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Environmental salinity regulates the *in vitro* production of [^{3}H]-1,25-dihydroxyvitamin D_3 and [^{3}H]-24,25 dihydroxyvitamin D_3 in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Previous studies have shown that specific binding of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and 24,25-dihydroxyvitamin D_3 ($24,25(OH)_2D_3$) to enterocyte basolateral membranes (BLM), as well as circulating concentrations, is affected in response to changes in environmental salinity. It is not known if the production of $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ is affected by environmental salinity. The aim of the present study was to measure the *in vitro* production of [^{3}H]-$1,25(OH)_2D_3$ and [^{3}H]-$24,25(OH)_2D_3$ in fresh water (FW) and after 1, 2, 3, and 7 days after transfer to seawater (SW). Pooled sub-cellular fractions (mitochondria and microsomes) from liver or kidney was incubated with [^{3}H]-$25(OH)D_3$ and the produced metabolites were separated using HPLC. Hepatic production of [^{3}H]-$1,25(OH)_2D_3$ was decreased after 24 h in SW. This was followed by an up-regulation after 48 h and a second, slower decrease in production rate which leveled out after 7 days in SW. The production rate in SW was lower than the original rate in FW-adapted fish. For hepatic [^{3}H]-$24,25(OH)_2D_3$ production the pattern was reversed. Renal production of [^{3}H]-$24,25(OH)_2D_3$ increased significantly during the period of SW acclimation. These results suggest that environmental salinity regulates the production rate of the two antagonizing calcium regulatory hormones; $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$. This gives further evidence to the hypothesis that there is a physiological regulation and a differentiated importance of $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ in relation to environmental calcium concentrations.

Keywords: Liver; Kidney; 1α-Hydroxylase; 24-Hydroxylase; $1,25(OH)_2D_3$; $24,25(OH)_2D_3$; Salinity; Calcium regulation; Vitamin D_3; Rainbow trout

1. Introduction

Teleost fishes, in contrast to land living vertebrates habituating a calcium free environment, encounter environments highly different in calcium concentrations. Some species live in fresh water (FW) with calcium concentrations ($[Ca^{2+}]$) ranging between 0 and 2 mM, whereas marine species encounter an environment with $[Ca^{2+}]$ of approximately 10 mM. Specialized roles for the two calcium regulating vitamin D_3 hormones 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and 24,25-dihydroxyvitamin D_3 ($24,25(OH)_2D_3$) may have been evolved in teleost fish, in response to inhabitation to variable environmental calcium concentrations (Larsson et al., 1995; Sundell et al., 1996; Larsson, 1999).

Rapid increases in intestinal and enterocyte calcium uptake after $1,25(OH)_2D_3$ exposure has been demonstrated in FW-adapted European eel (*Anguilla anguilla*; Chartier et al., 1979) and in FW-acclimated rainbow trout (*Oncorhynchus mykiss*; Larsson et al., 2003). On the other hand, no rapid effects of $1,25(OH)_2D_3$ on intestinal or enterocyte calcium transport were observed in Atlantic cod, *Gadus morhua* (Sundell and Björnsson, 1990; Larsson et al., 1995) or SW-acclimated rainbow trout (Larsson et al., 2003). Instead, $24,25(OH)_2D_3$ rapidly decreased intestinal and enterocyte calcium uptake in both species

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(Sundell and Björnsson, 1990; Larsson et al., 1995, 2001, 2002, 2003).

Specific binding of [^{3}H]-1,25(OH)$_2$D$_3$ to enterocyte basolateral membrane (BLM) has been reported from FW Carp (*Cyprino carpio*) and rainbow trout, but could not be demonstrated in SW-acclimated rainbow trout or the marine teleost Atlantic cod (Nemere et al., 2000; Larsson et al., 2003). On the other hand, specific binding of [^{3}H]-24,25(OH)$_2$D$_3$ to enterocyte BLM was demonstrated in carp, marine Atlantic cod and SW-acclimated rainbow trout, but could not be demonstrated in FW-acclimated rainbow trout (Larsson et al., 2001, 2003). Thus, this suggests that 1,25(OH)$_2$D$_3$ is the pre-dominant biologically active metabolite in a FW environment (Larsson et al., 1995, 2003; Sundell et al., 1996).

In mammals, vitamin D$_3$ is metabolized to 25(OH)D$_3$ in the liver and transported in the blood bound to the vitamin D binding protein (DBP). This metabolite serves as a substrate pool for renal 1α-25(OH)D$_3$-hydroxylase (1α-hydroxylase) and 24R-hydroxylase (24-hydroxylase) metabolizing 25(OH)D$_3$ into 1,25(OH)$_2$D$_3$ and 24,25(OH)$_2$D$_3$ (Jones et al., 1998). In teleosts, the liver has been suggested as the most important site for 1α-hydroxylation of 25(OH)D$_3$ (Yanda and Ghazarian, 1981; Hayes et al., 1986; Takeuchi et al., 1991; Sundell et al., 1992; Sunita Rao and Raghuramulu, 1998). Large amounts of vitamin D$_3$ are stored in the liver (Takeuchi et al., 1986) concomitant with much lower circulating levels of 25(OH)D$_3$ compared to mammals (Takeuchi et al., 1991; Sundell et al., 1992). Interestingly, in teleost fishes adapted to SW, 24,25(OH)$_2$D$_3$, and not 1,25(OH)$_2$D$_3$, was shown to be the dominating vitamin D$_3$ metabolite produced (Graff et al., 1999), which supports the view of a differentiated importance of the two hormones depending on environment.

This was further demonstrated by transferring anadromous rainbow trout from FW to SW which rendered a shift in specific binding of the respective hormones to enterocyte BLM. In FW, specific binding was only observed for 1,25(OH)$_2$D$_3$, whereas after SW transfer this binding was rapidly down-regulated and specific binding for 24,25(OH)$_2$D$_3$ was up-regulated (Larsson et al., 2003). During this transfer, Larsson et al. (2003) observed responses in the circulating levels of the two metabolites 1,25(OH)$_2$D$_3$ and 24,25(OH)$_2$D$_3$, in patterns that were essentially reversed to each other (Larsson et al., 2003). As plasma hormone levels are dependent upon both hormone production and clearance (Fox et al., 1990), changes in plasma 1,25(OH)$_2$D$_3$ and 24,25(OH)$_2$D$_3$ concentrations during SW acclimation is most likely a combined effect of the observed changes in receptor density for the two metabolites (Larsson et al., 2003) and changes in hormone production. However, it is to date not known if the production rates of 1,25(OH)$_2$D$_3$ and 24,25(OH)$_2$D$_3$ are affected by environmental salinity.

The present study aims at investigating the enzymatic production of the two antagonizing calcium regulatory hormones, 1,25(OH)$_2$D$_3$ and 24,25(OH)$_2$D$_3$, in response to environmental salinity. Furthermore, the relative importance of hepatic versus renal production of 1,25(OH)$_2$D$_3$ and 24,25(OH)$_2$D$_3$ was investigated.

2. Materials and methods

2.1. Experimental design

Rainbow trout (*O. mykiss*) of both sexes (210–430 g) were purchased from a local hatchery (Antens fiskodling AB, Alingsås, Sweden) and kept in recirculating, aerated FW at 10 °C and 12 h light and 12 h darkness photoperiod. The fish were acclimated for 3 weeks before the start of the experiment and fed *ad libitum* daily. At the start of the experiment (day 0) nine fish were sacrificed by a blow to the head and sampled for approximately 1 g of liver and kidney, respectively. Another 36 rainbow trout were transferred, in groups of nine, to four identical tanks supplied with recirculated and aerated SW (salinity 30‰) at 10 °C. Feeding and light regimes adopted in FW were retained in SW. A group of nine fish were sacrificed at day 1, 2, 3, and 7, respectively, and sampled for liver and kidney. All experimental procedures and animal care were approved by the Göteborg University ethical committee according to Swedish law (dnr: 265/02).

2.2. Cell fractionation

After sampling, liver and kidney were immediately transferred to separate tubes with ice-cold phosphate buffer containing sucrose (PBS$_S$) (0.08 M Na_2PO_4, 0.02 M KH_2PO_4, 0.15 M KCl, 0.25 M Sucrose, pH 7.2) in 1:5 w:v ratio. Sub-cellular fractionation (modified method from Förlin, 1980) was performed in order to obtain mitochodrial and microsomal fractions of the liver and kidney cells. Briefly, tissues were homogenized on ice using a glass-Teflon homogenizer attached to an electronic rotator set at moderate speed. The crude homogenate was centrifuged at 600*g* for 10 min, this first pellet (P1) was discarded and the supernatant (S1) further centrifuged for 20 min at 10,000*g* (Beckman Coulter Avanti J-20, rotor; JA-21, CA, USA). The resulting pellet (P2; containing mitochondrial membranes) was washed once (5 ml PBS$_S$, 10,000*g* for 20 min) and stored on ice for later pooling with the microsome fraction obtained. The supernatant from the second centrifugation (S2) was further centrifuged at 105,000*g* for 60 (Optima LE 80-K, rotor; 50.4 Ti, CA, USA) min revealing a microsome containing pellet (P3) which was pooled with P2 and resuspended in PBS$_S$ in 1:1 w/vol ratio. The obtained sample was used for assessment of 1α- and 24-hydroxylase activity.

2.3. 25-hydroxyvitamin D_3 1α- and 24-hydroxylase activity

2.3.1. Incubation

The enzymatic production of [^{3}H]-1,25(OH)$_2$D$_3$ and [^{3}H]-24,25(OH)$_2$D$_3$ were assessed by adding 900 μl of sample (i.e., the pooled membrane fractions) to test tubes containing 2.0 pmol [^{3}H]-25(OH)D$_3$ (25-Hydroxy[26,27-methyl-^{3}H]cholecalciferol; specific activity 186 Ci mmol^{-1}, Amersham Pharmacia Biotech, UK), glucose-6-phosphate dehydrogenase; 1 U/ml, NADP 2.7 mM, glucose-6-phosphate 2.5 mM and ATP; 10 mM. Final incubation volume was 1 ml with a protein concentration of approximately 15 mg protein ml^{-1}. All protein concentrations were determined with a commercial kit (Bio-Rad Protein Assay, Bio-Rad) according to Bradford (1976), using bovine serum albumin as standard. The incubation was terminated, after selected time-frames as described below, by addition of 2 ml methanol. Pre heated homogenate (100 °C for 15 min) was used as control of enzymatic production (Takeuchi et al., 1991).

2.3.2. Isolation of vitamin D metabolites

The produced metabolites were isolated and separated by a method slightly modified from Aksnes (1980). Briefly, 4 ml acetonitrile was added to the samples, vortexed for 10 s and centrifuged 10 min at 10,000*g*. The pellet was discarded and 7 ml K_2HPO_4 (0.1 M, pH 10.5) was added to