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Fuentes J, Guerreiro PM, Modesto T, Rotllant J, Canario AV, Power DM. A PTH/PTHrP receptor antagonist blocks the hypercalcemic response to estradiol-17β.*Am J Physiol Regul Integr Comp Physiol* 293: R956–R960, 2007. First published May 30, 2007; doi:10.1152/ajpregu.00111.2007.—Estradiol (E_2) increases circulating calcium and phosphate levels in fish, thus acting as a hypercalcemic and hyperphosphatemic factor during periods of high calcium requirements, such as during vitellogenesis. Since parathyroid hormone (PTH)-related protein (PTHrP) has been shown to be calciotropic in fish, we hypothesized that the two hormones could be mediating the same process. Sea bream (*Sparus auratus*) juveniles receiving a single intraperitoneal injection of piscine PTHrP(1-34) showed an elevation in calcium plasma levels within 24 h. In contrast, injections of the PTH/PTHrP receptor antagonist PTHrP(7-34) decreased circulating levels of calcium in the same period. Intraperitoneal implants of estradiol-17β (E_2; 10 μg/g) evoked significant increases of circulating plasma levels of calcium and phosphorus and a sustained increases of circulating plasma levels of PTHrP. However, a combined treatment of E_2 and PTHrP(7-34) evoked a markedly lower calcium response compared with E_2 alone. We conclude that PTHrP or a related peptide that binds the PTH/PTHrP receptor mediates, at least in part, the hypercalcemic effect of E_2 in calcium and phosphate balance in fish.

fish; calcium; phosphate

PARATHYROID HORMONE (PTH)-related protein (PTHrP) is linked to calcium metabolism in higher vertebrates and is associated with bone turnover (20). In preclinical trials, PTHrP has been shown to evoke increased bone mass (18). Moreover, recent reports show that the anabolic nature of PTHrP in the bone is achieved by altering osteoblast recruitment and survival in mice models for osteoporosis (21, 22). Estradiol-17β (E_2) has been shown to upregulate PTH and PTH/PTHrP receptors in human U2OS osteosarcoma cells (42) and in MCF-7 breast carcinoma cells to upregulate PTHrP mRNA expression and induce PTHrP release in a dose-dependent manner (13). The action of E_2 on renal calcium retention may be through its capacity to upregulate renal expression of PTHrP mRNA without changing PTHrP receptor expression and function (5). In contrast, E_2 decreased cell growth and PTHrP production in estrogen receptor (ER)-negative human breast cancer cells (MDA-MB-231) and cells transfected with full-length cDNA encoding ER(S-30) (37).

Vitellogenesis, or the production of nutrient-rich proteins in the liver and their accumulation in the oocytes of oviparous animals, is under the hormonal control of E_2 produced in the granulosa cells of the ovary and is accompanied by a marked increase of plasma calcium and phosphate, which can reach blood plasma levels of 10 mM. Phosphate binds covalently to serine residues of the phosvitin moiety of vitellogenin to which Ca^{2+} is ionically bound (44). During vitellogenesis, plasma levels of E_2, alkali labile phosphorus (phosphate bound to serine groups in proteins released by mild alkali solution), and total calcium are positively correlated, and the latter has been often used as an indirect measurement of vitellogenesis (7, 14, 26).

E_2 administration to freshwater and seawater fish, such as killifish (*Fundulus heteroclitus*), sea bream (*Sparus auratus*), rainbow trout (*Oncorhynchus mykiss*), and goldfish (*Carassius auratus*), increases plasma levels of calcium, phosphate, and vitellogenin (14, 15, 26, 28, 31, 43). Although E_2 has also been shown to promote whole body calcium uptake in fish (15, 31), the recent discovery of PTH and PTHrP with hypercalcemic actions in fish (3, 10, 12, 16, 36) suggests that the action of the two hormones may be required for mineral mobilization during vitellogenesis. A recent in vitro study indicates that although PTHrP on its own does not affect vitellogenin production by sea bream hepatocytes, it synergizes the E_2-induced synthesis of vitellogenin (2).

The objective of the present study was to test the hypothesis that PTHrP is involved in the E_2-related hypercalcemia and to establish the relationship with E_2 in this process. For these purposes we have administered E_2, PTHrP(1-34), or a combination of E_2 and the PTHrP(7-34) antagonist to sea bream *Sparus auratus* in vivo, and analyzed the hormonal and mineral responses.

MATERIALS AND METHODS

Animals. Sea bream (*Sparus auratus*) juveniles were used from a stock raised at Ramalhete Marine Station (University of Algarve, Algarve, Portugal). Experiments were conducted in June in open-water circuits under natural conditions of water temperature (18–20°C), photoperiod, and salinity (37 ppt). Fish were fed once a day (10:00–11:00 AM) at a ration of 2% of the estimated body weight with commercial dry pellets (Provimi) with the exception of the day of the experiments or sampling when food was withheld 24 h prior to the procedure. The experimental procedures comply with the Guidelines of the European Union Council (86/609/EU) and Portuguese legislation for the use of laboratory animals.

Experiment 1: effect of PTHrP on plasma calcium in sea bream. The experiment was designed to test the effect of PTHrP(1-34) and PTHrP(7-34) on plasma calcium in sea bream. Eight groups of eight fish each (n = 64, body mass 15 ± 1 g; mean ± SE) were created, each receiving a single intraperitoneal injection of 0.9% NaCl (2 μl/g

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wet weight) containing either 0, 0.2, 1, or 5 μg/g wet weight per sea bream PTHrP(1-34) (16) or similar doses of PTHrP(7-34) (38). All peptide solutions were prepared fresh. Fish were anesthetized with 2-phenoxyethanol (1:10,000; Sigma-Aldrich, Madrid, Spain) and weighed to the nearest gram before treatment and then placed in 600-liter tanks supplied with well-aerated seawater. The procedure took <3 min per group with two operators. Twenty-four hours after treatment, fish were anesthetized and 0.1 ml blood collected by caudal vessel puncture into heparinized (ammonium heparin, 30 U/ml; Sigma-Aldrich) 1-ml syringes fitted with 23-gauge needles. Plasma was obtained by centrifugation of whole blood (10,000 rpm for 5 min), aliquoted, snap-frozen in liquid N_2, and stored at −80°C for later analysis.

Experiment 2: interaction between E_2 and PTHrP in calcium metabolism. In this experiment, the effect of the PTH/PTHrP receptor antagonist PTHrP(7-34) on the calciotropic action of E_2 in sea bream was studied. The load of peptide in the miniosmotic pumps was established from a test in which 10,000 cpm ^{125}I-labeled PTHrP(1-34) was injected intraperitoneally, and the amount of peptide transferred to the plasma, whole blood, and packed blood cells was counted after 1 h. The average transfer rate of labeled PTHrP from the peritoneal cavity to the bloodstream estimated by this method was of 1.5 ± 0.1% of the injected hormone per milliliter of blood after 1 h. For the experiment, three groups of 25 fish each (n = 75, mean body mass 70 ± 1 g) received a miniosmotic pump (model 2001; Alza, Palo Alto, Ca) and a coconut oil implant in the following combination: pump with saline (0.9% NaCl) plus coconut oil alone (control group); pump with saline plus coconut oil implant with E_2 (10 μg/g; E_2 group); and pump with piscine PTHrP(7-34) plus coconut oil implant with E_2 (10 μg/g) [E_2 + PTHrP(7-34) group]. Fish were anesthetized (1:10,000, 2-phenoxy ethanol), transferred to a humid operating table with the gills irrigated with aerated seawater containing anesthesia (1:20,000, 2-phenoxyethanol). A blood sample was immediately collected and processed as described above to obtain plasma. The fish were placed ventral side up and a lateral incision 1-cm long was opened in the peritoneum behind the pectoral fins with a scalpel. A prefilled miniosmotic pump was fitted in the peritoneal cavity, and the incision was closed with three sutures with nonabsorbable line. The fish received an additional implant of coconut oil with or without E_2 (as described earlier). Pumps were estimated to deliver 5 ng PTHrP(7-34)·ml plasma^{-1}·h^{-1}, taking into consideration the fish osmolarity (341 ± 3 mOsmol/kg), water temperature (20°C), and the peritoneal/circulation transfer rate. After surgery, fish were distributed in 100-liter tanks (n = 5 per tank). Fish were not fed during the experiments. At *days 1*, *4*, and *8* after implantation, a blood sample was collected from each fish under anesthesia into heparin (30 U/ml, ammonium salt; Sigma-Aldrich) and EDTA-aprotinin (500 KIU/ml; Trasylol, Bayer)-treated tubes, taking care to avoid sample contamination with seawater. Plasma was separated by centrifugation, frozen in liquid nitrogen, and stored at −80°C for subsequent analysis.

Plasma analysis. Plasma E_2 was analyzed in duplicate by specific radioimmunoassay following extraction with diethyl ether and resuspension in assay buffer as previously described (15). Results are shown as nanograms per milliliter.

Plasma PTHrP was analyzed by a specific radioimmunoassay as previously described (40) with antisera against sea bream PTHrP(1-34), with the same peptide as standard curve ligand and ^{125}I-labeled PTHrP(1-36) as label. Bound and free label was separated by immunoprecipitation. The detection limit of the assay was 1.25 pg/tube. The intra- and interassay coefficients of variation were 3.9% and 8.8%, respectively (n = 6). The assay did not cross-react with related mammalian peptides (40) and cross-reaction to fugu PTH or PTH-L(1-34) peptides was <0.1%.

Total or ultrafiltered plasma calcium was measured in triplicate by colorimetric assay with the Arsenazo method (Sigma-Aldrich procedure no. 587). Plasma ultrafilterable fractions correspond to the ionized free calcium fraction and were measured in the fraction of plasma obtained after centrifugation 12,000 rpm 10 min at 4°C through a 10-kDa cut-off filter (Millipore). Values are shown as mmol/l. Total plasma phosphorus was measured in triplicate in undiluted plasma using an end point colorimetric assay (Sigma procedure No 360). Values are shown as millimole per liter.

Statistics. All values are shown as means ± SE, unless otherwise stated. Effects of treatment on plasma parameters were analyzed by one-way ANOVA in *experiment 1* and two-way ANOVA in *experiment 3*, after testing for homogeneity and normality of variances. The Bonferroni multicomparison a posteriori test was applied to determine significantly different groups. The experiment-wise error rate was 0.05.

RESULTS

Effect of PTHrP on plasma calcium. The effect of a single intraperitoneal injection of PTHrP(1-34) on plasma calcium 24 h later in sea bream juveniles is shown in Fig. 1*A*. At doses of 0.2 and 5 μg/g, a significant ($P < 0.05$, one-way ANOVA) increase of filtered plasma calcium was observed. Total calcium increased significantly ($P < 0.05$, one-way ANOVA) only in response to 0.2 μg/g PTHrP(1-34). In contrast, a single 0.2 μg/g injection of PTHrP(7-34) significantly decreased the filtered (free) calcium fraction without affecting total plasma calcium ($P < 0.05$, one-way ANOVA; Fig. 1*B*). Higher doses of 1 μg/g and 5 μg/g of PTHrP(7-34) did not have a significant effect on the total or filtered plasma calcium.

Effect of E_2 on plasma calcium, phosphate, and E_2. Plasma calcium increased significantly in response to E_2 at *days 4* and *8* postimplantation (Fig. 2*A*) ($P < 0.05$, two-way ANOVA).

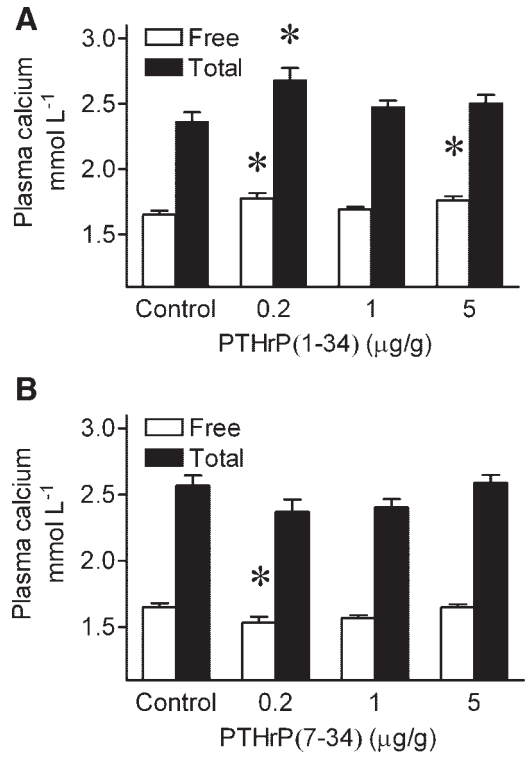

Fig. 1. Effect of single intraperitoneal injection of saline (control), 0.2, 1, and 5 μg/g PTHrP(1-34) (*A*) and PTHrP(7-34) (*B*) on blood plasma levels of free and total calcium in sea bream juveniles sampled 24 h after injection. PTHrP, parathyroid hormone-related protein. Results are shown as means ± SE (n = 8). *Significant difference from controls ($P < 0.05$).

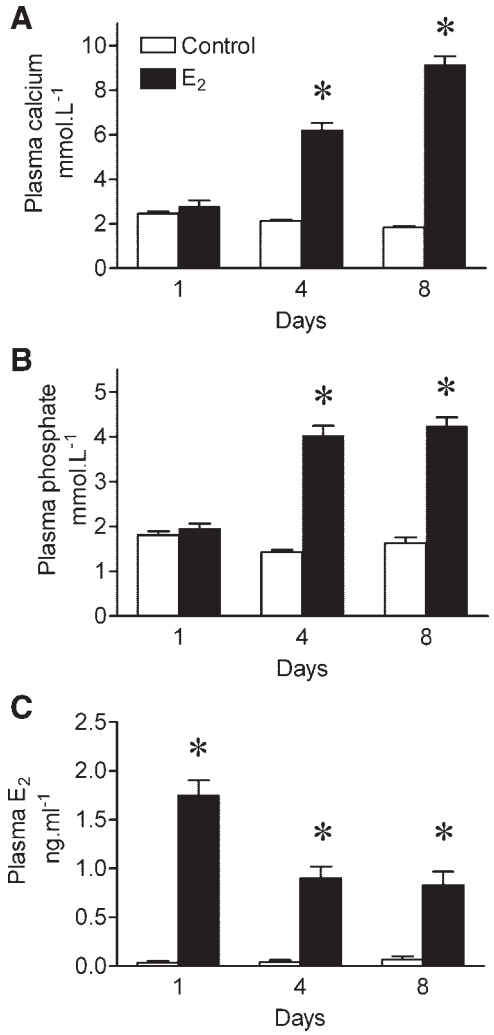

Fig. 2. The effect of estradiol-17β (E_2) implants (10 μg/g) in sea bream juveniles (30 g) at different times (1, 4, and 8 days) in total calcium (*A*), phosphate (*B*), and E_2 (*C*). Results are shown as means ± SE ($n = 7$). *Significant differences from controls at a given time ($P < 0.05$).

Plasma levels of phosphate followed the same pattern as for calcium (Fig. 2*B*).

The changes in plasma E_2 as a result of administration of E_2 implants to sea bream are shown in Fig. 2*C*. E_2 plasma levels were significantly greater than in controls at all sample points in E_2-treated fish ($P < 0.05$, two-way ANOVA). A maximal concentration of plasma E_2 was observed 1 day postimplantation, and then it declined slowly.

Effect of PTHrP antagonist on E_2-induced hypercalcemia. E_2 treatment significantly increased circulating plasma levels of PTHrP over control at *day 4* but not at any other times (Fig. 3*A*). No significant differences in plasma levels of PTHrP were found between E_2 and E_2 + PTHrP groups.

While plasma total calcium levels in the control group were stable over time, E_2 treatment, as expected (see also, Fig. 2), significantly increased plasma levels of total calcium from *day 4* onward (Fig. 3*B*). However, PTHrP(7-34) strongly inhibited this calcium increase when administered in conjunction with E_2 (Fig. 3*B*). The pattern for plasma phosphate was similar to that of total plasma calcium but the inhibitory effect of PTHrP(7-34) was not statistically significant (Fig. 3*C*).

DISCUSSION

In this study, we have demonstrated that an antagonist of the PTH/PTHrP receptor strongly inhibits the hypercalcemic action of E_2, indicating that PTHrP or a related peptide of the same family (3) mediates this inhibition.

One of the functions of E_2 is to promote protein synthesis in the liver, including vitellogenin and egg shell protein components (1, 8). Vitellogenin is highly phosphorylated and calcium binds to the ionized phosphate groups (23). Phosphate levels in the water are generally low and not a source for body functions. Thus, phosphate is obtained mostly from the food through the type II sodium-phosphate cotransporter in the intestinal brush border, and its excretion is regulated through the kidney (24, 25). The source of calcium for fish is largely

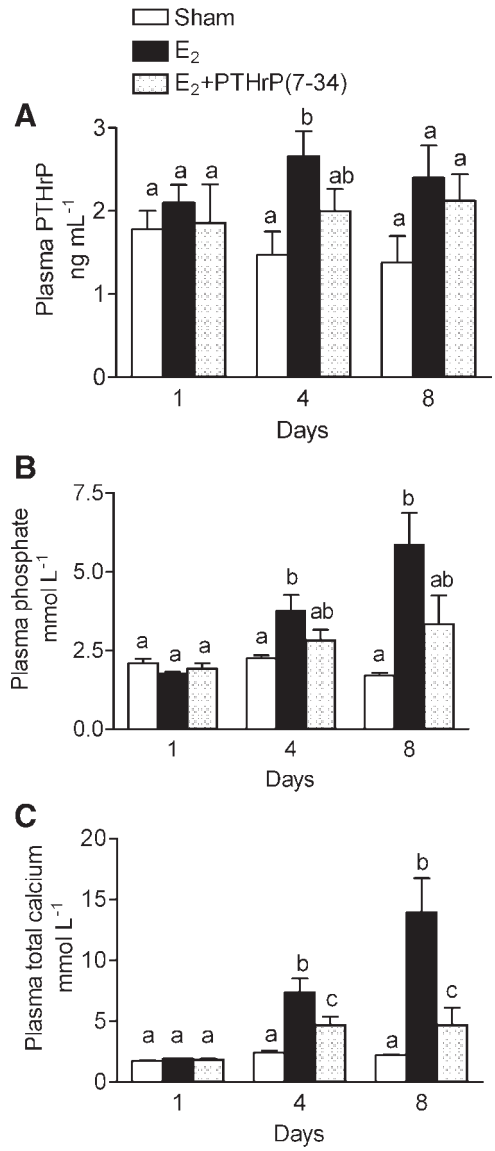

Fig. 3. The effect of E_2 implants (10 μg/g) alone or in combination with PTHrP(7-34) (PTHrP antagonist) in sea bream juveniles (70 g) at different times (1, 4, and 8 days) in plasma PTHrP (*A*); plasma phosphate (*B*), and plasma total calcium (*C*). Each point represents means ± SE of 6–9 fish. [a,b,c]Different letters indicate significant differences ($P < 0.05$, Bonferroni test) between treatments at a given time.

from the surrounding water, and E_2 administration increases whole body net calcium uptake via intestine and gill (15, 31). If required, calcium and phosphate can also be obtained from the mobilization of internal stores, such as bone and scales (30). The fact that a PTH/PTHrP receptor antagonist can inhibit, at least to some extent, the hypercalcemic action of E_2, indicates a role for PTH, PTHrP, or a related factor (see below) that binds to the PTH/PTHrP receptor. A recent in vitro study has also indicated a synergistic effect of PTHrP with E_2 on vitellogenin synthesis (2).

The gill and intestine are the sites for calcium uptake in fish, although the gills are proposed to make the greatest contribution (11). In the sea bream the hypercalcemic effects of E_2 are also achieved via both gill and intestine (15). Therefore, the hypercalcemic effect of E_2 can be either mediated directly through its action in the gills or intestine, indirectly via another endocrine factor responsive to E_2, or both. Support for direct estrogen effects come from the presence of ER mRNA in fish gills (9, 19) and intestine (4, 9, 45), including in the sea bream (33, 35, 41). However, in the present study we also show that a large part of the E_2-induced hypercalcemia is largely indirect possibly via PTHrP or a related factor.

Inhibition of E_2-induced hypercalcemia was achieved by using as antagonist the PTHrP(7-34) peptide, based on the sea bream sequence, which was shown earlier to bind to the PTH/PTHrP receptor and block PTHrP-induced second messenger cascades (3, 38, 39). The incomplete inhibition of calcium and phosphate increase in response to the combination of E_2 and PTHrP antagonist compared with E_2 alone is probably related to the dose of antagonist delivered and the different release/uptake kinetics of the two hormones from the implants.

Previous studies in sea bream have demonstrated that PTHrP on its own is able to increase net whole body calcium uptake in larvae (16) and in vitro intestinal uptake (12). Here we show that treatment with a single injection of 0.2 μg/mg PTHrP(1-34) translates into an increase in free and bound plasma calcium levels 24 h later. Furthermore, the PTH/PTHrP receptor antagonist PTHrP(7-34) caused a reduction in free calcium at the same concentration. These results together with relatively high levels in fish plasma (40) support a role for PTHrP as a hypercalcemic hormone in the sea bream and possibly other teleosts. Despite the blocking effect of PTHrP(7-34) placing PTHrP downstream of E_2 in the hypercalcemic response, the response to PTHrP alone was much lower than to E_2. A likely explanation is that E_2 has a simultaneous inhibitory effect on counteracting hypocalcemic factors. Recently, 5 genes for PTH-related peptides, encoding at least a similar number of cDNAs, have been identified in teleosts fish (3, 6, 29). Four correspond to duplicated PTH and PTHrP genes and the fifth encodes a novel peptide (named PTH-L) with hybrid structural characteristics between PTH and PTHrP, sharing a highly similar NH_2-terminal domain and strong calciotropic activity, suggesting action through common receptors (3). Considering the calciotropic actions of PTHrP and potential similar actions for PTH-L, we cannot decide whether E_2 is acting via PTHrP or PTH-L. However, the fact that PTHrP is elevated in response to E_2 may indicate that it is the mediator. Only when an immunoassay for PTH-L becomes available can we address this question.

While in the sea bream E_2 caused an elevation of PTHrP levels, and in mammalian breast cancer ER-negative cells transfected with ER, the secretion of PTHrP was substantially decreased (37). The difference could be explained either by differential regulation of gene expression in different tissues (34) and/or taking into account a possible contrasting physiological action of PTHrP in lower and higher vertebrates. In mammals PTHrP is essentially a paracrine/autocrine factor (32), while the effects in sea bream appear to be more of an endocrine nature with the pituitary as a possible glandular source (17, 40).

Perspectives

Although the facts that PTHrP(7-34) can effectively block E_2-induced hypercalcemia and that E_2 administration to some extent elevates PTHrP plasma levels are strong evidence for PTHrP mediation of E_2 effects, it is intriguing that E_2 is able to induce higher levels of calcium, especially calcium bound to protein. This may be due to the fact that one of the effects of E_2 is to induce synthesis of calcium-binding proteins (such as vitellogenin). We suggest additionally, that E_2, via its nuclear receptors, may be acting on the promoters of hypocalcemic hormones, such as stanniocalcin, to downregulate their production, thus reducing the counteracting effects of this hormone to maintain homeostatic levels of calcium. E_2 appears to upregulate stanniocalcin in cancer cells (46) but to downregulate luteal cell stanniocalcin expression and secretion (27). Future studies will have to address regulation of calciotropic hormone genes in relation to calcium homeostatic mechanisms.

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GRANTS

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