Oogenesis in Atlantic salmon (Salmo salar L.) occurs by zonagenesis preceding vitellogenesis in vivo and in vitro

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Abstract

Fish oogenesis represents pleiotropic cytodifferentiative programs including hepatic synthesis of the molecular components for both the eggshell and the oocytic energy deposits. Both hepatic processes are directly controlled by plasma levels of estradiol (E2), and injected E2 induces both biogenetic processes in prepubertal fish of both sexes. This work compares the temporal pattern of E2-induced biosynthesis of zona radiata proteins (zr-proteins) and vitellogenin in Atlantic salmon (Salmo salar L.) in vivo and in vitro. We monitored the presence of plasma zr-proteins and vitellogenin, using homologous polyclonal antiserum to zr-proteins and a monoclonal antibody to vitellogenin. We monitored the presence of plasma zr-proteins and vitellogenin, using homologous polyclonal antiserum to zr-proteins and a monoclonal antibody to vitellogenin. Zr-proteins were induced by all E2 concentrations (0·001–1·1 mg/kg body weight (bw)) within one week of exposure while vitellogenin was not induced until two weeks post-injection and then only in plasma from fish injected with high E2 concentrations (0·4 mg or 1·1 mg/kg bw). After E2 treatment, hepatocytes isolated from male fish synthesized zr-proteins and vitellogenin in vitro. However, zr-proteins were secreted into the medium two days before vitellogenin, as measured by ELISA. The data indicate a preferential induction of zr-proteins compared with vitellogenin, both with regard to E2 sensitivity and response time to E2 treatment. These findings suggest an obligate sequence in salmon oogenesis. During sexual maturation low E2 levels at first induce only zonagenesis, while increasing levels of E2 subsequently induce both zonagenesis and vitellogenesis. In nature, the interval between zonagenesis and vitellogenesis may, therefore, be considerable. The data suggest new control mechanisms in fish oogenesis.

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Introduction

Sexual maturation in female fish has been extensively reviewed (Guraya 1986). The rapid growth of the oocyte prior to ovulation in most oviparous vertebrates is mainly due to the incorporation of the yolk protein precursor vitellogenin (Wallace 1985). Vitellogenin is synthesized by the liver (Chen 1983, Bidwell & Carlson 1995) and transported to the ovaries where it is sequestered to serve as an energy reserve for the developing embryo. Another feature of oocyte development is the formation of an extracellular eggshell, or vitelline envelope (Dumont & Brummet 1985). Zona radiata constitutes the major part of the fish eggshell, with the outer zona pellucida as the minor part (Lønning 1972, Oppen-Berntsen et al. 1991, Murata et al. 1991, Olsen & Walther (1993) reported that hepatocytes from E2-treated fish synthesized zr-proteins when further treated with E2 in vitro. Olsen & Walther (1993) reported that plasma levels of zr-proteins correlated with activity of pituitary gonadotropins and pituitary contents of GtH I. Salmon plasma levels of zr-proteins are broadly correlated to plasma levels of both E2 and GtH I (but not GtH II) during sexual maturation (Oppen-Berntsen et al. 1994).
Sumpter et al. (1984) reported that significant increases in plasma levels of GtH, vitellogenin, E₂, and testosterone occurred at least one year before ovulation in trout. Formation of yolk vesicles in oocytes (termed endogenous vitellogenesis) began a full year before ovulation, while ovarian uptake of liver-derived vitellogenin (exogenous vitellogenesis) started three months later, or about ten months before ovulation. Hyllner & Haux (1992) reported that the amount of vitelline envelope proteins in the plasma of maturing rainbow trout varied during the year, in a manner that was similar to vitellogenin. In Atlantic salmon, Lindhom (1997) observed parallel changes in plasma levels of zr-proteins and vitellogenin during a 21-month period before ovulation. Both data sets were interpreted in terms of a similar hormonal regulation of liver synthesis of vitelline envelope proteins and vitellogenin. However, Hyllner et al. (1994) later reported that zr-proteins were detected in plasma nearly a year before vitellogenin, and that the initial formation of the eggshell started before the active uptake of vitellogenin. They also stressed that the low pre-vitellogenic plasma levels of E₂ in females are of physiological significance. These observations in rainbow trout appear to differ from the findings of Lindhom (1997) in Atlantic salmon, where monthly sampling of plasma did not distinguish the chronology of appearance of zr-proteins and vitellogenin during sexual maturation. The reason for this seeming discrepancy is not known.

Selective uptake of vitellogenin by trout oocytes is thought to occur by receptor-mediated endocytosis in a process involving specific cell surface receptors (Tyler et al. 1988, 1990). Tyler et al. (1991) reported that in rainbow trout, receptor-mediated uptake of vitellogenin is developmentally regulated and that oocytes must reach a certain size before they are able to sequester vitellogenin. However, Lancaster & Tyler (1994) demonstrated that vitellogenin receptors are expressed at the oocyte surface in follicles as small as 0.3 mm, although they were not shown to be active. Any presence of vitellogenin in the oocyte would, therefore, seem to suggest both its synthesis by the liver and its uptake by the oocyte.

In order to resolve the apparent discrepancies in the literature, we investigated the E₂ induction of salmon zr-proteins and vitellogenin, in vivo and in vitro. Specifically, we compared the inducibility of biosynthesis of zr-proteins and vitellogenin, both in terms of E₂ concentration, and in terms of the lag time needed for E₂ to induce biosynthesis of zr-proteins and of vitellogenin.

Materials and Methods

Chemicals

Estradiol-17ß (E₂), 4-chloro-1-naphthol tablets, O-phenylenediamine dihydrochloride, EGTA, HEPES, BSA, and collagenase were purchased from Sigma (St Louis, MO, USA). Other chemicals for Western blotting and ELISA analysis were from Bio-Rad Laboratories (Hercules, CA, USA). Diethyl ether and heptane were from Merck (Darmstadt, Germany). [³H]E₂, for determination of recovery in E₂ extraction, was obtained from Amersham International (Amersham, Bucks, UK), and soybean oil was from Idun (Oslo, Norway). Monoclonal antibody against vitellogenin (KB-1) was from Biosense (Bergen, Norway). AutoDELFIA Estradiol kit was from Wallac Oy (Gaithersburg, MD, USA). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), glutamine and antibiotic/antimycotic solution (AAS) were obtained from GIBCO BRL (Life Technologies Ltd, Paisley, Renfrewshire, UK). Secondary antibodies for Western blotting and ELISA analysis were purchased from Bio-Rad Laboratories. Primaria plates for cell cultures were from Falcon, Becton Dickinson Labware, Oxnard, CA, USA. Other chemicals were of the highest commercial purity.

Fish and treatments

Juvenile Atlantic salmon were obtained from Salar Álvik (Álvik, Norway), belonging to the Mowi salmon strain (Bergen, Norway). A total of 36 individuals (an even mix of both sexes and weighing 89 ± 12 g) was used for the experiment. Fish were kept in continuously running sea water at a constant temperature of 8.5 °C (± 0.5 °C) at the Industrial Laboratory (ILAB), HIB, Bergen. The experiment was performed in January; six groups of six fish each were injected once with a single intraperitoneal dose of various amounts of E₂ (0·001, 0·01, 0·1, 0·4, and 1·1 mg/kg body weight (bw)) and subsequently maintained in separate tanks for two weeks. E₂ was dispersed in soybean oil and sonicated for 10 min (50% duty cycle and an output of 60 W) with an Ultrasonic Homogenizer (Cole Palmer Instrument Co, Chicago, IL, USA) just prior to injection. The experiment also contained a control group of fish injected with soybean oil only. The sex of all fish was determined at the end of the experiment.

Collagenase perfusion of liver

Hepatocytes were isolated from prepubertal Atlantic salmon (male, ≈ 300 g) by a two-step perfusion method of Berry and Friend (1969) as modified for fish by Andersson et al. (1983). The liver was perfused using a peristaltic pump at a flow rate of 22 ml/min. First, the liver was perfused in situ for 10 min with a calcium-free solution containing: NaCl (7·14 g/l); KCl (0·36 g/l); MgSO₄ (0·15 g/l); Na₂HPO₄ (1·6 g/l); NaH₂PO₄ (0·4 g/l); NaHCO₃ (0·31 g/l) and EGTA (20 mg/l) at ambient temperature, until all blood was washed out. The whitened liver was then perfused in situ for about 10 min in the same buffer but with calcium (0·22 g/l) instead of...
EGTA, and collagenase (80 mg/150 ml). All glassware and instruments were autoclaved before use. Solutions were sterilized by filtration using a 0·22 µm millipore filter (Millipore AS, Oslo, Norway).

**Preparation and culture of hepatocytes**

After perfusion the liver was dispersed in a plate together with calcium-containing buffer supplemented with BSA (0·1 g/100 ml). The suspension was filtered through a 150 µm nylon monofilament filter and centrifuged at 50 g for 2 min. Cells were washed with medium three times, and finally resuspended in medium. Viability of the hepatocytes used for experiments was always over 90%, as determined by Trypan blue exclusion. After measuring cell yield, cells were plated on 24-well multiwell Primaria plates (≈ 0·5 × 10⁶ cells per well) in DMEM medium (without phenol red) containing 0·5% (v/v) FBS, and 15 mmol/l HEPES, glutamine (0·3 g/l medium) and AAS (1000 units/l penicillin, 1 µg/ml streptomycin, and 0·75 µg/ml amphotericin). Cells were kept at a constant temperature of 10·0 °C (± 0·5 °C) in ambient atmosphere. After plating, cells were cultured for two days in DMEM medium. Then the medium was removed and replaced by new medium containing different concentrations of E₂ (0·1, 1·0, 10·0 nmol/l), changed daily for five days. Cells were plated and treated in triplicate. Collected culture media were stored at −20 °C until analyzed.

**Collection of plasma and extraction of E₂**

Blood was collected from the caudal vein by heparinized syringes, both from treated and control fish. Blood was centrifuged at 5000 r.p.m. for 5 min, and the collected plasma was stored at −45 °C. E₂ was extracted using a modified method of Methven et al. (1992) with an average recovery of 84%. E₂ was extracted from aliquots of plasma (150–200 µl) by adding 4 ml of a mixture of diethyl ether:heptane (4:1), followed by vigorous vortex mixing for 1 min. Test tubes containing the mixture were placed in liquid nitrogen until the bottom water phase was frozen. The top organic phase was decanted to a new test tube and the extraction repeated. The resulting diethyl ether:heptane extracts, containing the extracted E₂, were evaporated to dryness on a heater (45 °C) under nitrogen gas flow. The dry sample was dissolved in PBS buffer (containing 0·50 mol/l NaH₂PO₄, 0·10 mol/l Na₂HPO₄, 1·01 mol/l NaCl, 2% BSA, pH 7·0) and frozen at −20 °C until analysis. Concentrations of E₂ were measured by AutoDELFIA Estradiol kit.

**Antiserum preparation and specificity**

Antiserum to zr-proteins from Atlantic salmon was prepared by Rong (1992) as follows: zr-proteins collected from plasma were purified by preparative SDS-PAGE analysis, and a polyclonal antiserum that recognized all three zr-proteins was raised in rabbit. The specificity of the polyclonal antiserum was checked by Western blotting, and no cross-reactivity with purified vitellogenin or other plasma proteins was seen (Oppen-Berntsen et al. 1992a, Lindhom 1997).

**SDS-PAGE and Western blotting**

Proteins were separated by SDS-PAGE as described by Laemmli (1970) using 4% stacking and 9% separation gels. Proteins were electrophoretically transferred onto a nitrocellulose membrane at 100 V, 400 mA for 1 h as described by Towbin et al. (1979). Salmon zr-proteins were probed with homologous antiserum (1:2000) prepared in rabbit, and salmon vitellogenin was probed with monoclonal antibody (1:500). Incubation with secondary antibodies was performed with goat anti-rabbit IgG horseradish peroxidase conjugate (1:3000) and goat anti-mouse IgG horseradish peroxidase (1:3000). Blots were developed with 4-chloro-1-naphthol (30 mg) dissolved in 10 ml methanol, and mixed with 50 ml TBS containing 0·03% H₂O₂.

**ELISA analysis**

Enzyme-linked immunosorbent assay (ELISA) was based on the method of Oppen-Berntsen et al. (1994), except that non-specific protein binding to the wells was blocked using 2% BSA (200 µl). The wells were incubated with 100 µl polyclonal antiserum against zr-proteins, diluted 1:1000 in 1% BSA, or 100 µl monoclonal antibody against vitellogenin at a concentration of 1:1000 in 1% BSA. Secondary antibodies were goat anti-rabbit IgG horseradish peroxidase conjugate (1:3000) and goat anti-mouse IgG horseradish peroxidase (1:3000). Absorbance was measured at 492 nm.

**Results**

Figure 1A documents the induction of zr-proteins at all E₂ concentrations. At 0·001 mg/kg only the β-monomer of the zr-proteins was induced. This monomer was also seen in plasma from control fish, but to a much lesser extent. In contrast with the induction of zr-proteins, vitellogenin was not detected at any E₂ concentrations after one week of exposure (data not shown). At the end of the experiment (14 days), fish were killed and plasma collected. Plasma samples were analyzed by Western blotting and ELISA analysis. Figure 1B presents a Western blot showing that the three monomers of zr-proteins are induced at all E₂ concentrations. At 0·001 mg/kg only the β-monomer of the zr-proteins was induced. This monomer was also seen in plasma from control fish, but to a much lesser extent. In contrast with the induction of zr-proteins, vitellogenin was not detected at any E₂ concentrations after one week of exposure (data not shown). At the end of the experiment (14 days), fish were killed and plasma collected. Plasma samples were analyzed by Western blotting and ELISA analysis. Figure 1B presents a Western blot showing that the three monomers of zr-proteins are induced at all E₂ concentrations. At 0·001 mg/kg only the β-monomer of the zr-proteins was induced. This monomer was also seen in plasma from control fish, but to a much lesser extent. In contrast with the induction of zr-proteins, vitellogenin was not detected at any E₂ concentrations after one week of exposure (data not shown). At the end of the experiment (14 days), fish were killed and plasma collected. Plasma samples were analyzed by Western blotting and ELISA analysis. Figure 1B presents a Western blot showing that the three monomers of zr-proteins are induced at all E₂ concentrations.
Figure 1 Western blots of plasma from Atlantic salmon injected with various concentrations of E2. Blot A: plasma sampled after one week of exposure. Lane 1, 1·1 mg/kg bw; lane 2, 0·4 mg/kg bw; lane 3, 0·1 mg/kg bw; lane 4, 0·01 mg/kg bw; lane 5, 0·001 mg/kg bw; lane 6, control. Blot B: plasma sampled after two weeks of exposure. Lane 7, 1·1 mg/kg bw; lane 8, 0·4 mg/kg bw; lane 9, 0·1 mg/kg bw; lane 10, 0·01 mg/kg bw; lane 11, 0·001 mg/kg bw; lane 12, control. Plasma was diluted 1:100 and 10 µl was applied to each lane. Western blots were probed with polyclonal antiserum to salmon zr-proteins (α, β, γ). Each lane shows representative plasma from one fish from an experimental group consisting of six fish, all of which were analyzed with the same results. The sexes of all fishes were characterized after the experiment, and each group contained at least two individuals of male or female, except for the control group which contained females only. Results are therefore representative for both sexes. F=female, M=male.

Figure 2 Western blot of plasma contents of vitellogenin from Atlantic salmon injected with various concentrations of E2, analyzed after two weeks of exposure. Lane 1, 1·1 mg/kg bw; lane 2, 0·4 mg/kg bw; lane 3, 0·1 mg/kg bw; lane 4, 0·01 mg/kg bw; lane 5, 0·001 mg/kg bw; lane 6, control (soybean oil only). Western blot was probed with monoclonal antibody to salmon vitellogenin (vtg). Each lane shows representative plasma (dilution 1:100 with 10 µl applied to each lane) from one fish from an experimental group consisting of six fish, all of which were analyzed. The sexes of all fishes were characterized after the experiment, and all groups contained at least two individuals of male or female, except for the control group which consisted of females only. Results are therefore representative for both sexes. F=female, M=male.

Figure 3 ELISA analysis of zr-proteins from Atlantic salmon plasma after two weeks of exposure to various concentrations (mg/kg bw) of E2. Control, fish treated with soybean oil. Absorbance was measured at 492 nm using a polyclonal antiserum to zr-proteins. Each experimental group consisted of six fish. The sexes of all fishes were characterized after the experiment, and all groups contained at least two individuals of male or female, except for the control group which contained females only. Results are therefore representative for both sexes. To calculate P values, Microsoft Excel 5·0 was used. *P<0·01, **P<0·001 compared with control (t-test).

Plasma was also investigated by ELISA analysis. Figures 3 and 4 present ELISA measurements of zr-proteins and vitellogenin respectively, after two weeks of exposure. Results show a dose-dependent induction of both proteins. Plasma sampled one week after injection was also analyzed by ELISA, yielding the same result except that the plasma contents of zr-proteins and vitellogenin were much lower (data not shown).
Measurements of plasma concentrations of E2 show that the levels of plasma E2 at the end of the experiment reflected the amounts injected and the consequent induction of zr-proteins and vitellogenin (Fig. 5). In vitro studies demonstrated that both zr-proteins and vitellogenin were synthesized and secreted during E2 treatment of primary hepatocyte cultures from male Atlantic salmon (Fig. 6A,B). ELISA analysis reveals induction of zr-proteins after 24-h treatment of cells, while vitellogenin was detected in the medium after 72 h of cell exposure. However, we were not able to detect vitellogenin in these samples by Western blotting.

Discussion

The present study reports different inducibilities of zr-proteins and vitellogenin in Atlantic salmon. This finding was observed both by in vivo and in vitro experiments. E2 induced zr-proteins more rapidly than vitellogenin. Also, at lower levels of E2 only zr-proteins were induced, and not vitellogenin. Similar results were also obtained when primary hepatocyte cultures from male salmon were treated with increasing concentrations of E2. This work suggests an ordered sequence of initiation of zonagenesis before initiation of vitellogenesis during salmon sexual maturation. However, the pattern of in vivo accumulation of zr-proteins and vitellogenin in various fish species may be distinct (Hyllner et al. 1994) or concerted (Lindhom 1997) depending on how rapid the plasma E2 concentration increases during sexual maturation.

Data on zonagenesis compared with vitellogenesis are at present limited. In our studies primary cultures of hepatocytes from male salmon showed differential inducibilities for zr-proteins and for vitellogenin. Secretion of both zr-proteins and vitellogenin was observed in culture media after E2 treatment. Zr-proteins were more responsive than vitellogenin to induction by E2, both in terms of E2 concentration and in terms of the lag time before zr-proteins were detected in the culture medium. Zr-proteins were present in the medium within 24 h of E2 treatment, while no vitellogenin was detected at this time using ELISA (Fig. 6B). Only after induction for 72 h was vitellogenin detected. Secretion of zr-proteins and vitellogenin into culture media may mimic secretion of the proteins into the blood stream in vivo.

The sequential appearance of zr-proteins and vitellogenin was also observed in vivo. We detected zr-proteins in plasma from juvenile salmon for all E2 concentrations given, within one week of exposure. Vitellogenin was detected only in plasma from fish injected with the two highest amounts of E2, and then only those sampled two weeks after treatment. The lack of detectable vitellogenin in our studies could possibly be due to insufficient sensitivity of the methods used. The absolute sensitivity of our ELISA assay was not determined. Sensitive radio-immunoassays have permitted measurements of very low...
levels of vitellogenin. Examining the earliest stages of vitellogenesis with this technique, Copeland et al. (1986) concluded that the sensitivity of the assay employed to measure vitellogenin determines the conclusion reached about its absence or presence in plasma.

The basis for a time- and concentration-dependent mechanism of E2 induction of different oogenetic parameters is not known. Sathya et al. (1997) reported that many estrogen-responsive genes possess multiple estrogen-responsive elements (EREs) that act synergistically to activate gene expression. They demonstrated that the synergism among EREs depends on the number of EREs, their spacing, and the distance of the EREs from the promoter. The differential induction of zr-proteins and vitellogenin seen in our study may potentially be explained by the vitellogenin gene requiring more E2 to activate multiple EREs than is necessary for activating the zr-protein genes. One of the alternative explanations is that two different estrogen receptors are responsible for activation of such genes. However, in pilot studies we tested the induction of zr-proteins and vitellogenin with E2 in concentrations from 1–10 mg/kg bw, and found that both were equally induced after two weeks exposure, as measured by Western blot and ELISA.

Previous work on the teleostean eggshells has revealed that formation of the eggshell starts from the outside. It is most peculiar that fish eggshells are composed of a thin outer layer (zona pellucida) and a thicker inner part, the zona radiata (Guraya 1986). Ovarian synthesis of zona pellucida occurs before the formation of the zona radiata takes place by deposition of bloodborne hepatic components on the oocytic aspect of the zona pellucida (Wourms...
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1976, Dumont & Brummet 1985, Guraya 1986, Matsuyama et al. 1991). It remains a mystery how the zona radiata becomes specifically deposited next to the oocytes, what causes soluble plasma proteins to become insoluble macromolecular aggregates, and why zonagenesis should occur before vitellogenesis.

Based on immunological methods this work suggests an ordered sequence of initiation of zonagenesis and of vitellogenesis during teleostean sexual maturation. Since synthesis and deposition of zonagenetic and vitellogenetic components take place in different cells, in vitro observations on zr-protein synthesis do not readily translate into the plasma zr-protein contents observed in vivo. In our experiments there is, nevertheless, a general correspondence between in vivo and in vitro observations. This would seem to strengthen the interpretation that the differential and sequential induction of zonagenesis and vitellogenesis may reflect a basic feature of salmon oogenesis. However, since the relative affinities of the two antibodies for their respective macromolecules have not been established, this conclusion should be confirmed in terms of transcriptional activation of the respective genes. Cloning and sequencing of increasing numbers of vitelline envelope (zr-proteins) genes will permit future studies on the basis for sequential and differential E2 inducibility of zr-proteins and vitello-genin. Work in progress to analyze mRNAs for vitellogenin and zr-proteins during E2 induction of juvenile salmon.

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