Estrogen receptor mRNA in mineralized tissues of rainbow trout: calcium mobilization by estrogen

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Received 25 April 1997; revised version received 27 May 1997

Abstract RT-PCR was undertaken on total RNA extracts from bone and scales of the rainbow trout, *Oncorhynchus mykiss*. The rainbow trout estrogen receptor (ER)-specific primers used amplified a single product of expected size from each tissue which, using Southern blotting, strongly hybridized with a ^{32}P-labelled rtER probe under stringent conditions. These data provide the first in vivo evidence of ER mRNA in bone and scale tissues of rainbow trout and suggest that the effects of estrogen observed in this study (increased bone mineral and decreased scale mineral contents, respectively) may be mediated directly through ER.

Key words: 17β-Estradiol; Estrogen receptor; Teleost; Bone; Scale

1. Introduction

The significantly elevated plasma 17β-estradiol levels detected in female teleost fish during the reproductive period are associated with greatly enhanced hepatic vitellogenin (Vg) production, increased physiological demands for calcium and elevated plasma calcium concentrations [1,2]. Radiotracer studies have indicated that the calcium demand is met in part by a mobilization of calcium from internal stores, particularly the scales and also muscle [3–5], and by an increased uptake from the environment [5]. However, the mechanism(s) by which 17β-estradiol exerts its effects on calcium mobilization in teleosts are unclear.

Estrogen receptor (ER) mRNA has been identified in several tissues of the rainbow trout, *Oncorhynchus mykiss*, including the liver, forebrain, pituitary, pineal gland and retina [6–8]. Sequencing of rainbow trout ER (rtER) cDNA has revealed two domains (DNA binding and steroid hormone binding) with strong homology to the human, avian and *Xenopus* ER [6,9]. The major transcript (3.5 kilobases) detected in each tissue codes for a 65 kDa ER protein [9,10] which is similar in size to the human ER [11]. To date, however, the presence of ER mRNA has not been demonstrated in teleost mineralized tissues which, as in other vertebrates, are important targets for estrogenic action.

Evidence for the expression of ER in mammalian bone was presented relatively recently. High-affinity ER were shown to be expressed in primary human osteoblast-like cells and in human and rat osteoblast-like cell lines [12,13]. While highlighting the relatively low expression of ER in bone cells compared with other target tissues (e.g. the uterus), these studies provided clear evidence that the effects of 17β-estradiol on bone could be mediated directly through a classical receptor mechanism. Recent work on ER-knockout transgenic mice, which lack functional ER and show significantly reduced bone mineral densities compared with wild-type controls, strongly supports a direct role for ER action in bone physiology [14]. Other studies have described ER expression and estrogenic effects in a variety of osteoblast-like and osteoclast-like cells derived from humans and animals (for a review, see [11,15]) and the presence of ER mRNA in vivo has also been reported in rat bone [16,17].

In the current study, ER mRNA expression in vivo is demonstrated in bone and scale tissues of the rainbow trout using RT-PCR and 17β-estradiol is shown to have differential effects on calcium and phosphate mobilization in these tissues.

2. Materials and methods

2.1. Animals and estrogen treatment

Juvenile rainbow trout (approximately 5 months old, weighing <75 g) were obtained from local suppliers and maintained in aquaria in aerated, running fresh water. Fish, in weight-matched groups, were injected intraperitoneally with either 17β-estradiol dissolved in ethanol (25 μg/g body weight) or injection vehicle alone (n=11, both groups). Fish were not fed immediately prior to and during the experimental period. Fourteen days after injection the fish were killed and bone, scales, muscle and liver were removed, rigorously cleaned of connective tissue, snap-frozen in liquid nitrogen and stored at −70°C.

2.2. RNA extraction

Equivalent amounts of bone, scales and muscle were ground to a fine powder in the presence of liquid nitrogen using a mortar and pestle. Total RNA was then extracted from the powdered samples following the standard method of Chomczynski and Sacchi [18]. Total hepatic RNA was extracted using LiCl-urea as previously described [9,19]. Liver and muscle were used as positive and negative controls, respectively.

2.3. Reverse transcription-PCR

10 μg aliquots of total RNA extracts were reverse-transcribed into complementary DNA (cDNA) using 7 μM random hexanucleotide primers and 1 U/ml AMV reverse transcriptase (Amersham, France). Samples were incubated for 1 h at 42°C in a 20 μl solution containing: 50 mM Tris HCl (pH 8.3), 50 mM KCl, 10 mM $MgCl_2$, 1 U/ml human placental ribonuclease inhibitor and 1 mM each of dATP, dCTP, dGTP and dTTP.

The primer sequences used for the PCR were: (1) 5′-ACATGTACCCTGAGGAGACA-3′, commencing at nucleotide 222, and (2) 5′-TCAGTACCTCGTCTCGTTGGC-3′, commencing at nucleotide 645 of the rtER. The primers amplified a 423-base-pair fragment, spanning exons 2 and 3, coding for the A/B region of the rtER. Aliquots of cDNA (corresponding to 500 ng total RNA) were added

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PII S0014-5793(97)00680-7

to a 100 μl reaction mixture containing: 1× buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3), 0.2 mM each of dATP, dCTP, dGTP and dTTP, 0.05 U/μl Taq DNA polymerase (Boehringer Mannheim, France) and 1 μM of each primer. Using a Techne thermocycler, PCR was initiated at 94°C for 1 min followed by 29 cycles of incubation at 94°C for 1 min, 52°C for 1 min and 72°C for 1 min, and a final incubation at 72°C for 10 min. RT-PCR was repeated 4 times on samples from different fish.

Agarose and denaturing gel electrophoresis along with Southern and Northern hybridizations were performed using standard methods [20–22]. Full-length rtER [9] and rtVg [23] cDNA probes were used for the hybridizations, the latter being used solely for liver Northern blots as a positive control.

2.4. Mineral analyses

Skin, containing the scales, was dissected from the body of the fish and analyzed separately from the skinned body. Organic matter was removed over 24 h at 60°C using 2 changes of 85% hydrazine hydrate [24]. The remaining mineralized tissue was washed repeatedly in distilled water, sequentially dehydrated in ethanol and xylene and then dried. Tissues were subsequently ashed at 900°C prior to mineral analyses. Calcium contents were measured by atomic absorption spectroscopy while phosphate concentrations were measured colorimetrically using an automatic analyzer.

2.5. Statistical analysis

The effect of estrogen treatment on calcified tissue mineral contents was assessed by analysis of variance and Bonferroni *t*-tests were used subsequently to allow for multiple comparisons. Value of $P < 0.05$ was taken as significant.

3. Results and discussion

RT-PCR has been used successfully to identify low-abundance ER transcripts in rat bone [17]. In the current study, a single band of expected size (~420 base-pairs) was visible on agarose gel electrophoresis of PCR-amplified products from liver, bone and scale. Southern blot analysis of the PCR products revealed strong hybridization with a ^{32}P-labeled rtER probe under stringent conditions (Fig. 1). This is the first report of ER mRNA expression in mineralized tissues of a teleost fish. The longer autoradiographic exposure time required to visualize the bone and scale hybridizations (48 h) compared with that for liver (24 h) suggests that ER expression in these tissues may be lower than in liver. A second primer pair, corresponding to the 5′-non-coding region of the rtER, was also used and again amplified a single band (~220 base pairs) from each tissue which hybridized with the rtER probe (not shown). No ER signal was detectable in muscle tissue which was used as a negative control. The detection of ER mRNA expression in bone and scale in vivo, while not proving that ER protein is present, strongly suggests that the effects of 17β-estradiol on calcium mobilization (see Fig. 3) may be mediated directly through ER expressed in these tissues.

Northern hybridization analysis of total RNA, with a full-length ^{32}P-labelled rtER probe, detected ER mRNA expression in liver which was upregulated following 17β-estradiol treatment, as has been previously described [23]. However, ER mRNA was not detectable by Northern analysis in muscle, bone or scale before or after 17β-estradiol treatment. Preliminary immunohistochemical staining of sectioned bone and scale, using a specific rabbit anti-rtER antibody previously used to detect immunoreactive ER in the brain of rainbow trout [10], also did not detect the presence of rtER protein (not shown). These results are, perhaps, not altogether surprising. From the literature it is clear that ER expression

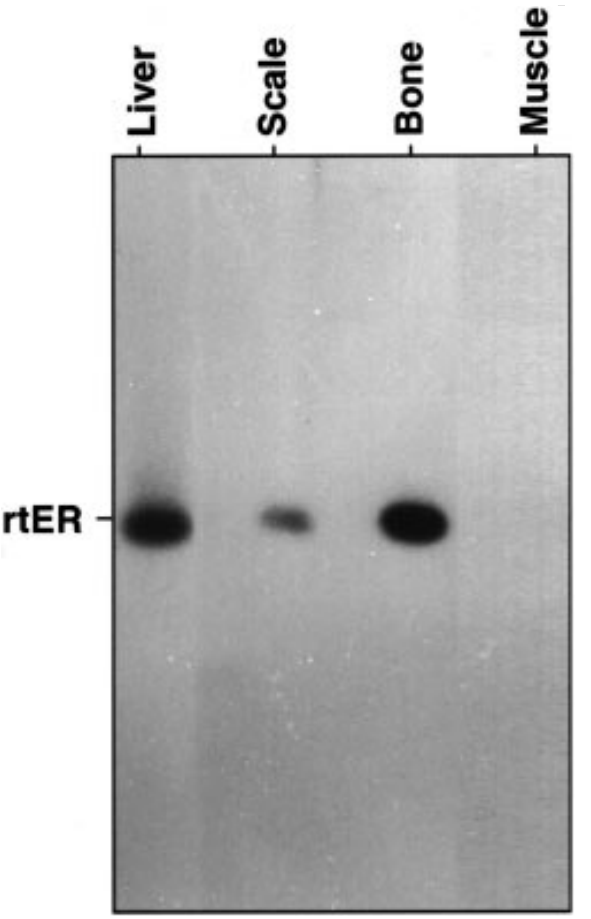

Fig. 1. Southern blot analysis of RT-PCR products from liver, bone, scale and muscle. PCR products were run on a 2% agarose gel, transferred to a nylon membrane overnight and hybridised under stringent conditions [20] with a ^{32}P-labelled rainbow trout ER probe. Autoradiography: liver, 24 h; bone and scale, 48 h; muscle, 72 h.

in mammalian bone is low compared with uterine tissue, which is a primary target for 17β-estradiol action [12,13,15,25,26]. The results of the current study suggest that ER mRNA levels in rainbow trout bone and scale may be low compared with liver, the primary target tissue for 17β-estradiol in teleosts [23]. Consequently, ER message and protein may be difficult to detect in these calcified tissues using conventional methods [26,27]. In contrast, vitellogenin mRNA expression in the liver (positive control) was greatly upregulated by 17β-estradiol [23] having been undetectable before treatment (Fig. 2).

Following 17β-estradiol treatment calcium and phosphate contents were significantly reduced ($P < 0.001$, both cases) in scales and significantly increased ($P < 0.01$, both cases) in bone (Fig. 3a,b). Previous studies on teleosts using radiolabelled calcium have not shown significant increases in bone calcium content in response to estrogen [3–5]. However, the consensus of opinion from these studies and the current work is that 17β-estradiol does not stimulate loss of calcium from teleost bone. In this regard, 17β-estradiol may be considered to have a bone-preserving effect in teleosts analogous to its role in female mammals. The mechanisms of 17β-estradiol action in mammalian bone are not fully understood but ER have been detected in osteoblasts and osteoclasts [12,13,15,25]

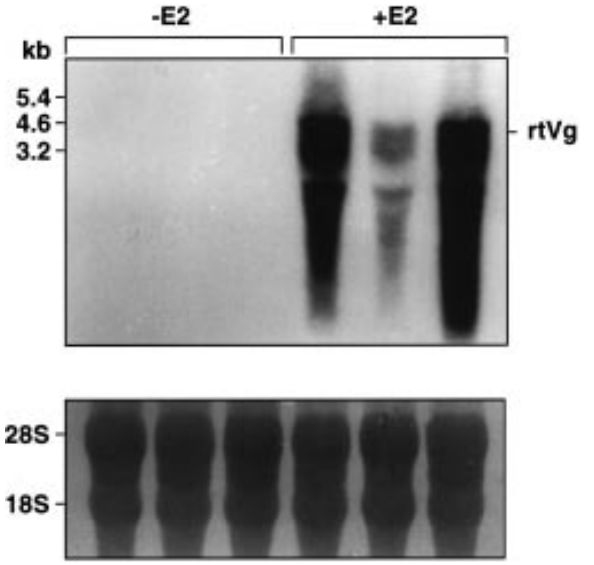

Fig. 2. Upper panel: Northern blot analysis of total RNA from trout liver. Total RNA was extracted from livers of estrogen-treated and untreated trout, then 30 μg was separated on a denaturing formaldehyde-agarose gel, transferred to a nylon membrane overnight and hybridised under stringent conditions [20] with a ^{32}P-labelled rainbow trout vitellogenin (Vg) probe prior to autoradiography for 4 h. Vg was significantly upregulated by estrogen. Lower panel: Methylene blue staining of ribosomal RNA shows uniform RNA loading of the gel.

and 17β-estradiol has been shown to affect both cell types [11]. The presence of osteoblasts and osteoclasts has also been reported in teleost bone [28–33] and these cells are considered to be similar to their mammalian counterparts. In addition, recent histomorphometric studies have shown that 17β-estradiol decreases osteoclast-mediated resorption of teleost bone (Persson, P., personal communication). It is probable, therefore, that the osteoblasts and osteoclasts present in teleost bone express ER and are targets for the direct actions of 17β-estradiol.

17β-Estradiol treatment causes a profound decrease in scale calcium content, but the mechanisms involved in this effect are unknown. Osteoblasts and osteoclasts have been identified in scales [31,32,34,35] and, as mentioned above, in mammals these cells are targets for the direct actions of 17β-estradiol. The presence of ER mRNA in scales suggests that 17β-estradiol may act directly through ER expressed by cells within the scale micro-environment. This is supported by the recent finding that 17β-estradiol markedly and dose-dependently down-regulated the mRNA expression of osteonectin, a collagen binding protein derived from osteoblasts in mammals, in a cell line derived from goldfish scales [36]. Persson et al. [35] have also shown recently that osteoclast activity in the scales of the rainbow trout is induced by 17β-estradiol, leading to increased scale resorption. Thus, it is possible that scale osteoclasts express ER and are targets for the direct actions of 17β-estradiol during vitellogenesis. The predominant role of 17β-estradiol in mammalian skeletal tissue is the maintenance of bone mass by down-regulation of osteoclastic resorption and suppression of bone remodelling [37]. So, clearly, a direct, osteoclast-mediated pro-resorptive effect of 17β-estradiol on teleost scales would be a contrasting and novel finding.

The presence of ER mRNA in bone and scale tissues of the rainbow trout supports the view that the effects of 17β-estradiol on these tissues are directly mediated through ER. Furthermore, the differential effects of 17β-estradiol on calcium mobilization in these tissues may reflect differences in the sensitivities of the osteoblasts and osteoclasts present to estrogen. Indeed, a second ER isoform with a lower affinity for 17β-estradiol has recently been identified in humans [27]. Pre-

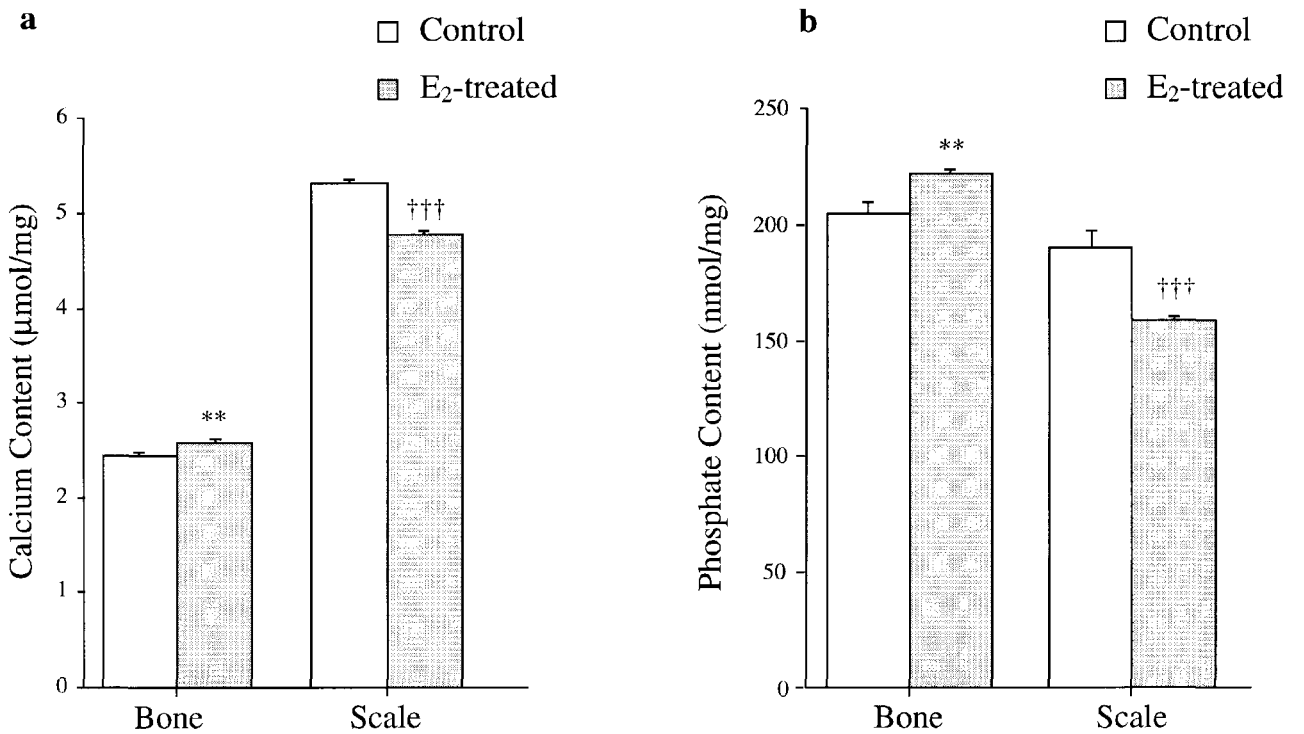

Fig. 3. Effect of 17β-estradiol on (a) calcium and (b) phosphate contents of bone and scale from rainbow trout. $^{**}P < 0.01$, significant increase compared with control; $P < 0.001$, significant decrease compared with control (Bonferroni t-test).

liminary work has shown that it is possible to grow primary cells derived from teleost bone and scale in vitro [32] and this approach may facilitate mechanistic studies of ER regulation in teleost mineralized tissues.

Acknowledgements: K.J.A. was funded by the Wellcome Trust and EMBO.

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