen, and osterix) were also detected in the scales of goldfish (Thamamongood et al., 2012). Therefore, we have developed an *in vitro* assay system with goldfish scales and have analyzed the influence of calcemic hormones (calcitonin: Suzuki et al., 2000; Sekiguchi et al., 2009; parathyroid hormone: Suzuki et al., 2011a; prostaglandin E_2: Omori et al., 2012) and environmental pollutants (bisphenol-A: Suzuki and Hattori, 2003; heavy metal: Suzuki et al., 2004a; Suzuki et al., 2011b; Yachiguchi et al., 2014a; tributyltin: Suzuki et al., 2006; polychlorinated biphenyl: Yachiguchi et al., 2014b) on osteoblasts and osteoclasts. Therefore, we strongly believe that scale can be utilized as a model for bone, and that our *in vitro* scale assay system is effective for analyzing fluoride in fish bone metabolism.

In the present study, we first examined the effects of sodium fluoride (NaF) on plasma calcium levels in an *in vivo* experiment with goldfish (fresh water teleosts). In the goldfish, thereafter, the detailed influence of NaF on bone metabolism was examined using the scale *in vitro* assay system.

2. Materials and methods

2.1. Animals

The Yamato strain of goldfish (*Carassius auratus*) that was purchased from a commercial source (Higashikawa Fish Farm, Yamatokoriyama, Japan) was artificially fertilized from a female and a male goldfish (20–30 g) in the Graduate School of Marine Science and Technology, Tokyo University of Marine Science and Technology. Fish were fed a commercial pellet diet for puffer fish (Feed One Co., Ltd. Yokohama, Japan) every morning and were maintained in fresh water at 26 °C. Growing fish were moved to Noto Marine Laboratory in Kanazawa University and used for the both *in vivo* and *in vitro* experiments.

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

2.2. Effects of NaF on plasma calcium level in the goldfish (in vivo experiment)

Immature goldfish (4–6 g), which do not have developed gonads, were used for the *in vivo* study. In the experimental group, goldfish were anesthetized with 0.03% ethyl 3-aminobenzoate, methanesulfonic acid salt (Sigma-Aldrich, Inc., MO, USA) and then NaF (purity: 99.0%, Wako Pure Chemical Industries Ltd., Osaka, Japan) (low dose: 500 ng/g body weight; high dose: 5 μg/g body weight) was injected intraperitoneally. The goldfish in the control group were injected with saline (0.9% NaCl) in the same manner as experimental goldfish. These goldfish were kept in the aquarium for 1 and 2 days (each $n = 10$). These experimental periods were adopted because hormonal and toxicological effects were influenced in goldfish during 2 days (Suzuki et al., 2004a; Suzuki et al., 2004b; Suzuki et al., 2011a; Omori et al., 2012; Yachiguchi et al., 2014b). During the experimental periods, these goldfish were fasted to exclude intestinal calcium uptake from diets. Each 1 and 2 day after injection, blood samples were collected from the caudal vessel using a heparinized syringe from individual, anesthetized goldfish (each n = 10). The collected blood was put into a 1.5 ml tube. Thereafter, the tube was 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 level (mg/100 ml) was determined using an assay kit (Calcium E, Wako Pure Chemical Industries).

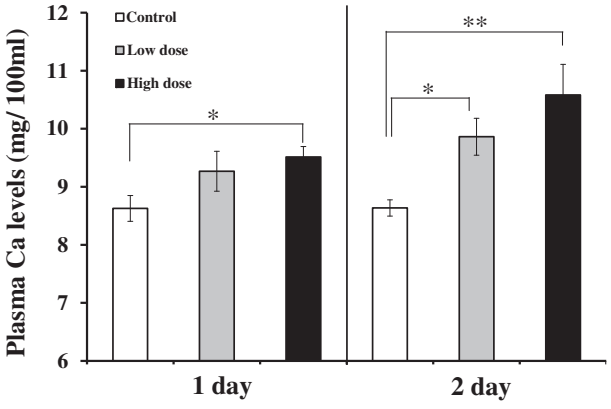

Fig. 1. Effects of NaF on plasma calcium level in the goldfish at 1 and 2 days after administration. NaF (low dose: 500 ng/g body weight; high dose: 5 μg/g body weight) was injected intraperitoneally. Thereafter, the blood sampling was performed at 1 and 2 days. * and **indicate statistically significant differences at $p < 0.05$ and $p < 0.01$, from the values in the control scales. $n = 10$ samples; one sample from one fish.

2.3. Effects of NaF on scale osteoblastic and osteoclastic activities using the cultured scales of goldfish (in vitro experiment)

Scales were collected from goldfish ($n = 16$) after anesthesia with 0.03% ethyl 3-aminobenzoate, methanesulfonic acid salt (Sigma-Aldrich). Using these scales, we examined the influences of NaF on the osteoblasts and osteoclasts with ALP and TRAP as markers because, in mammals, the effects of hormones and some bioactive substances on osteoblasts and osteoclasts have been investigated using ALP and TRAP as respective markers (Vaes, 1988; Dimai et al., 1998; Suda et al., 1999). These scales were incubated for 6 h in

Table 1
Primer sequences for real-time quantitative PCR.

Name	Forward primer	Reverse primer	Accession no.
RANK	GGGAGATGCTGCGAAAAATG	TTTAGGGTTGTGTGGACGAGTG	AB894121
RANKL	CGAGTGTGGCGATTTTGTTG	ATGGGCGTCTTGATTGGAAG	AB894120
TRAF6	TCTGATGGGTCTTCGCTCGGCT	ACTGGACATTTCTGCCCCGTGT	LC149878
NFATc1	CTGTGGCTTTGCTTGTGGATGTC	GATGCTGGTGTTTTGGCTGTAACC	AB685221
MMP-9	GCTTCTGCCCCAGTGAGCTT	GTGGAGCACCAGCGATACCC	AB889498
CTSK	TGGGAGGGCTGGAAACTCAC	CATGAGCCGCATGAACCTTG	AB236969
OPG	CGTGAACACGGTGTGCGAGTGT	CCTCTGCGCAGGCCTCACA	AB970727
EF1α	ATTGTTGCTGGTGGTGTTGG	GGCACTGACTTCCTTGGTGA	AB979720

RANK: Receptor activator of nuclear factor-κB, RANKL: Receptor activator of nuclear factor-κB ligand, TRAF6: TNF receptor associated factor 6, NFATc1: Nuclear factor of activated T-cells cytoplasmic 1, MMP-9: Matrix metallopeptidase-9, CTSK: Cathepsin K, OPG: Osteoprotegerin, EF1α: Elongation factor 1α.