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Effect of glutamate and somatostatin-14 on basal and cAMP-stimulated steroidogenesis by rainbow trout (*Oncorhynchus mykiss*) ovarian follicles, in vitro

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Abstract

The effects of glutamate and somatostatin-14 (SRIF) on the in vitro basal and cAMP-stimulated steroid production of mid-vitellogenic rainbow trout (*Oncorhynchus mykiss*) ovarian follicles were investigated. cAMP-stimulation was achieved by the addition of the adenylyl cyclase activator, forskolin (FS), or a membrane permeate cAMP agonist, 8-bromo-cAMP (BA), to the incubation medium. Testosterone (T) and 17β-estradiol (E_2) secretion was measured using radioimmunoassay. Solid phase extraction (SPE) was used to measure the relative formation of unconjugated and conjugated steroids, and high performance liquid chromatography (HPLC) was used to examine the steroid metabolites formed from the metabolism of a tritium labelled precursor, pregnenolone (P_5). The accumulations of T and E_2 in the medium were suppressed in the presence of the glutamate agonists, *N*-methyl-D,L-aspartate (NMA) or L-glutamic acid (GA), and by the presence of SRIF. The suppression was evident for both basal and cAMP-stimulated steroidogenesis except for T concentrations of GA treatments following basal steroidogenesis, when there were no treatment effects. No significant effects of treatment on conjugated:unconjugated steroid ratios were found. For all treatments E_2 was the major end product steroid synthesized from P_5, and the steroid profiles were similar except for trace amounts of radiolabelled androgens in the medium following cAMP-stimulated steroidogenesis that were not present following basal steroidogenesis. The findings suggest that glutamate and SRIF reduce end point steroid production, possibly by reducing P_5 production. However, since the inhibitory affect was found for basal and cAMP-stimulated steroidogenesis, the response does not appear to be due to the inhibition of cAMP synthesis.

Keywords: Forskolin; 8-Bromo-cAMP; Testosterone; 17-βEstradiol; High performance liquid chromatography; Conjugated steroids; Unconjugated steroids; *N*-Methyl-D,L-aspartate; L-Glutamic acid; Somatostatin-141

1. Introduction

We recently reported that the glutamate agonist, *N*-methyl-D,L-aspartate (NMA) and L-glutamic acid (GA) exerted a dose-dependent suppression of the in vitro basal production of testosterone (T) and 17β-estradiol (E_2) by mid-vitellogenic stages of ovarian follicles of rainbow trout, *Oncorhynchus mykiss* (Leatherland et al., 2004). However, although T and E_2 secretion was reduced in the presence of glutamate, there was no effect of glutamate on either the rates of biotransformation of exogenous radioactively labelled pregnenolone (P_5), or the nature of the steroid metabolites formed. These observations suggested that glutamate reduces the synthesis of endogenous P_5, which is a major rate-limiting step in the steroidogenic cascade, possibly by affecting the synthesis of steroid acute regulatory (StAR) protein (Stocco, 2001). In mammals, it is known that the synthesis of StAR and other possible protein candidates is at least partly regulated by cAMP through its interaction with, and the phosphorylation of, a member of the cAMP response element binding (CREB)

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protein family (Manna et al., 2002). Thus, if glutamate suppresses steroid synthesis by suppressing cAMP expression (and thereby possibly reducing StAR expression) this response should be abolished when intracellular cAMP levels are experimentally enhanced. Thus, in the present study, we examined the action of NMA and GA on steroid production by ovarian follicles incubated in vitro in the presence of the adenylyl cyclase activator, forskolin (FS), and the membrane permeant cAMP agonist, 8-bromo-cAMP (BA).

The peptide, somatostatin-14 (SRIF) has also been implicated in the regulation of gonadal steroidogenesis in vertebrates. SRIF immunoreactivity has been demonstrated in the granulosal cells of the rat (McIntyre et al., 1992), Japanese quail (*Coturnix japonica*) (Mori et al., 1984a), and African lungfish (*Protopterus annectens*) (Masini et al., 1999), and has been measured by radioimmunoassay (RIA) in porcine ovarian tissues from which ovarian fluid had been removed (Mori et al., 1984b). SRIF is also present in human ovarian follicular fluid at higher concentrations than in serum (Holst et al., 1994). This might suggest a local production of the peptide by granulosal cells, although McIntyre et al. (1992) were unable to identify preprosomatostatin mRNA in rat ovaries. Whether SRIF is synthesized locally in mammalian ovary, perhaps at only some stages of maturation, or whether the neuropeptide is released from neural tissue within the ovary remains to be determined. In the ovary of African lungfishes, however, Masini et al. (1999) found evidence of the expression of genes encoding for SRIF precursor protein suggesting a local production of peptide.

In mammals, SRIF or SRIF analogues have been shown to modify gonadal and adrenal steroidogenesis (Hausdorff et al., 1989; Rajkumar et al., 1992; Andreani et al., 1995; Holst et al., 1995; Gerendai et al., 1996; Mimuro et al., 1998). In some instances this was associated with a suppression of cAMP production (Hausdorff et al., 1989; Rajkumar et al., 1992). However, not all studies found a consistent suppressive action of SRIF on gonadal steroidogenesis. For example, Mimuro et al. (1998) described a suppression of progesterone (P_4) secretion by human granulosal cells, in vitro, in the presence of a SRIF analogue alone, but P_4 secretion was enhanced when the SRIF analogue was applied in combination with gonadotropin (GtH). Similarly, Gerendai et al. (1996) reported a suppressive effect of SRIF on serum T concentrations, when it was administered intratesticularly to adult rats with two intact testes, but a stimulatory effect of SRIF in immature hemi-castrated rats. Taken together, the various studies in mammals suggest a complex modulatory role for SRIF in the local regulation of steroidogenesis.

The expression of genes encoding for SRIF and the presence of immunoreactive SRIF in the ovary of African lungfishes (Masini et al., 1999) raises the possibility that this peptide influences ovarian steroidogenesis in fish, as it appears to do in mammals. Indirect evidence for such an effect based on an inverse correlation in plasma SRIF and E_2 concentrations in rainbow trout sampled during the vitellogenic period (Holloway et al., 1999) is indicative of a possible modulatory action of the neuropeptide on ovarian steroid production. However, these data do not necessarily demonstrate a cause–effect relationship, and in a previous study using rainbow trout ovarian follicles at an early stage of vitellogenesis, we were unable to show any effects of SRIF on in vitro basal or GtH-stimulated steroidogenesis (Reddy et al., 1999). In subsequent trials using the same model to compare the response of follicles to several secretagogues at different stages of vitellogenesis, we began to find evidence of an effect of SRIF on basal steroidogenesis by thecal and granulosal cells at some maturational stages (data unpublished). Therefore, in the present study, we re-examine the possibility that SRIF affects basal steroidogenesis, and in light of the suggestion that the SRIF effect is mediated via a reduction in cAMP synthesis, we extended the study to determine if any actions of SRIF could be negated by experimentally elevating follicle cell cAMP.

For both parts of the study, we used RIAs to measure secretion rates of E_2, the main end-product steroid, and its intermediate precursor, T. We also used solid phase extraction (SPE) to examine the relative production of conjugated and unconjugated steroids produced from the metabolism of tritium-labelled P_5, and high performance liquid chromatography (HPLC) to separate and identify the conjugated and unconjugated steroids to determine if the post-P_5 production steroidogenic pathways were affected by the treatments.

2. Materials and methods

2.1. Animals

Six sexually mature rainbow trout (*O. mykiss*) (3+year class), two for each of the three trials, were obtained from the Alma Aquaculture Research Station, University of Guelph.

2.2. Chemicals

[7-^{3}H]Pregnenolone ([^{3}H]P_5) was purchased from New England Nuclear (NEN). Non-radioactive testosterone (T) and 17β-estradiol (E_2), Medium 199 (M199), bovine serum albumin, β-D-glucose, bovine β-glucuronidase, *N*-methyl-D,L-aspartate (NMA), and L-glutamic acid were purchased from Sigma (Sigma-Aldrich Canada, Oakville, ON, Canada). Forskolin (SKF38393) and 8-bromo-cAMP were purchased from Research Biochemicals, Natick, MA, USA).

2.3. Ovarian follicle incubations

Three separate trials (NMA, GA and SRIF) were run using follicles harvested from fish at the mid-vitellogenic