Stimulation of estrogen receptor accumulation by estradiol in primary cultures of salmon hepatocytes

Thomas P. Mommsen* and Catherine B. Lazier

*Departments of *Biology* and *Biochemistry Dalhousie University, Halifax, Nova Scotia B3H 4H7, Canada*

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Hepatocytes of *Salmo salar* in primary culture form confluent monolayers and can be maintained at 11°C in serum-free medium for 8 days with minimal cell loss. Cultured hepatocytes from immature male salmon contain estrogen receptor both in nuclear and cytosol fractions (2000 and 2400 sites/cell, respectively). A single addition of estradiol results in an increase in the nuclear receptor to a level of 23 000 sites/cell after 24 h. This nuclear receptor concentration is similar to that in liver of estrogen-treated salmon in vivo, and is much higher than has been found for any other egg-laying vertebrate. The cultured salmon hepatocytes thus represent a highly sensitive system for the study of estrogen receptor dynamics and vitellogenesis in vitro.

(Salmon hepatocyte culture) *Estrogen receptor* *Estradiol*

1. INTRODUCTION

The induction of vitellogenin by estrogen in lower vertebrates is a useful system for the study of hormonal regulation of gene expression [1]. In this context, fishes have only quite recently attracted increased attention [2–4]. We have found that immature salmon (*Salmo salar*) liver constitutes the richest source of estrogen receptor to date for any vitellogenic species [5]. Since it is inherently difficult to analyze regulation and dynamics of estrogen receptors in vivo, we decided to explore the suitability of salmon hepatocyte cultures in this respect. Studies on estrogen receptors in hepatocyte monolayer cultures from other egg-laying vertebrates have been limited by the small quantity of assayable receptor or by poor responsiveness to estrogen added in vitro [1,6].

Here, we describe for the first time a primary cell culture system for salmonid hepatocytes and show that the cells cultured in serum-free medium respond to added estradiol with accumulation of high levels of nuclear estrogen receptor.

* Present address: Department of Zoology, University of British Columbia, Vancouver, BC V6T 2A9, Canada

2. MATERIALS AND METHODS

Immature, salt water-adapted Atlantic salmon were maintained as described earlier [5]. Hepatocytes were prepared by collagenase/hyaluronidase perfusion in situ as detailed in [7] with several important alterations. Solutions were gassed with 1% CO_2, 99% oxygen, and a modified, glucose-free Hank's medium was used (176 mM NaCl, 5.4 mM KCl, 0.81 mM $MgSO_4$, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 10 mM Hepes, 10 mM $NaHCO_3$ adjusted to pH 7.75 at 22°C). The collagenase concentration was lowered to 0.35 mg/ml, which decreased the yield but improved the viability of the cells. After disintegration of the liver, cells were washed twice with Hank's medium containing 2% defatted bovine serum albumin and 1 mM $CaCl_2$, and then washed twice with modified Waymouth's medium (MB 752/1, Flow Laboratories), adjusted to 176 mM NaCl, 10 mM

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$NaHCO_3$ and 25 mM Hepes, pH 7.8, at 22°C. The cells were counted, dye-exclusion tested, diluted with modified Waymouth's medium containing 10 mU bovine insulin, 5.8 μg streptomycin, 60 μg penicillin and 50 μg gentamycin/ml, plated out at 1.8×10^7 cells/dish on Falcon Primaria culture dishes (25 cm^2) and maintained at 11 ± 1°C. Medium (3 ml/dish) was changed every 24 h.

Cells were treated with estradiol [6] after a minimum of 48 h in culture. At different intervals, the cells were harvested by scraping into buffer containing 250 mM sucrose, 3 mM $MgCl_2$, 10 mM Tes (2-[tris(hydroxymethyl)methylamino]-1-ethanesulfonic acid), 10 mM thioglycerol and 50 μg phenylmethylsulfonyl fluoride/ml, pH 7.85 at 22°C (1 ml/dish). The cells were then homogenized and cytosol and nuclear salt extracts isolated and assayed for specific [^{3}H]estradiol binding exactly as in [5], using either Scatchard analysis or duplicate determinations with 10 nM [^{3}H]estradiol and correction for the degree of saturation using an experimentally determined value for the K_d of 3.4 nM. The protein concentration of both nuclear and cytosol fractions was 1–2 mg/ml.

Table 1

Estrogen receptor levels in cultured salmon hepatocytes

Time in culture at harvest (days)	Time of estradiol addition (h before harvest)	Estrogen receptor (sites/cell)		
		Nuclear extract	Cytosol	Total
3	–	2090	2300	4390
3	24	8200	1600	9800
4	–	2090	2100	4190
4	24	23000	1420	24420
4	48	9700	1400	11100
5	–	2200	2300	4500
5	48	10100	1170	11270
5	72	3300	1160	4460
8	48	12000	2400	14400

Liver cells isolated from immature Atlantic salmon were cultured for varying periods at 11°C as described in section 2 and estradiol (10^{-6} M) was added at the indicated time. Upon harvest, cytosol and nuclear extracts were prepared and estrogen receptor levels measured by the charcoal adsorption method

3. RESULTS AND DISCUSSION

The isolated salmon hepatocytes formed confluent monolayers by 24 h in the modified Waymouth medium at an optimal density of 7×10^5 cells/cm^2. The longest culture period attempted was 9 days at 11°C, after which 80% of the cells still excluded trypan blue. Culture at higher temperatures was detrimental to cell survival.

Our main priority was to study estrogen receptor in the cultured cells with an emphasis on the nuclear receptor and the effect of estradiol added in vitro. Table 1 shows the results of experiments in which estradiol was added to cells which had been in culture for increasing periods. The nuclear estrogen receptor concentration in untreated cells did not change significantly with time in culture and averaged 64 ± 16 fmol/mg protein. This value is several-fold higher than that found in vivo [5], possibly because of selection of parenchymal cells on culturing. Increased nuclear estrogen receptor levels were seen in cells harvested after 3 days in culture and to which estradiol had been added 24 h previously. A greater response was found if the cells were allowed to remain in culture for 1 more day prior to hormone exposure, which might reflect the phenomenon of 'culture shock' described by Wolffe et al. [8]. After the initial 3 day period the cells remained responsive to added estradiol, even up to 8 days in culture (table 1, comparing results for 4, 5 and 8 days in culture with 48 h estradiol treatment).

As can be seen from both table 1 and fig.1, accumulation of nuclear receptor is maximal after 24 h of hormone exposure. Receptor levels fall gradually thereafter, possibly due to estradiol metabolism [9].

The peak level of nuclear estrogen receptor found in the estrogen-treated cultured hepatocytes (711 fmol/mg protein) is equivalent to 4830 fmol/mg DNA or 9.66 pmol/g liver (assuming about 2 mg DNA/g liver [5]). Each gram of packed isolated hepatocytes used in these experiments contained 250×10^6 cells. Thus, the concentration of nuclear estrogen receptor sites per cell is 38.6×10^{-6} fmol or approx. 23000 molecules. Using the same assumptions, the mean receptor concentration in nuclei from cells not previously exposed to estrogen is about 2000 molecules/cell. Similar

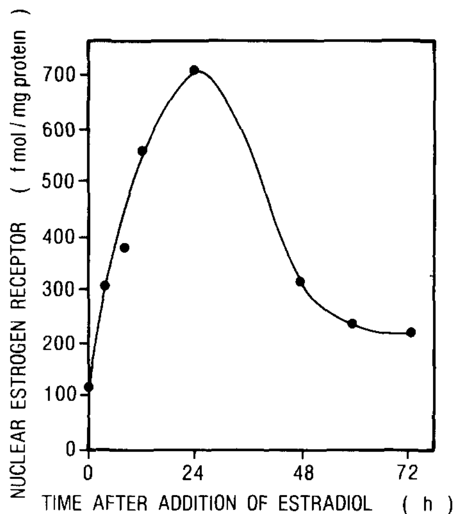

Fig.1. Time course for the increase in nuclear estrogen receptor concentration after a single addition of estradiol. Cells isolated from liver of an immature male salmon were supplemented with estradiol (10^{-6} M) after 3 days in culture and were harvested at the intervals indicated. Nuclear salt extracts were prepared and assayed in duplicate for receptor activity as described in section 2.

values are obtained if the calculations are made on the basis of the number of cells per culture dish.

Cytosol receptor concentrations were a little higher than those found in vivo [5] and did not appear to vary with time in culture. The mean value for all control cultures was 71.1 ± 16 fmol/mg protein or 2400 sites/cell. After estrogen treatment, this level fell slightly in most cases. Thus the total receptor level in untreated hepatocytes is about 4100–4500 sites/cell, compared to 24420 sites/cell in the 4-day cultures treated for 24 h. The mechanism responsible for this 5–6-fold increase in receptor concentration is unknown, but indirect evidence with inhibitors suggests that it might involve receptor synthesis [6,10].

The notably high level of estrogen receptor found in vivo in the salmon liver compared to other oviparous vertebrates is still evident under the appropriate conditions in tissue culture. The cultured salmon hepatocytes could be a better system than those currently used for the analysis of the molecular mechanisms of receptor regulation and estrogen responsiveness associated with vitellogenesis.

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