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Inhibition of cortisol metabolism by 17α,20β-P: Mechanism mediating semelparity in salmon?

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ABSTRACT

In vitro experiments were conducted to test the hypothesis that 17α,20β-dihydroxy-4-prengnen-3-one (17,20-P) regulates cortisol metabolism in Pacific salmon. In both rainbow trout and coho salmon, cortisol metabolism was significantly higher in the kidney compared to the liver. The rainbow trout kidney converted cortisol primarily into an unidentified water-soluble metabolite with a molecular mass of 354. The coho salmon kidney converted cortisol primarily into cortisol-21-sulfate. High physiological concentrations of 17,20-P had no effect on cortisol metabolism by the rainbow trout kidney, but almost completely inhibited the production of cortisol-21-sulfate by the coho salmon kidney. This was accompanied by a coincident increase in the production several neutral cortisol metabolites, including cortisone. Cortisone was also found to inhibit renal sulfotransferase (SULT) activity suggesting that there could be a local positive feedback mechanism initiated by the rise in 17,20-P that quickly reduces SULT activity as follows: the pre-spawning rise in 17,20-P inhibits SULT, cortisol is metabolized to cortisone instead of cortisol-21-sulfate, cortisone further inhibits SULT, more cortisone is produced, and so on. If SULT normally acts as a gatekeeper enzyme to protect the cell from cortisol excess, this mechanism would rapidly remove enzymatic protection and expose tissues to high local concentrations of cortisol. In addition, the inhibition of peripheral cortisol metabolism by 17,20-P could increase circulating concentrations of the corticosteroid. These events could be a part of the mechanism that leads to the symptoms of cortisol excess associated with the post-spawning mortality of semelparous Pacific salmon.

1. Introduction

The die-off of Pacific salmon shortly after they spawn is one of the most dramatic but poorly understood phenomena in biology (Dickhoff 1989). Cortisol excess of unknown etiology mediates this programmed death by causing tissue degeneration, suppressing the immune system, and impairing various homeostatic mechanisms (Robertson and Wexler, 1960; reviewed by Dickhoff, 1989; Stein-Behrens and Sapolsky, 1992). Evidence that gonadectomy blocks the normal increase of cortisol in pre-spawning salmon suggests that a gonadal factor regulates cortisol excess (Fagerlund and Donaldson, 1970; Donaldson and Fagerlund, 1972). Given the temporal disassociation between elevated androgen and estrogen levels with pre-spawning cortisol excess, it is unlikely that these steroids are the responsible factors. The steroid 17α,20β-dihydroxy-4-prengnen-3-one (17,20-P) is produced by the gonads of salmonid fish in response to a pre-spawning rise in gonadotropin, and regulates final gamete maturation in both sexes (reviewed by Scott and Canario, 1987; Nagahama, 1987; Nagahama et al., 1993). In salmonids, blood levels of 17,20-P rise markedly prior to spawning, and in some species remain elevated for several days thereafter (Scott et al. 1983). Typically, peak 17,20-P levels are higher in females (~300 ng/ml) than males (10–100 ng/ml) (Ueda et al., 1984). Only very low concentrations of 17,20-P are required to stimulate final gamete maturation (100-fold less than peak plasma concentrations) in both sexes, and thus it has been postulated that 17,20-P plays other physiological roles at spawning (Scott and Canario 1987).

Given the close temporal relationship between the rise in 17,20-P and the death of spawning salmonids, we hypothesize that cortisol excess in Pacific salmon is regulated by the pre-spawning rise of 17,20-P. Specifically, we postulate the following: Under normal physiological conditions, even in the face of elevated stress-induced cortisol levels, peripheral targets are protected from cortisol excess by cortisol-metabolizing enzymes, which inactivate cortisol before it can bind to cellular receptors and initiate a biological response. At spawning, however, 17,20-P inhibits peripheral cortisol metabolism, and thus, cortisol receptors in various targets are exposed to high concentrations of cortisol only when 17,20-P levels

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are elevated at the time of spawning. The purpose of the present investigation was to use an *in vitro* tissue culture system to characterize cortisol-metabolizing activity in the liver and kidney of the semelparous coho salmon (*Oncorhynchus kitsuch*) and the iteroparous rainbow trout (*Oncorhynchus mykiss*), and investigate the effects of 17,20-P, and other sex steroids, on cortisol metabolism in these species. If our hypothesis regarding the role of cortisol-metabolizing enzymes in mediating post-spawning mortality is correct, we expected to observe differences in cortisol metabolism between these two species of Pacific salmon.

2. Materials and methods

2.1. Fish

Kamloops strain rainbow trout were obtained from commercial sources in Wisconsin. Coho salmon were obtained from the Lake Mills State Fish Hatchery, Lake Mills, Wisconsin. The fish were reared in 750-L flow-through tanks under ambient temperature conditions (11–16 °C) and a constant 16-h light/8-h dark photoperiod. The fish were fed Silver Cup trout feed (Murray, UT) once daily at approximately 1% of their body weight. Food was withheld 24 h prior to sacrifice. The experimental fish were mixed-sex and ranged in size from 80 to 263 g. All were sexually immature as evidenced by macroscopic examination of the gonads following sacrifice.

2.2. In vitro cultures

In vitro cultures of rainbow trout and coho salmon liver and kidney tissues were conducted using a modification of the method of Barry et al. (1997). In brief, three to five fish were sacrificed using a lethal dose of MS-222. The livers and kidneys were removed and pooled in a 50 ml polypropylene culture tube containing 20 ml of ice-cold physiological saline (Kobayashi et al., 1986). The tissues were minced with a Polytron homogenizer set at the lowest speed until the tissue was finely fragmented. The tissue fragments were washed for 30s by gentle vortexing in 10 ml of physiological saline, centrifuged at 2000*g*, and the supernatant withdrawn with a glass pipette. This washing procedure was repeated a total of four times using fresh saline. After the final wash, the saline was withdrawn and fresh culture medium (approximately 5× tissue vol.) was added to the tube. The tissue fragments were allowed to loosely settle to the bottom of the tube, and 50-μl aliquots of tissue were removed and added to individual 150 × 20-mm culture tubes. Tissue was transferred using a 1-ml pipette tip whose tip was cut off to increase the size of the orifice to ~4 mm. Culture medium and 10 μl of concentrated test solution(s) were then added to each well to a final incubation volume of 1 ml. Cultures were conducted under ambient air at 15 °C for 1–24 h with constant shaking in a temperature-controlled incubator. All treatments were carried out in duplicate or triplicate within each experiment, and each experiment was repeated at least twice with tissues from different pools of fish. To obtain sufficient cortisol metabolites for identification by LC–MS, some larger-scale (10-fold the conditions described above) incubations were conducted with rainbow trout and coho salmon kidney tissues.

2.3. Cortisol metabolism by the liver and kidney

Cortisol metabolism was analyzed by measuring the formation of ether- and water-soluble products after incubation of kidney and liver fragments of both species in the presence of [4-^{14}C]-cortisol (150 nM total cortisol concentration) for 1–24 h at 15 °C. [4-^{14}C]-Cortisol (53.5 mCi/mmol) was purchased from NEN-Perkin-Elmer (Boston, MA). Cultures were conducted in triplicate in pre-weighed (±0.1 mg), 150 × 20-mm test tubes. Controls were incubates without tissue. The effects of various steroid hormones on cortisol metabolism was evaluated by conducting the cultures in the presence of 17,20-P, estradiol-17β (E_2), testosterone (T), 11-ketotestosterone (11-KT), 17α,20α-dihydroxy-4-prengnen-3-one (17,20α-P), cortisol, cortisone, or the 11β-hydroxsteroid dehydrogenase inhibitor, 18β-glycyrrhetinic acid (GA). All steroids and GA were tested at 1.5×10^{-6} M except for 17,20-P, which was tested at various doses (detailed below). Steroids were obtained from Sigma Chemical Co. (St. Louis, MO) or Steraloids (Newport, RI). Concentrated steroid stock solutions were prepared in ethanol and subsequently diluted in physiological saline.

Neutral steroid metabolites were extracted from the medium three times with diethyl ether (5× vol.). The ether fractions were collected by rapidly freezing the aqueous layer in a dry ice/ethanol bath, pouring the ether into a clean 16 × 120-mm test tube, and dried under a stream of nitrogen gas at 40 °C. The products were separated using 20 × 20 cm, 60 Å, 19-channel, preadsorbent plates with fluorescent indicator (Whatman No. 4866 821, Clifton, NJ), and the solvent system chloroform:methanol (97:3). Radioactivity on the plates was imaged and quantified using an InstantImager (Perkin-Elmer, Wellesley, MA). Recovery of [^{14}C]-cortisol from the no tissue control incubations was used to estimate and correct for procedural loss. The radioactivity remaining in the culture medium after ether extraction was quantified by liquid scintillation counting. Cultures were standardized by dry tissue weight, which was determined by drying the test tubes in a vacuum oven overnight and weighing to the nearest 0.1 mg.

2.4. Characterization of cortisol metabolites

In both rainbow trout and coho salmon, cortisol was primarily converted into water-soluble metabolites (WSMs), which were characterized both by liquid chromatography–mass spectroscopy (LC–MS) and biochemically.

2.4.1. LC–MS

Total metabolites (neutral and conjugated) were extracted using Oasis C-18 cartridges (Waters, Inc., Milford, MA). Chromatography of cortisol and its neutral metabolites was performed according to Marwah et al. (2002) on C_{18} analytical columns (Zorbax-SB, 3.0 × 150 mm, 3.5 μm, Mac-Mod, and Novapack, 3.9 × 75 mm, 4.0 μm, Waters Inc., Milford, MA) protected with matching C_{18} guard columns at a flow rate of 0.4–0.8 ml/min and a temperature of 20–40 °C. The solvent system was acetonitrile/water with a linear gradient of 20–45% acetonitrile in 25 min, 94% at 32 min and back to 20% at 34 min, followed by a 10 min post-run time. Water-soluble conjugates were resolved on a C_{18} analytical column (Zorbax-SB, 3.0 × 150 mm, 3.5 μm) at a flow rate of 0.4 ml/min using acetonitrile–water and acetonitrile containing 3% acetic acid and 3% acetic acid gradients. The gradients started with 10% acetonitrile to reach 40% in 30 min, and 96% acetonitrile in 38 min and back to 10% at 40 min. Radioactivity was quantified using an inline radioactivity detector.

2.4.2. Biochemical characterization

Ether-extracted culture media was dried under a stream of nitrogen gas at 40 °C, and incubated for 18 h at 50 °C in 5 ml trifluoroacetic acid/ethyl acetate (1/100, v/v) to cleave glucuronic acid and/or sulfate groups from the steroid conjugates and release free steroids (Scott and Canario, 1992). The trifluoroacetic acid/ethyl acetate solution was dried under a stream of nitrogen at 40 °C. The free steroids were dissolved in methanol and separated and quantified by TLC as described above. Further analysis was conducted to determine if the WSMs were primarily steroid glucuronides or sulfates. Acetylation transforms water-soluble glucuronides