IN VITRO CORTISOL METABOLISM BY EMBRYONIC TISSUES OF ARCTIC CHARR AND RAINBOW TROUT

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Introduction:

Fish eggs contain several hormones of maternal origin, including thyroid (see reviews by Leatherland, 1994; Lam, 1994) and steroid hormones such as cortisol (Feist et al., 1990; de Jesus and Hirano, 1992; Hwang et al., 1992; Khan et al., 1995). Cortisol is the major interrenal steroid hormone of most teleosts and has been implicated in several physiological processes, including osmoregulation, stress response, and intermediary metabolism. However, little is known about any possible roles of this hormone in the early embryonic development of fish. In mammals, cortisol appears to be needed for differentiation of neuronal and somatic tissues during early ontogeny (Sapolsky and Meaney, 1986; Ballard, 1989; Muggia et al., 1995), but it is neurotoxic at high concentrations (Bohn, 1984; DeKloet et al., 1988; Sapolsky and Meaney, 1986). Therefore, at least in mammals, the level of cortisol to which the fetus is exposed during early development is crucial for normal organogenesis and development (Langlois et al., 1995).

Developing fish embryos, which are dependent on yolk for their nutritional requirements, are probably exposed to the steroid hormones (including cortisol) that are present in the yolk. Whereas in mammals there is an intricate control mechanism involving differential expression of various cortisol-regulating steroidalogenic enzymes (Langlois et al., 1995; Nagaya et al., 1995), there is no such information available regarding the the ability of fish embryos to metabolize endogenous cortisol. The purpose of the present study is to determine whether the enzymes involved in cortisol metabolism are expressed in the early developmental stages of embryos of two salmonid fish species.

Although there are no reports on cortisol metabolism in fish embryos, some studies have described the profiles of yolk cortisol during development in various teleosts including Japanese flounder (Paralichthys olivaceus) (de Jesus et al., 1991), chum salmon, (Oncorhynchus keta) (de Jesus and Hirano, 1992), tilapia (Oreochromis mossambicus) (Hwang et al., 1992), rainbow trout (Oncorhynchus mykiss) (Yeoh, 1993) and Asian seabass (Lates calcarifer) (Sampath-Kumar et al., 1995). There is a decline in the cortisol levels concomitant with the absorption of yolk indicating that the hormone is being eliminated by the embryo as the yolk is absorbed.
The presence of cortisol in yolk could potentially affect the development of the hypothalamic-pituitary gland-interrenal tissue (HPI) axis. The HPI axis in rainbow trout is reported to become responsive to stressor challenge two weeks after hatching and one week before the onset of exogenous feeding (Barry et al., 1995a). Pottinger & Mosuwe (1994) postulated that the appearance of a functional HPI axis is developmentally delayed to avoid stress-induced disruption of other physiological processes during key ontogenetic events. This hypersensitiveness to stress may be homologous to a similar 2-week postnatal period in rodents, the function of which may be to maintain low, constant corticosteroid levels during a critical development period when these steroids could exert a permanent deleterious effect on neuronal organization (Barry et al., 1995).

We have recently shown that Arctic charr (Salvelinus alpinus) embryos are able to metabolize various steroid hormones such as progesterone (P), 17α-hydroxyprogesterone (17αOHP), androstenedione (A.), testosterone (T), 17β-estradiol (E2) and estrone (E1), in vitro (Khan et al., 1995; M.N. Khan, R. Renaud and J.F. Leatherland, unpublished). These studies reveal that several key steroidogenic enzymes are expressed early in ontogeny and that steroid hormones are rapidly metabolized. In the present report we present the first evidence of cortisol metabolism by developing embryos of two salmonid species, Arctic charr and rainbow trout.

**Materials and methods:**

[3H]cortisol incubation

Yolk-sac embryos of Arctic charr [41 days post fertilization (dpf)] and rainbow trout (53 dpf) were killed by decapitation and the yolk sac was removed. The embryos (three per well) were placed in individual tissue culture wells (24-well plate, 3.5 ml well capacity, M/S Flow Laboratories Inc., VI, USA). Each well contained 1 ml of modified Medium 199 (M-3274 M/S Sigma Chemical Company) with Hank’s salts, glutamine, sodium bicarbonate, bovine albumin, antibiotics and glucose, without phenol red, pH 7.2 (Venkatesh et al., 1992). The radiolabelled substrate [3H]cortisol made in the same medium was added to each well (0.56 nM). Duplicate control wells with no tissue and three replicates (wells) containing fish embryos were used for each incubation time and for each species separately. The plates were gently agitated on a shaker during incubation which lasted for 20 min and 24 h. At the completion of each incubation period the embryos from each well were removed and the incubation media was stored at -20°C until analysed for steroid hormones.

Extraction of steroid hormones

The extraction procedure for HPLC was based on the methods described by Payne et al. (1989), Venkatesh et al. (1989), Kime et al. (1991) and Scott and Canario (1992) with some modifications. The incubate along with embryos was applied to a Sep-Pak C18 cartridge which had been primed by washing with 5 ml of methanol and 5 ml of distilled water. The incubate was passed through the Sep-Pak column by syringe and the column was again washed with 5 ml of water and 5 ml of hexane. Subsequently, the free steroid hormones were washed from the column using 5 ml of diethyl ether and the conjugated steroids were eluted with 5 ml of 100 % methanol. The ether and methanol extracts were dried separately under nitrogen at 45°C. The free fraction having radiolabelled metabolites was dissolved in 200 μl of acetonitrile containing a mixture of 21 unlabelled steroid hormones (as internal standards); 20 μl of this mixture was injected onto the HPLC column. The dried conjugated steroid fraction was processed for acid solvolysis and enzyme hydrolysis to obtain the free steroids for HPLC (see below).
Acid solvolysis of sulphates

The dried methanol fraction containing the conjugated steroids was incubated overnight at 45°C with 5 ml of trifluoroacetic acid/ethylacetate (TFA/EA 1/100; v/v) to convert sulphated forms of steroids into free hormones. The solvent was then evaporated and the residue was dissolved in 5 ml of water. The free (formerly sulphated) and glucuronide conjugated steroids were concentrated and separated as described above using Sep-Pak cartridge. The sulphated steroids now present as free steroids, were extracted with 5 ml of diethyl ether. The glucuronide conjugates were extracted with 5 ml of 100% methanol as above and subjected to enzyme hydrolysis.

Enzyme hydrolysis of glucuronides

The dried methanol fraction from the second extraction was reconstituted with 1.0 ml of 0.5 M sodium acetate buffer (pH 5.0) and the glucuronides were hydrolysed at 37°C using 20µl of β-glucuronidase (snail juice, containing 2000 I.U. of enzyme activity). The free steroids resulting from enzymic hydrolysis were taken up in 5 ml of water and extracted from a Sep-Pak column with 5 ml ether (see above).

HPLC Analysis

The apparatus consisted of a Waters Associates (Milford, MA, USA) Liquid Chromatography System comprising two 510 pumps, a WISP 710B autoinjector and a solvent programmer (Waters Baseline 810 controller Software), coupled with a dual channel model 441 UV detector. The NOVA.Pak C18 (8 mm X 100 mm, 4 µm) column was also purchased from Waters Associates. For optimum separation of steroids Waters Baseline 810 controller Software was used to create a binary solvent gradient of acetonitrile-water. The multistep gradient mobile phase consisted of 29.5, 35, 39, and 75% acetonitrile in water at time 0, 10, 12.5, and 32 min, respectively, in the run. In order to obtain better yield of metabolites off the HPLC column, the incubation replicates were pooled together after extraction, in acetonitrile containing 21 steroid standards and injected into column as described above. Fractions were collected from the HPLC column at 0.5 min intervals for a total of 40 min. To each fraction was added 4 ml of scintillation fluid and the radioactivity was counted on scintillation spectrometer. Retention times for each steroid hormone was determined from the elution time of the internal standards and the absorbance was monitored at 254 nm and 280 nm. The counts (CPM) in each fraction and absorbance of 21 internal standards was plotted against retention time.

Results:

Both Arctic charr (41 dpf) and rainbow trout (53 dpf) embryos converted [3H]cortisol to 11β-hydroxyandrostenedione (11β-OHA). Another metabolite, more polar than cortisol, probably tetrahydrocortisol or tetrahydrocortisone, was also found eluting immediately after the solvent front. The elution profiles of cortisol and its metabolite as separated by HPLC for both species are shown in Table 1 and Figure 1-2. The metabolite yields were maximal after 20 min of incubation and remained in the same range throughout the incubation time, indicating that 11β-OHA is the end product of cortisol metabolism in salmonid embryos. In addition to 11β-OHA, a small fraction of cortisol was also converted to its sulphated and glucuronated metabolites.
Table 1. *In vitro* metabolism of [3H] cortisol (0.56 nmol/well) by yolk sac embryos of Arctic charr (41 dpf) and rainbow trout (53 dpf) after 20 min and 24 h of incubation at 10°C. The radioactivity (CPM) measured under the respective peak area and expressed as % of total counts.

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Time</th>
<th>Arctic charr (41 dpf)</th>
<th>Rainbow trout (53 dpf)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol (substrate)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20 min</td>
<td>14.5</td>
<td>31.0</td>
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<tr>
<td></td>
<td>24 h</td>
<td>21.62</td>
<td>26.2</td>
</tr>
<tr>
<td>11β-OHA*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20 min</td>
<td>52.4</td>
<td>44.5</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>54.9</td>
<td>46.8</td>
</tr>
<tr>
<td>Tetrahydrocortisol (?)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>16.1</td>
<td>13.6</td>
</tr>
<tr>
<td>Sulphates</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20 min</td>
<td>11.4</td>
<td>7.0</td>
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<tr>
<td></td>
<td>24 h</td>
<td>5.7</td>
<td>8.9</td>
</tr>
<tr>
<td>Glucuronides</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20 min</td>
<td>3.0</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>1.6</td>
<td>4.5</td>
</tr>
</tbody>
</table>

*11β-hydroxyandrostenedione*
Figure 1 (upper) 2 lower. HPLC profiles of radiolabelled steroid hormones produced by Arctic charr (Fig. 1) and rainbow trout (Fig. 2) embryos after incubation for 24 h with \(^{3}H\) cortisol. THC = tetrahydrocortisol or tetrahydrocortison (?); 11β-OHA = 11β-hydroxyandrostenedione.

**Discussion:**

Arctic charr and rainbow trout embryos appear to convert \(^{3}H\) cortisol almost exclusively to 11β-OHA, with the exception of a smaller peak of unknown metabolite probably a tetrahydro derivative of cortisol or cortisone. This finding is consistent with the report of Kime (1978) who demonstrated that cortisol, incubated in vitro with liver slices of rainbow trout, pike (Esox lucius) and perch (Perca fluviatilis), was converted into cortisone, 11β-OHA and 11-oxoandrostenedione.

Cortisol is known to be secreted as part of the physiological stress response in fish, and may have inhibitory effects on steroidogenesis (Pankhurst and Dedual, 1994). Similarly, in mammals, glucocorticoids are reported to be neurotoxic at high concentrations during fetal development (Sapolsky and Meaney, 1986). Therefore, it seems plausible to speculate that the same hormone may also elicit a deleterious stress-response in the sensitive early embryonic stages in fish. The fact that exogenous cortisol was converted to 11β-OHA further indicates that fish embryos may
be able to inactivate cortisol and thus avoid these effects. In this context, it is interesting to note that appearance of a functional HPI axis in salmonids has been reported to be delayed possibly an adaptation to avoid the stress-induced disruption of other physiological processes during the key ontogenetic events (Pottinger and Mosuwe, 1994). A parallel situation has been reported in mammals in which a nadir in responsiveness to adrenocorticotropic hormone (ACTH) acts to lower the exposure of the developing fetus to bioactive glucocorticoids (Langlois et al., 1995). In our experiments, the (yolk sac) embryos of rainbow trout and Arctic charr were incubated with cortisol at a time in development when the HPI axis is thought not to be functional (Barry et al., 1995).

We have recently found that in Arctic charr embryos there are various steroidogenic enzymes expressed very early in the development (Khan et al., 1995) even before the reported maturity of the HPI axis in similar salmonids (Saga et al., 1993; Barry et al., 1995). One of these enzymes, 17, 20-desmolase (lyase) [also called mitochondrial cytochrome P450 enzyme (side chain cleavage, P450ccc)], converts 17α-OHP to A4, and can also cleave the cortisol molecule to form A4. The A4 produced as an intermediate metabolite can then be converted to 11β-OHA by 11β-hydroxylase [also called mitochondrial cytochrome P450 enzyme (11hydroxylase, P450c11)]. These two enzymes are possibly being expressed de novo in response to [1H]cortisol challenge before the maturation of the HPI axis; therefore, the catabolism of the cortisol may reflect the need of the embryo to excrete the hormone to protect itself from the adverse effects of this biologically potent steroid hormone. This finding is in agreement with our recent work which has established that Arctic charr embryos can inactivate several bioactive steroid hormones such as P1, 17α-OHP, T, E2 and E1 (Khan et al., 1995; M.N. Khan, R. Renaud, and J.F. Leatherland, unpublished). The catabolism of cortisol to an inactive metabolite, 11β-OHA, therefore may represent another example of this inactivation process. This hypothesis is further supported by the finding in mammals that the expression of the same enzymes, P450ccc and 11β-hydroxylase regulates the cortisol levels in neonatal rats (Nagaya et al., 1995). Similarly, the expression of two isozymes of 11β-hydroxysteroid dehydrogenase (11β-HSD) which convert bioactive cortisol to the less active cortisone in developing ovine fetal liver and kidney, is thought to protect the fetus from elevated levels of glucocorticoids (Langlois et al. 1995). Another enzyme, 20β-reductase, has also been shown to be expressed in the chorioallantoic membrane of chick embryos (McNatt et al., 1992); this enzyme metabolizes cortisol to produce 20β-dihydrocortisol, and both of these hormones are angiotastic (i.e., they inhibit neovascularization of the chorioallantoic membrane); the expression of this enzyme perhaps controls the development of the vascular capillary network in chick embryo. The expression of P450ccc and 11β-hydroxylase in fish embryos that are exposed to exogenous (maternal) cortisol, and the resulting conversion of cortisol to 11β-OHA may represent a parallel of the phenomena seen in mammalian and avian models.

The other minor, as yet unidentified, metabolite in the present experiment may be one of tetrahydro derivatives of cortisol or cortisone. McKerns (1969) reported that in human beings the major excreted metabolite of cortisol is tetrahydrocortisol or tetrahydrocortisone. Similarly Pottinger et al. (1992) found that the predominant steroid metabolite in the bile of stressed fish is tetrahydrocortisone. The four hydroxyl groups would make this metabolite more polar than cortisol, and it would therefore elute from the HPLC column before the cortisol/cortisone.

However, the definite identity of this metabolite could not be ascertained due to lack of a proper standard.

Conjugation as a form of inactivation does not seem to be the preferred mode of cortisol
metabolism in either Arctic charr or rainbow trout embryos. It is possible that unlike other steroids, for example T, which is suitable substrate for conjugation without prior transformation (Förlin and Haux, 1985; Andersson et al., 1985), cortisol undergoes extensive metabolism without conjugation (Truscott, 1979; Pottinger et al., 1992). Therefore, in embryos of the two salmonid species examined here, the main route of detoxification of cortisol appears to be via the formation of 11β-OHA and possibly tetrahydrocortisol/tetrahydrocortisone, rather than by conjugation.

REFERENCES:


Kime, D.E. (1978). The hepatic catabolism of cortisol in teleost fish-Adrenal origin of 11-


