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STIMULATION OF *IN VITRO* OVARIAN ESTRADIOL-17β SYNTHESIS IN THE RAINBOW TROUT BY THE CARBOHYDRATE-POOR PROTEIN FRACTION FROM SOCKEYE SALMON PITUITARY GLANDS

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Summary—The role of carbohydrate-poor (Con A I) and carbohydrate-rich (Con A II) pituitary protein fractions, isolated from sockeye salmon (*Oncorhynchus nerka*), were investigated pertaining to *in vitro* estradiol-17β (E_2) production by rainbow trout (*Oncorhynchus mykiss*) ovarian follicles. During the early vitellogenic phase of the reproductive cycle, using defolliculated ovarian follicle preparations (outer epithelium–thecal layer absent), it was demonstrated that the Con A I fraction was capable of increasing E_2 production, in the presence of exogenous testosterone (T) as the substrate. Under similar conditions the Con A II fraction (containing the maturational gonadotropin) was inactive. However the Con A II fraction or T, separately, increased E_2 production by intact ovarian follicles, whereas the Con A I fraction did not. A mechanism proposed to explain the regulation of ovarian E_2 synthesis involves the Con A I fraction enhancing aromatase activity in granulosa cells permitting an increased conversion of T to E_2.

INTRODUCTION

In salmonids the pituitary regulation of ovarian estradiol-17β (E_2) synthesis has been attributed to the maturational gonadotropin (mGTH), distinct from the vitellogenic gonadotropin (carbohydrate-poor or Con A I type) [1, 2]. A number of experimental studies, both *in vivo* [3, 4] and *in vitro* [5–9], have demonstrated that mGTH can increase ovarian E_2 production. In contrast, accumulated evidence from studies on natural blood levels of mGTH and E_2 in female salmonids during the reproductive cycle do not indicate an interdependence of one with the other [3, 10, 11]. During the vitellogenic period when plasma E_2 levels increase progressively in female rainbow trout (*Oncorhynchus mykiss*) to their highest levels [10, 12] plasma mGTH levels remain very low (<0.5 ng/ml) and unchanged [13]. It has been reported that mGTH may inhibit E_2 synthesis *in vitro* [14, 15]. Never-the-less *in vitro* E_2 production by ovarian follicles has been used as a bioassay for different forms of salmonid pituitary gonadotropin [16, 17].

The site of E_2 biosynthesis in the ovary of salmonid fish is the granulosa cell layer within the ovarian follicle [5]. It follows from the "two-cell" model first proposed in mammals by Falck [18], whereby testosterone (T) synthesized in the outer thecal cell layer is aromatized by the inner granulosa cell layer to E_2. However, in the amago salmon (*Oncorhynchus rhoduras*) mGTH was shown to be inactive in enhancing *in vitro* E_2 production by ovarian granulosa cell preparations incubated with exogenous T as aromatizable substrate [5, 7]. The inability of mGTH to affect granulosa cell aromatization persists throughout the reproductive cycle of this salmon species [9]. Clearly discrepancies remain concerning the role of mGTH in regulating E_2 production in the salmonid ovary.

E_2 is important reproductively in female salmonids, like other oviparous teleosts, for the induction of hepatic vitellogenin synthesis, a major yolk precursor protein (for review see [19]). In rainbow trout the increase in blood levels of T and E_2, and subsequently vitellogenin, signal the onset of the vitellogenic phase of reproduction, characterized by rapid

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Abbreviations: Con A I = pituitary protein fraction not adsorbed on Concanavalin A Sepharose; Con A II = pituitary protein fraction adsorbed on Concanavalin A Sepharose; mGTH = maturational gonadotropin (carbohydrate-rich or Con A II type); TBSS = trout balanced salt solution.

ovarian growth due to the accumulation of yolk protein [10, 11].

Previous studies in male teleosts have documented the steroidogenic capabilities of the carbohydrate-rich pituitary fraction (Con A II; containing mGTH) but have also reported some androgen stimulating activity in the carbohydrate-poor pituitary fraction (Con A I) [20, 21]. In this study we have examined the regulation of *in vitro* ovarian E_2 synthesis during the early vitellogenic phase in female rainbow trout, by these two established teleost pituitary protein fractions, Con A I and Con A II, isolated from the closely related sockeye salmon (*Oncorhynchus nerka*). Using ovarian follicle preparations with (intact) and without the surface epithelium—thecal layer (defolliculated) it was demonstrated that ovarian aromatase activity could be enhanced, measured in terms of increased E_2 synthesis, by the Con A I pituitary fraction.

EXPERIMENTAL

Fish

Female rainbow trout were used from a stock of winter spawning fish maintained at the Marine Sciences Research Laboratory. Fish had been held in flowing freshwater, at seasonally ambient temperature and natural photoperiod and fed a commercial trout food (Rainbow Trout Brood Stock Food, No. 8 Gr., Martin Feed Mills, Elmira, ON) daily. Females were selected over a period of approx. 6–8 months prior to spawning for their first (age 4+) or second time (age 5+), during which time their gonadosomatic indices (GSI) ranged between 0.5 and 2.5%. Once the GSI in female rainbow trout reaches 0.16% and an oocyte diameter of 0.6 mm is attained vitellogenesis is expected to begin [11].

Follicle preparation and in vitro incubation

Fish were subdued in a solution of 2-phenoxyethanol (0.4 ml/l; Sigma, St Louis, MO) and quickly killed by decapitation, the ovaries were removed and immediately placed in cold incubation medium. The incubation medium used was trout balanced salt solution (TBSS), pH 7.5, [22, 23] prepared with Type II grade deionized water (Milli-R/Q system, Millipore, Bedford, MA). Intact follicles (1.0–2.5 m) were dissected from the ovaries using fine forceps (N5, Dumont, Switzerland) and spring scissors (IR-116-11.5 cm, IREX, Toronto, ON) under a stereomicroscope (Wild M5A) with a water cooled stage. Defolliculated follicles (following the terminology of Scheutz and Lessman [24]) were prepared from intact follicles which had been separated from the ovary the previous day and left in TBSS at 4°C. A small nick was made in the surface epithelium–thecal layer with scissors and by holding one of the edges and gradually enlarging the opening this layer could be removed. The defolliculated preparation remaining consisted of an intact oocyte surrounded by a relatively undisturbed granulosa (follicular epithelium) cell layer, verified by observations using a scanning electron microscope (Hitachi S570).

Groups of 5 follicle preparations (either intact or defolliculated) were placed in 12 × 75 mm borosilicate glass tubes containing 0.5 ml TBSS. Triplicate tubes were used for each treatment. Incubations were conducted under darkness with 100% O_2 at 15°C in a shaking water bath. The duration of incubation was 24 h. All follicle preparations in each assay were derived from a single fish and all assays were repeated with a different fish. Results between assays in a given experiment were always reproducible, however control levels and the magnitude of response varied significantly between fish preventing the pooling of assays from each experiment. A representative assay is therefore shown for each experiment.

Hormone preparations

Pituitary protein fractions were prepared from sockeye salmon since the quantity required was unobtainable from rainbow trout and both species belong to the same genus. Sockeye salmon pituitary glands (80 g) were collected from mixed sexes of fish caught commercially in the Strait of Juan de Fuca (BC, Canada), during August. Fish were approx. 2 months from spawning and females ($n = 4$) sampled had GSIs of about 4%. Pituitaries were kept on dry ice following removal until they could be stored at −60°C. A crude pituitary extract was derived by homogenizing pituitaries in 4 vol of Buffer B [25] with 3% aprotinin (Sigma). The pituitary extract was stored at 4°C for 24 h, followed by centrifugation at 14,600 *g* for 1 h, and collection of the supernatant. Con A I (unadsorbed) and Con A II (adsorbed) protein fractions were isolated from the supernatant by affinity chromatography on a 5 × 25 cm Concanavalin A-Sepharose (Con A, Pharmacia, Uppsala, Sweden) column [25]. The Con A I

fraction was re-run on Con A to reduce any residual Con A II contamination. Using a chum salmon (*Oncorhynchus keta*) mGTH radioimmunoassay (RIA) [26] only 2.8 ng mGTH/μg Con A I (i.e. 0.28% of the total) was measured after the second pass on the Con A column. To confirm that the Con A II fraction contained mGTH subsequent purification of mGTH was conducted following the procedure in Ref. [27]. Purified mGTH significantly stimulated *in vitro* cyclic AMP and E_2 production in intact rainbow trout ovarian follicles, and *in vitro* germinal vesicle breakdown in preovulatory ovarian follicles from landlocked Atlantic salmon (*Salmo salar ouananiche*) (data not shown). The Con A I and Con A II fractions were dialyzed against TBSS for incubations. Protein concentrations of the Con A I and Con A II fractions were determined by trichloroacetic acid precipitation followed by the Lowry method [28], using bovine serum albumin (Pierce, Rockford, IL) as a standard. Testosterone (Steraloids, Wilton, NH) was dissolved in 100% ethanol and diluted in TBSS, resulting in a final ethanol content of $<0.01\%$.

E_2 RIA

Following incubation all media was recovered and either extracted immediately or frozen at $-20°C$. Media was extracted twice with diethyl ether, evaporated to dryness under N_2 and reconstituted in 0.5 ml 100% ethanol. Tritiated E_2 purchased from New England Nuclear (Dupont, Boston, MA), was cleaned up by paper chromatography prior to use as both label and recovery tracer. Extraction efficiency was calculated by recovery of added tritiated E_2. Antisera specific for E_2 (Steraloids) were raised in male New Zealand white rabbits injected with E_2 (3-*O*-carboxy-methyl)-oxime conjugated to bovine serum albumin, as described by Idler and Ng [20]. The antisera developed for E_2 had 0% cross-reaction with T; even at the high concentrations of T used in some experiments (i.e. 10 μg T/ml media) there was no interference in the E_2 RIA. However, there was significant cross-reaction of the E_2 antisera with estrone and estriol. It was determined, by chromatography of media extracts on Sephadex LH-20 to separate E_2 fractions from those of estrone and estriol [29], that follicular preparations in these experiments produced predominately E_2 (78%). This confirmed that E_2 is the major estrogen produced by the rainbow trout ovary, similar to conclusions reached by Sire and Dépêche [14]. All media extracts were assayed direct and values reported are expressed as E_2.

In the RIA aliquots (50 μl) of the ethanol extract, in duplicate, were evaporated under N_2 and reconstituted in 200 μl RIA buffer [30] prepared without sodium azide. Tritiated E_2 (100 μl) was added, vortexed, and 100 μl of E_2 antisera (1:6000 dilution) added, vortexed, and left overnight on ice. The following day addition of dextran-coated charcoal (600 μl), followed by centrifugation was used to separate antibody-bound from free tracer [30]. An aliquot (800 μl) added to liquid scintillation fluid (Ready-Safe, Beckman) was then counted in a scintillation counter (Packard Tri-Carb Model 300C). Serially diluted E_2, to provide a standard curve, was similarly carried through the above procedures. Intra- and interassay coefficients of variation were 6.1 and 6.3%, respectively. The E_2 RIA's lower limit of sensitivity was 2 pg. Serially diluted ethanol extracts (volumes between 25 and 125 μl) paralleled the standard curve.

Statistical analyses involved one-way ANOVA followed by Tukey's multiple comparisons test to determine significant differences between treatment groups [31].

RESULTS

In experiment 1 the response by intact follicles to produce E_2 in the presence of either Con A I or Con A II was examined. In this experiment the Con A II preparation significantly increased E_2 levels above the control levels (Fig. 1). A dose response was observed, at Con A II doses of 20 and 200 μg/ml, E_2 levels rose to 0.8 and 1.22 ng/ml, respectively compared to 0.08 ng/ml for the control. Incubations treated with Con A I were no different from the

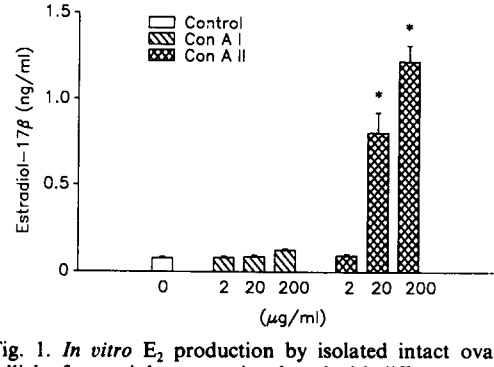

Fig. 1. *In vitro* E_2 production by isolated intact ovarian follicles from rainbow trout incubated with different concentrations of sockeye salmon pituitary protein Con A I or Con A II fractions. Each bar represents the mean ± SEM; * = significantly different from the control at $P < 0.001$.

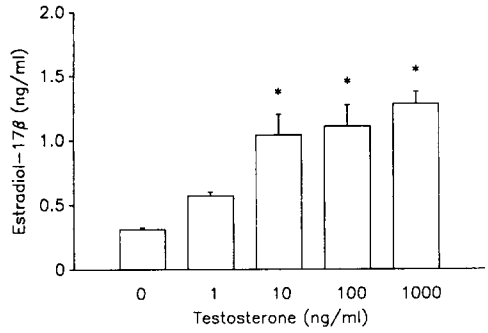

Fig. 2. *In vitro* E_2 production by isolated intact ovarian follicles from rainbow trout incubated with different concentrations of T. Mean ± SEM as in Fig. 1; * = significantly different from the control at $P < 0.005$.

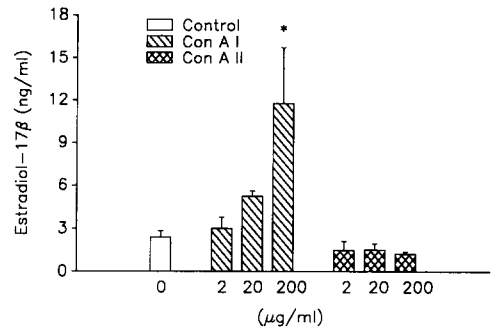

Fig. 4. *In vitro* E_2 production by isolated defolliculated ovarian follicles from rainbow trout incubated with T (1 μg/ml), and different concentrations of sockeye salmon pituitary Con A I or Con A II fractions. Mean ± SEM as in Fig. 1; * = significantly different from the control at $P < 0.025$.

control level at any of the doses tested. From previous studies the increase in E_2 can be attributed to actions of mGTH (present in the Con A II) on thecal cells that produce T which is in turn aromatized to E_2 by granulosa cells within the follicle [5].

To confirm that elevated levels of T can increase E_2 production different amounts of T were incubated with intact follicles in experiment 2 (Fig. 2). T added to the incubation media dramatically increased the amount of E_2 normally produced. The doses of 10–1000 ng T/ml produced incremental increases in E_2 statistically significant from the control level. This indicated that increased levels of T can enhance the amount of E_2 produced by intact follicles, similar to effects of the Con A II fraction.

When the thecal cell layer, the major T synthesizing tissue of salmonid ovarian follicles [5, 15], is manually removed (=defolliculated follicle) E_2 production by granulosa cells can be assessed free of thecal cell influence [7]. T is not produced by salmonid granulosa cells in response to mGTH [5, 15]. To determine the response to exogenous T, different doses of

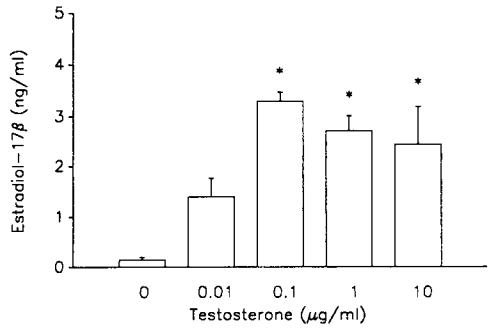

Fig. 3. *In vitro* E_2 production by isolated defolliculated ovarian follicles from rainbow trout incubated with different concentrations of T. Mean ± SEM as in Fig. 1; * = significantly different from the control at $P < 0.025$.

T were incubated with defolliculated follicles (Fig. 3). All doses of T beyond 0.1 μg/ml significantly elevated E_2 production. However, beyond the dose of 0.1 μg T/ml increasing amounts of T caused no further increase in E_2 production and appeared to decrease production. Although not statistically significant high levels of T may inhibit E_2 production under these conditions. The level of 1 μg T/ml was chosen for future experiments, since it achieves maximum E_2 production similar to 0.1 μg T/ml, yet provides an excess of exogenous T in the medium available for further aromatization.

The fourth experiment tested the effect of Con A I and Con A II on E_2 production by defolliculated follicles incubated in the presence of exogenous T, at 1 μg T/ml, as determined in experiment 3 (Fig. 3). The rationale being that Con A I and/or Con A II might enhance the aromatase activity within granulosa cells, resulting in further aromatization of available exogenous T and higher E_2 levels. Only the Con A I preparation was able to significantly elevate E_2 production (Fig. 4). Although a dose response is suggested only the Con A I level of 200 μg/ml significantly ($P < 0.025$) increased E_2 levels to 11.75 ng/ml relative to 2.4 ng/ml for the control. All doses of Con A II resulted in E_2 levels no different from the control. Assays conducted later in the reproductive cycle, during mid to late vitellogenesis (intact follicle dia >3 mm) demonstrated a diminished effect of the Con A I fraction in stimulating E_2 synthesis under the above conditions.

The fifth experiment tested whether Con A I in the presence of T (1 μg T/ml) could enhance E_2 production by intact follicles. There was no significant difference ($P > 0.05$) between the

amount of E_2 (ng/ml) produced by the control group (11.1 ± 0.9) and groups of intact follicles treated with 2 (12.1 ± 1.7), 20 (10.4 ± 1.2), or 200 μg/ml (10.7 ± 0.8) Con A I.

DISCUSSION

These results demonstrate that a component(s) of the sockeye salmon pituitary Con A I fraction, but not Con A II, is capable of enhancing aromatase activity in the rainbow trout ovarian follicle during the early vitellogenic phase of the reproductive cycle. A proposed mechanism for the regulation of salmonid E_2 synthesis can be generated from these results and earlier studies [5, 7]. Maturational gonadotropin induces T synthesis by the thecal cells which in turn is aromatized to E_2 by granulosa cells, the aromatase enzyme being regulated by a component(s) of the Con A I pituitary fraction. On this basis mGTH has an indirect affect on E_2 synthesis by providing T produced by the thecal cells as aromatizable substrate. Endogenous levels of ovarian aromatase are sufficient to produce elevated levels of E_2 as observed in Fig. 1. This explains the action observed in many studies that mGTH, *in vitro*, is able to increase E_2 synthesis by intact follicles [5–9, 16, 32]. Alternatively, the presence or absence of mGTH made no difference to E_2 production by follicle preparations containing only granulosa cells incubated with exogenous T in this study and others [5, 7].

There was a dramatic influence of the surface epithelium–thecal layer on whether the Con A I fraction was able to significantly increase E_2 production. The presence of this layer (i.e. intact follicles) prevented the increase in E_2 noted under similar conditions using defolliculated follicles (Fig. 4). These results can be explained by a reduced accessibility of the Con A I fraction to the granulosa cell layer in experiments with intact follicles. The surface epithelium–thecal cell layer may provide a barrier that prevents the active component(s) from reaching the underlying granulosa cells.

As mentioned earlier mGTH levels are very low throughout the vitellogenic period in the rainbow trout [13]. Therefore a question can be raised pertaining to the above explanation of mGTH prevailing to stimulate T as an E_2 precursor at this time. Recently two "glycoprotein" gonadotropins, designated GTH I and II, have been isolated from chum and coho (*Oncorhynchus kisutch*) salmon pituitaries [17, 33]. Both GTH I and II have steroidogenic biological activities [32] analogous to mGTH. It has been shown that the highest levels of GTH I in rainbow trout may be found in the blood during the vitellogenic phase, while GTH II similar to mGTH is only apparent at spawning time [34]. If this is the case GTH I could play a role in the initiation of ovarian T synthesis during the early vitellogenic period.

The level of ovarian aromatase activity appears to undergo a seasonal cycle that peaks during the mid-vitellogenic phase and decreases toward spawning time in the amago salmon [7, 9]. It was apparent from control levels in this study that aromatase enzyme is present in the granulosa cells of the ovarian follicle from rainbow trout during the early vitellogenic period (Fig. 3). The mechanism by which Con A I enhances ovarian E_2 production is not understood, but it may function to increase the amount of aromatase. A maximum amount of aromatase is probably attained by the mid-point of the vitellogenic phase since it was found that the ability of Con A I to enhance E_2 diminished once follicle dia >3 mm were attained.

The high concentration (200 μg) of Con A I required to significantly increase E_2 production by ovarian follicle preparations presumably results from this fraction being partially purified and characterized by considerable extraneous protein. We have found that proteins with M_ws $<100{,}000$ only make up about 20% of the Con A I fraction from sockeye salmon. With this fact in mind it suggests that the bioactive component(s) within the Con A I is likely present in conservative quantities. Further purification of the sockeye salmon Con A I pituitary fraction will be undertaken and incubations performed during the appropriate phase of the reproductive cycle to determine the component(s) responsible for the demonstrated enhancement of ovarian aromatase.

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