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Regulation of branchial zinc uptake by 1α,25-$(OH)_2D_3$ in rainbow trout and associated changes in expression of ZIP1 and ECaC

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Abstract

Zinc is a vital micronutrient to all organisms, but is also a toxicant to aquatic species. It is therefore of importance to determine the mechanisms by which zinc uptake is modulated. In the present study, we investigated the regulatory effects of the vitamin D metabolite, 1α,25-$(OH)_2D_3$, on branchial zinc influx in rainbow trout, *Oncorhynchus mykiss*. Our results showed that branchial zinc uptake in rainbow trout was stimulated 7 days after a single intraperitoneal injection of 1α,25-$(OH)_2D_3$ (0.01 μg/g fish). To understand the molecular components of zinc uptake regulation by 1α,25-$(OH)_2D_3$, a ZIP zinc transporter (*Om*SLC39A1) and a partial vitamin D receptor (*Om*VDR) were molecularly cloned from rainbow trout gill, and the transcriptional expression of *OmSLC39A1*, epithelial calcium channel (*OmECaC*) and *OmVDR* genes in the gill was subsequently analyzed in response to 1α,25-$(OH)_2D_3$. *OmECaC*, *OmSLC39A1* and *OmVDR* were all upregulated following treatment with 1α,25-$(OH)_2D_3$, but the effect was observed at different time points. *OmECaC* expression was significantly increased by 1α,25-$(OH)_2D_3$ on Days 3 and 5 after the injection, and expression of *OmVDR* was stimulated on Day 5. There was also an increased abundance of *Om*SLC39A1 mRNA on Day 7 following the injection with 1α,25-$(OH)_2D_3$, but given the late response the effect of 1α,25-$(OH)_2D_3$ on this gene might be indirect. The results from the present study provide strong evidence that administration of 1α,25-$(OH)_2D_3$ results in enhanced zinc uptake across rainbow trout gill and that this effect is associated with an increased expression of transporters that mediate zinc uptake. The implications of our findings, in terms of aquatic toxicology, are that vitamin D status influences zinc accumulation in gill and body of fish.

Keywords: Calcitriol; SLC39A1; ZIP; ECaC; TRPV5; TRPV6; VDR

1. Introduction

Excessive zinc accumulation can be toxic and has been linked to deleterious effects, such as hypocalcaemia (Spry and Wood, 1985) and neurodegeneration (Frederickson and Bush, 2001). Furthermore, zinc is an environmental hazard, frequently violating water quality criteria in Europe and the USA. Zinc deficiency can also be a serious dilemma for organisms. It has been found that even moderate zinc deficiency can cause problems including anaemia, loss of appetite, immune system defects, developmental impairment and teratogenesis (Castillo-Duran and Weisstaub, 2003; Walker and Black, 2004).

In fish, zinc is not only absorbed in the gut, but also across the gills and the latter pathway is of high significance in terms of zinc toxicity. Spry and Wood (1989) showed that calcium competitively inhibited zinc uptake across rainbow trout gill. Later, it was found that zinc was a competitive inhibitor for branchial calcium uptake, indicating that zinc and calcium may share a common uptake site in the gills (Hogstrand et al., 1994). The nature of this site was revealed by the discovery of epithelial calcium channels (ECaC aka TRPV5/6) in *Fugu* pufferfish (*Takifugu rubripes*), zebrafish (*Danio rerio*), and rainbow trout (*Oncorhynchus mykiss*; Qiu and Hogstrand, 2004; Qiu, 2004; Pan et al., 2005; Shahsavarani et al., 2006). In post-larval stages of fish, ECaC is highly expressed in the gill with low mRNA levels present in other tissues (Qiu and Hogstrand, 2004; Qiu, 2004; Shahsavarani et al., 2006). Functional characterization

Abbreviations: ZIP, ZRT1-, IRT1-like protein; ECaC, epithelial calcium channel; ZnT, zinc transporter; MT, metallothionein; aa, amino acid; VDR, vitamin D receptor; VDRE, vitamin D responsive element

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showed that *Fugu* pufferfish and zebrafish ECaC (*Fr*ECaC and *Dr*ECaC) facilitated uptake of calcium and zinc when functionally expressed in Madin-Darby Canine Kidney (MDCK) cells and *Xenopus laevis* oocytes, respectively (Qiu and Hogstrand, 2004; Qiu, 2004). Interestingly, calcium and zinc also seem to compete for a common pathway across the brush border membrane of rat and piglet intestine (Rothbassell and Clydesdale, 1991; Gunshin et al., 1991; Bertolo et al., 2001), but it remains unknown if mammalian ECaCs transport zinc.

In addition to zinc uptake mediated by ECaC, there are other zinc transport pathways in fish gill. This was previously postulated because branchial zinc influx cannot be completely blocked by raising the concentration of calcium in the water (Spry and Wood, 1989; Hogstrand and Wood, 1996). Recently, members of the large ZIP (Zrt, Irt-like proteins) family of metal transporters (SLC39A, Eide, 2004) were cloned from gills of zebrafish (SLC39A1) and *Fugu* pufferfish (SLC39A1 and SLC39A3) (Qiu et al., 2005; Qiu and Hogstrand, 2005; Feeney et al., 2006). Functional characterization suggested that SLC39A1 in zebrafish (*Dr*ZIP1) and pufferfish (*Fr*ZIP1) represent a high-affinity zinc uptake transporter (Qiu et al., 2005), while the pufferfish SLC39A3 (*Fr*ZIP2) mediates low-affinity zinc uptake (Qiu and Hogstrand, 2005). The identification of ZIP uptake transporters suggests the existence of alternative, and probably more specific, pathways of cellular zinc regulation in fish gill compared with that mediated by ECaC.

Hormones are important regulatory factors controlling numerous physiological processes, including ion homeostasis. In vertebrates, the divalent metal ion, Ca^{2+}, is under tight regulation by hormones, *i.e.* parathyroid hormone, vitamin D system, calcitonin and stanniocalcin (Wendelaar Bonga and Pang, 1991). Among these, the active metabolite of vitamin D, 1α,25-$(OH)_2D_3$, is an important hypercalcaemic hormone controlling calcium homeostasis in both mammals and fish (Sundell et al., 1996; DeLuca, 2004). In mammals, 1α,25-$(OH)_2D_3$ acts *via* its nuclear receptor (VDR) to stimulate calcium absorption or reabsorption in the intestine and kidney, by transcriptional upregulation of epithelial calcium channels, *ECaC1* (*TRPV5*) and *ECaC2* (*TRPV6*)- the 'gatekeepers' of calcium absorption or reabsorption in intestinal and renal routes (Den Dekker et al., 2003). Recently, two vitamin D responsive elements (VDRE) were identified in the promoter of the *FrECaC* gene (Qiu and Hogstrand, 2004), suggesting that like its mammalian counterparts, this fish *ECaC* gene may be transcriptionally controlled by 1α,25-$(OH)_2D_3$. Further, 1α,25-$(OH)_2D_3$ stimulated the transepithelial transport of both calcium and zinc in human intestinal cell line Caco-2 cells (Fleet et al., 1993; Fleet and Wood, 1999). Thus, we hypothesized that in fish gill, calcium and zinc uptake mediated by ECaC may be regulated by 1α,25-$(OH)_2D_3$.

To understand the regulation of branchial zinc uptake, we investigated the effects of 1α,25-$(OH)_2D_3$ on branchial zinc acquisition in rainbow trout, molecularly cloned a novel ZIP transporter (*Om*SLC39A1) and a partial sequence of the vitamin D_3 receptor (*Om*VDR). The latter overlapped with a VDR fragment previously sequenced from rainbow trout (Accession number: AAS99156; Sugiura and Ferraris, 2004). We then analyzed mRNA levels of *Om*SLC39A1, ECaC (*OmECaC*, Accession number: AY256348; Shahsavarani et al., 2006) and *Om*VDR in response to 1α,25-$(OH)_2D_3$ in the gill.

2. Materials and methods

2.1. Animals

Animal care and handling was performed in accordance with, and with approval given under, the Animal (Scientific Procedures) Act (UK) 1986. Juvenile rainbow trout (*Oncorhynchus mykiss*, 2–5 g) were obtained from Houghton Springs fish farm in Dorset, England. Fish were maintained in 300–400 l fiberglass tanks supplied with flowing and aerated City of London tap water ($[Na^+] = 0.53$ mM; $[Ca^{2+}] = 0.92$ mM; $[Mg^{2+}] = 0.14$ mM; $[K^+] = 0.066$ mM; $[NH_4^+] = 0.027$ mM) that was passed through activated carbon, mechanical, and biological filters, and treated with UV-light and ozone. Water temperature was kept between 11 and 13 °C, varying with season. Fish were fed commercial pellets (Houghton Springs Fish Farm, Dorset, UK) to satiation three times a week.

2.2. Hormone treatment and branchial $^{65}Zn^{2+}$ influx assay

1α,25-$(OH)_2D_3$ (Sigma–Aldrich, Poole, UK) was thoroughly dissolved in ground nut oil to a final concentration of 0.01 μg/μl, and an intraperitoneal injection of 1α,25-$(OH)_2D_3$ (0.01 μg/g fish) was administered to juvenile rainbow trout with a Hamilton syringe. As a control, fish were injected with vehicle (ground nut oil). The injected fish were maintained without feeding throughout the experiment.

After 1, 3 and 7 days following the hormonal injection, unidirectional Zn^{2+} influx across the gill was assayed as previously described (Hogstrand et al., 1996). This time-course was chosen because effects of a single 1α,25-$(OH)_2D_3$ i.p. injection on abundance of ECaC1 and 2 in mice culminated within 24 h following the treatment (Song et al., 2003) and it was assumed that the response would be somewhat slower in rainbow trout. Briefly, eight fish were incubated in 3 l of aerated aquarium water (12 °C) containing 0.52 μCi/ml (19 kBq/ml) $^{65}Zn^{2+}$ (Perkin-Elmer, Bucks, UK) plus either 0.5 or 20 μM $ZnSO_4$ for 6 h. The lower zinc concentration was chosen to correspond to high-affinity zinc transporters, such as SLC39A1 (Qiu et al., 2005), while Zn^{2+} influx across rainbow trout gill approaches J_{max} at the higher zinc concentration (Spry and Wood, 1989). Afterwards, fish were anaesthetized in 0.1 M 3-aminobenzoic acid ethylester (MS222; Sigma–Aldrich), and rinsed in water containing excessive non-radiolabelled $ZnSO_4$ (2 mM) to displace surface-bound ^{65}Zn. Blood was collected by a Hamilton syringe from the caudal vessels, and transferred to heparinized tubes on ice. The blood was centrifuged for the separation of plasma from blood cells. Fish were then sacrificed by overdose of MS222, and gills and carcasses (whole body minus gill and withdrawn plasma sample) were sampled. Plasma, gill and carcass were weighed, and radioactivity of each sample was counted in a LKB1282 CompuGamma counter.