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Effects of Stannius Corpuscle Extracts and 17β-Estradiol on the Concentration of Gallbladder Bile Calcium in the Rainbow Trout, *Oncorhynchus mykiss*

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Abstract To examine a role of calcium excretion into gallbladder bile in calcium homeostasis, a single administration of Stannius corpuscle extracts from rainbow trout or 17β-estradiol was given the rainbow trout, *Oncorhynchus mykiss*, and serum and bile calcium concentrations were analysed by flame photometry. Calcium concentrations were approximately 3.7 times higher in bile than serum in the control fish. Calcium-roaded fish showed marked increases in serum and bile calcium conentrations. The simultaneous administration of the extracts effectively reduced the hypercalcemia after 3 h. However, the extracts did not have any effects on bile calcium concentrations at any examination times. 17β-estradiol induced marked hypercalcemia with a peak 10 days after administration. In contrast, bile calcium concentrations decreased to a minimum level on the day showing a mirror profile to changes in serum calcium concentrations ($r=-0.95$). These results inidcate that gallbladder bile calcium is not involved in the hypocalcemic regulation by Stannius corpuscle extracts, but decreases in concentration as a result of vitellogenin synthesis in hepatocytes.

Teleosts regulate their plasma calcium levels within a physiological range of 4 to 6 meq/*l*. The corpuscles of Stannius (CS) release the hypocalcemic hormone, stanniocalcin, which acts on gills and suppresses the branchial uptake of calcium (Lafeber et al., 1988). Calcium load resulted in the degranulation of the corpuscle cells (Yamada et al., 1982; Hanssen et al., 1991) and stimulated calcium excretion into gallbladder bile to reduce the hypercalcemia (Mugiya and Takayama, 1992). However, a causative relationship between these results has not been studied.

Gallbladder bile is a fluid produced by hepatocytes and contains much higher calcium concentrations than serum. In rats, the hypocalcemic hormone, calcitonin, has been suggested to control the excretion of plasma calcium into bile (Yamaguchi and Yamamoto, 1981). In fish, which have significantly different mechanisms of calcium homeostasis from terrestrial vertebrates (Simkiss, 1974), the hypocalcemic effect of calcitonin is not necessarily consistent and, instead, stanniocalcin seems to function universally as a hypocalcemic hormone. However, we have as yet no information as to the effect of stanniocalcin on calcium excretion into the bile of fish.

Estrogen is well known to induce hypercalcemia as a result of the accumulation of the calcium-bound protein, vitellogenin, which is synthesized by hepatocytes (Kwon et al., 1993). Therefore, the cells would require much calcium to integrate the molecule during vitellogenesis, resulting in a possible reduction in calcium excretion into bile. Mugiya and Takayama (1992) have preliminarily reported an inverse relationship between serum and bile calcium concentrations in estrogenized rainbow trout.

The present study was undertaken to examine the effects of CS extracts and 17β-estradiol on calcium excretion into gallbladder bile using calcium-loaded and unloaded rainbow trout.

Materials and Methods

Rainbow trout, *Oncorhynchus mykiss*, were obtained from a commercial dealer and reared in outdoor ponds with running water at about 14°C. They were fed fish food pellets once a day except for a one-day's fasting before bile collection. Their gonads were found to be immature at autopsy.

Stannius corpuscle extracts

A few CS (2.5 on the average) were found in the kidney of an individual. They were dissected out

under a binocular microscope and pooled from 40 fish after weighing. The corpuscles were homogenized in 0.05 M CH_3COONH_4 (pH 7.4) using a glass homogenizer, extracted overnight at 4°C, and centrifuged at 9000 rpm for 10 min. The supernatant was freeze-dried and kept frozen (−80°C) for 3 to 100 days before use. Protein content of the extracts was determined with Coomassie brilliant blue G (595 nm) using bovine serum albumin as a standard (Bradford, 1976).

The CS extracts were analysed by SDS-polyacrylamide gel electrophoresis (PAGE) according to the method of Laemmli (1970). Gels were stained with Coomassie brilliant blue R-250. Standard proteins used for determination of molecular weight (MW) were carbonic anhydrase (MW 29 kDa), egg albumin (45 kDa), and bovine serum albumin (66 kDa) (Sigma, MW-SDS-200 kit).

Stannius corpuscle extract experiment

Thirty-two rainbow trout weighing about 150 g were divided into two groups. One group (16 fish) was given a single intraperitoneal injection of CS extracts dissolved in 0.9% NaCl containing $CaCl_2$. The amount of administration was 10 mg CS extracts +12 mg Ca+0.5 m*l* NaCl per 100 g body weight. Fish were injected at 10 a.m. and sampled after 3, 6, and 10 h. The other group (16 fish) was only given the calcium-containing solvent and sampled as the control at the same time schedule. The experimental and control fish were injected and sampled alternately.

After fish were netted one at a time, blood was collected from the caudal vessels by cutting the tail of the fish and draining it into plastic tubes. After centrifugation, the separated sera were stored at −80°C for calcium analysis. Bled fish were transported to the laboratory and their gallbladder bile was collected by suction as described by Mugiya and Takayama (1992).

Serum and bile calcium concentrations were determined by flame photometry. The SDS-PAGE of CS extracts was conducted to examine the effect of calcium load on stanniocalcin-like proteins in the CS. Electrophoretic procedures were the same as previously described but the gels after electrophoresis were stained with 12 mM silver nitrate (Merril et al., 1981).

17β-estradiol experiment

Forty-six rainbow trout weighing about 30 g were intraperitoneally injected with 17β-estradiol (Sigma Chemical Co.) dissolved in propylene glycol at a dose of 1 mg hormone/0.5 m*l* solvent/100 g body weight and were sampled after 5, 10, 12, 15, 18, 22, 25, and 30 days. Another five fish were only injected with the solvent and sampled on day 18 as the control. Blood and bile collections and calcium analyses were performed by the same procedures as described in the former experiment. From a comparative point of view, sodium concentrations were also determined by flame photometry using the remainder of the diluted samples used for calcium determination. Since the size of the fish used was small and the amount of bile collected from one individual was not enough for a single determination, all bile samples collected each time were pooled for determination.

Statistics

A one-way ANOVA was applied to assess statistical significance of differences between mean values. Significance was accepted at $p<0.05$.

Results

The wet weight of the CS collected from 40 fish totalled 118.9 mg and the protein content of the CS extracts was 219.1 μg/mg-CS. SDS-PAGE of the extracts showed a main band with a MW of slightly less than 29 kDa (Fig. 1). Several other minor bands were also visualized.

A single administration of calcium chloride resulted in increases in calcium concentrations in serum and gallbladder bile (Fig. 2). Such hypercalcemia decreased toward the control levels (4.7 and 17.0 meq/*l* in serum and bile, respectively) with time. The simultaneous administration of CS extracts effectively ($p<0.05$) suppressed serum calcium concentrations after only 3 h (Fig. 2). The extracts did not have any effects on bile calcium concentrations at any examination times.

The CS extracts from the calcium-loaded fish were analyzed by SDS-PAGE together with those from unloaded fish. Calcium-load obviously resulted in a disappearance of stanniocalcin-like protein (slightly

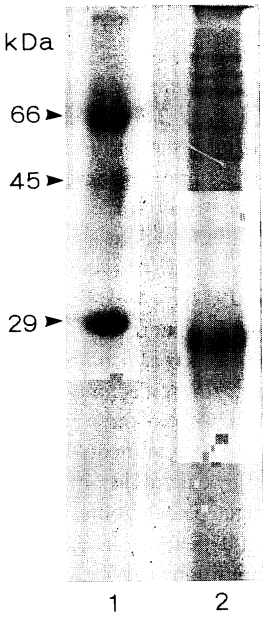

Fig. 1. SDS-PAGE of the corpuscles of Stannius extracts (lane 2) in rainbow trout. A main protein band occurred slightly less than 29 kDa. Lane 1: morecular weight markers.

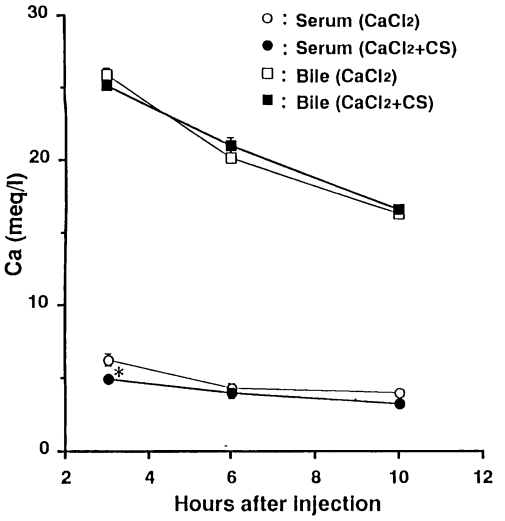

Fig. 2. Effects of the corpuscles of Stannius (CS) extracts on serum and bile calcium concentrations in calcium-loaded rainbow trout. Each plotted value represents mean ± SE of either seven fish at 3 h and 6 h or two fish at 10 h. $*p<0.05$ for the control value.

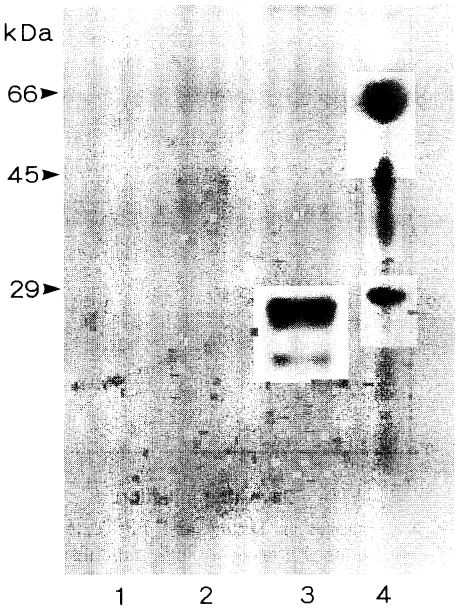

Fig. 3. SDS-PAGE of the corpuscles of Stannius extracts in calcium-loaded rainbow trout. Calcium-unloaded fish show two or more protein bands (lane 3) but calcium-loaded fish (lanes 1 and 2) lack these bands. Such disappearance occurred within 3 h after calcium load (data omitted). Lanes 1 and 2: experimental (extract-treated) and control fish sampled after 6 h, respectively. Lane 4: molecular weight markers.

less than 29 kDa) in the CS (Fig. 3), regardless of CS extract administration.

The administration of 17β-estradiol induced a marked increase in serum calcium concentrations, reaching a peak on day 10 and recovering to the control level on day 18 (Fig. 4). In contrast, bile calcium concentrations characteristically changed in a mirror image to the profile of changes in serum calcium concentrations ($r=-0.95$), representing a minimal level on day 10. The relationship between the two fluids are expressed in terms of ratios of bile to serum calcium concentrations (Fig. 5). A minimum ratio occurred on day 10 after estradiol administration, when vitellogenin concentrations in the serum was the highest (unpublished data). Ratios in sodium concentrations between serum and bile were not affected by vitellogenesis, showing a stable level throughout the experiment (Fig. 5).

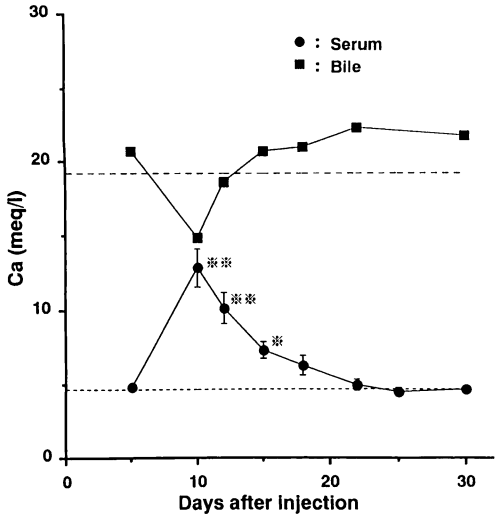

Fig. 4. Changes in serum and bile calcium concentrations following a single administration of 17 β-estradiol in rainbow trout. Dotted lines show the control level of serum and bile calcium. Each plotted symbol represents mean± SE of five to seven fish for serum and the value of a single determination of the samples pooled from the same individuals for bile. A correlation coefficient of −0.95 was obtained between serum and bile calcium concentrations. * and ** $p<0.05$ and $p<0.01$, respectively, for the control level ($n=5$).

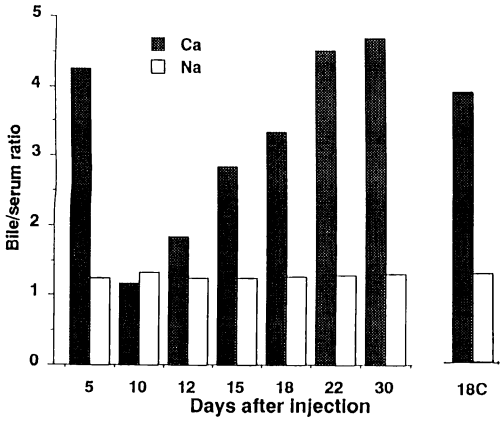

Fig. 5. Ratios of bile to serum calcium and sodium concentrations in estrogenized rainbow trout. 18C: control ratios examined on day 18.

Discussion

Gallbladder bile contains 3–4 times higher calcium concentrations than serum in rainbow trout (Mugiya and Takayama, 1992). Therefore, if this calcium is excreted into the intestine and then discharged outside through the rectum, this route of calcium excretion would be important for calcium homeostasis. There is no doubt that calcium load stimulated calcium excretion into the bile of rainbow trout (Mugiya and Takayama, 1992). Likewise, calcium-loaded rats showed a marked increase in bile calcium concentrations but this increase completely disappeared following thyroparathyroidectomy (Yamaguchi and Yamamoto, 1981). From these results, it has been suggested that hepatocyte-mediated calcium excretion is controlled by calcitonin. In fish, however, the hypocalcemic effect of calcitonin has been reported to be inconsistent depending on fish species used and experimental conditions (Wendelaar Bonga and Pang, 1991), and its physiological function remains obscure (Chakrabarti and Mukherjee, 1993). Instead, stanniocalcin is recognized as a hypocalcemic hormone in fish, which are capable of absorbing necessary calcium from the environmental water via gills. This hormone is known to be a 56 kDa glycoprotein consisting of a homodimer of 28 kDa in rainbow trout (Flik et al., 1990) and to regulate blood calcium levels by suppressing the branchial uptake of calcium. We anticipated that stanniocalcin was also concerned with calcium homeostasis through calcium excretion into gallbladder bile, like calcitonin in rats. However, in spite of a significant decrease in serum calcium concentrations after the administration of CS extracts, no change was found in bile calcium concentrations in the present calcium-loaded experiment. Therefore, this decrease in serum calcium concentrations is not due to the stanniocalcin-stimulated excretion of calcium from serum to bile, but to a possible suppression of calcium uptake via gills.

Electrophoresis of CS extracts revealed the absence of stanniocalcin-like proteins in the calcium-loaded fish, suggesting that calcium load induced the release of endogenous stanniocalcin into the blood regardless of CS extract administration. This may lead to the unclear interpretation of the present

results. However, considering a significant decrease in serum calcium concentrations without an increase in bile calcium concentrations in the CS extract-treated group, it seems reasonable to conclude that stanniocalcin does not stimulate calcium excretion into gallbladder bile.

In addition to the steady excretion of intracellular calcium into gallbladder bile, hepatocytes synthesize the calcium-bound protein, vitellogenin, in response to estrogen and release the protein into the blood of fish. This dual function of hepatocytes arouses interest in the effect of vitellogenesis on calcium excretion into bile. Mugiya and Takayama (1992) examined such an effect in rainbow trout and reported an inverse relationship between serum and bile calcium concentrations on day 10 after estradiol administration. In the present study, we were able to show such a relationship over an experimental period of 30 days after estrogenization. This inverse relationship likely resulted from the fact that, since a considerable amount of calcium (7 mg Ca/g-protein in rainbow trout, Fremont and Riazi, 1988) is bound to vitellogenin for its molecular integrity probably in hepatocytes, extra calcium to be excreted into bile decreased to the equivalent extent, showing a mirror image between serum and bile calcium concentrations. A physiological role of calcium in vitellogenin integrity is under examination using cultured hepatocytes.

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ニジマス (*Oncorhynchus mykiss*) の胆汁カルシウム濃度に及ぼすスタニウス小体抽出物および 17β-エストラジオールの影響

麦谷泰雄・裕　香織

ニジマスの肝細胞を通じて胆汁中に排出されるカルシウムが，血液カルシウムの恒常性の維持に機能しているか否かを調べた．ニジマスに塩化カルシウムを投与することにより高カルシウム

血症を誘起し，同時にスタニウス小体抽出物を投与した．抽出物投与群では，対照群に比べて血中カルシウム濃度は低下したが，胆汁カルシウム濃度は両群間で差がなかった．またエストラジオール投与により，10日目をピークとして顕著な高カルシウム血症が認められたが，胆汁カルシウム濃度はむしろ逆位相の関係で変動し，両者の相関係数は −0.95 であった．これらの結果から，胆汁カルシウムはスタニウス小体抽出物による低カルシウム調節には関係していないが，エストラジオールによる肝細胞でのビテロゲニン合成に伴って減少することが明らかになった．

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