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Steroidogenic acute regulatory protein transcript abundance in the eel, *Anguilla australis*: Changes during the induced reproductive cycle and effects of follicle-stimulating hormone during previtellogenesis

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ABSTRACT

Steroidogenic acute regulatory protein (StAR) mRNA levels in the eel ovary were assayed by quantitative PCR and related to plasma steroid levels throughout oogenesis in order to shed light on the previously considered 'aberrant' prematurational increase in plasma levels of estradiol-17β (E2). Total ovarian StAR transcript abundance mirrored circulating levels of E2, but not of 11-ketotestosterone (11KT). The study was complemented by evaluation of in vitro effects of follicle-stimulating hormone (FSH) on ovarian StAR transcript abundance and on short-term ('acute') radiolabelled pregnenolone-supported steroid metabolism by ovarian fragments to understand how the production of steroids during previtellogenic oocyte growth is regulated. We observed a significant effect of FSH on StAR mRNA levels within 24 h of incubation, but these were no longer evident by 4 days of culture. Unexpectedly, FSH had no effect on substrate-supported steroidogenesis, as comparable yields of steroid products were detected using semi-quantitative HPLC and scintillation counting. We conclude that the eel ovarian follicle can respond to FSH from a very early stage of development (early oil droplet stage) by increasing StAR mRNA levels, but that there is no evidence for acute effects of FSH on bioactive steroid production downstream of cytochrome P450 side-chain cleavage. Furthermore, the prematurational increase in StAR mRNA in vivo is in keeping with general teleost models and is likely to be a 'normal' response to reaching advanced stages of development.

1. Introduction

Sex steroid hormones are textbook mediators of sex differentiation, reproduction, expression of secondary sexual characteristics and behaviour [1]. In vertebrates, sex steroids are produced primarily by specialized somatic cells in the gonads, i.e. granulosa and theca cells in the case of females [2]. These cells have the machinery that is required to modify cholesterol into a range of bio-active products through a complex series of oxidoreductase (including: 3β-hydroxysteroid dehydrogenase (3β-HSD), 17β-HSD and 11β-HSD) and mono-oxygenase (e.g. cytochrome P450 side-chain cleavage (P450scc), 17α-hydroxylase and aromatase) reactions. However, in order for these enzymes to generate sex steroids and their intermediates, it is vitally important that cholesterol is made available to the first enzyme, P450scc, in the steroidogenic cascade.

P450scc is encoded by the *CYP11A* gene in the nuclear genome, and following synthesis, is processed to find its destination on the inner mitochondrial membrane [3]. Cholesterol, which is virtually insoluble in water, somehow needs to move through the aqueous environment of the cell to the inner mitochondrial membrane in order for P450scc to initiate the series of bio-conversions needed for the production of bioactive steroids [4]. Existing evidence on intracellular availability and transport of cholesterol has recently been reviewed by Miller and Bose [2] and indicates that cholesterol used for steroidogenesis is most likely made available by non-vesicular transport on lipid–protein rafts. Cholesterol then needs to cross the mitochondrial membranes, which is facilitated by steroidogenic acute regulatory protein (StAR), augmenting the synthesis of steroid product 5- to 10-fold [5]. The

Abbreviations: StAR, steroidogenic acute regulatory protein; FSH, follicle-stimulating hormone; LH, luteinizing hormone; P450scc, cytochrome P450 side-chain cleavage; HSD, hydroxysteroid dehydrogenase; E2, estradiol-17β; T, testosterone; 11KT, 11-ketotestosterone; 17,20β-P4, 17,20β-dihydroxy-4-pregnen-3-one; 11-OHA, 11β-hydroxyandrostenedione; qPCR, quantitative polymerase chain reaction; HPLC, high pressure liquid chromatography; PV, previtellogenic; EV, early vitellogenic; MV, midvitellogenic; MN, migratory nucleus; SPH, salmon pituitary homogenate; Ef1a, elongation factor-1α.

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activity of this protein has been associated with its binding to the outer mitochondrial membrane [6]; there, StAR interacts with other proteins [2] to ultimately see to the thoroughfare of cholesterol to the inner mitochondrial membrane, considered a main rate-limiting step in steroidogenesis [7]. Cholesterol availability subsequently enables P450scc to cleave off a 6-carbon residue to yield pregnenolone, the 'first' steroid in the steroidogenic cascade. The importance of StAR in controlling steroidogenesis has been demonstrated in a range of studies and is perhaps best illustrated in human subjects with dys – or non-functional mutations in the *StAR* gene – in these subjects, cholesterol accumulates in both the adrenal and the gonads as it cannot make its way to P450scc in the mitochondria, and this often manifests as corticosteroid insufficiency and feminization of 46, XY foetuses [reviewed in 2].

Gonadotropic hormones, i.e. follicle-stimulating hormone (FSH) and luteinizing hormone (LH), are major known regulators of StAR expression in a range of vertebrate species, including the domestic hen [8], coho salmon, *Oncorhynchus kisutch* [9], and domestic pig [10]. The expression profile of StAR throughout oogenesis differs considerably between fish species. In the zebrafish, *Danio rerio*, StAR expression does not change during primary and vitellogenic growth, but does decrease significantly at maturation [11]. In rainbow trout and brook trout ovarian follicles, levels of StAR are relatively low for the greater part of vitellogenesis but increase dramatically around ovulation [12], mirroring the dramatic increase in circulating levels of the maturation inducing steroid, 17, 20β-dihydroxy-4-pregnen-3-one (17,20β-P4) [13].

Whilst the importance of sex steroids in fish reproduction is well established during periods of rapid gonadal growth and during final maturation, their involvement during previtellogenesis mostly remains obscure. Indeed, only a few studies on function of sex steroids during early oogenesis in vitro have recently appeared, and these studies were conducted on very few species, i.e. eel (*Anguilla* spp.), coho salmon and Atlantic cod, *Gadus morhua*. What we have learnt from these studies so far is that in salmon, estradiol-17β (E2) strongly stimulates cortical alveolus synthesis, whereas androgens increase oocyte size [14]. Similarly, in cod [15] and eel [16,17], androgens increased oocyte diameter. Furthermore, in the latter, compelling data [16,17] strongly point towards a role for androgens in lipid uptake. However, essentially nothing is known about the regulation of steroid synthesis in previtellogenic ovarian follicles.

To date, the inter-relationships between FSH, steroid hormone levels and StAR transcript abundance have really only been explored in coho salmon. In this species, FSH levels increase as previtellogenic oocyte growth advances in vivo, and the increase is associated with increases in levels of E2 and ovarian StAR mRNA [18]. It is reasonable to consider the increase in FSH levels to be at least partly responsible for the increase in StAR message, given that FSH could increase StAR transcript abundance in vitro [9].

In the eel, circulating concentrations of steroid hormones are low, but detectable, during previtellogenesis [19], yet the factors that control the synthesis of these steroids are not known. Likewise, the mediators responsible for the increase in levels of both E2 and 11-ketotestosterone (11KT) at the migratory nucleus stage in artificially maturing eels, and that have been dubbed 'artefactual' [20] and possibly impact on oocyte quality, have not been fully elucidated. To address these issues, we (i) quantified StAR mRNA levels throughout reproductive development in wild or artificially maturing shortfinned eels (*Anguilla australis*), and (ii) subsequently focused on effects of FSH on StAR transcript abundance and pregnenolone-supported steroidogenesis by previtellogenic eel ovarian tissues incubated in vitro.

2. Materials and methods

2.1. Experimental procedures

2.1.1. Plasma steroid levels and ovarian StAR mRNA levels in previtellogenic and early vitellogenic eels captured from the wild

Eight previtellogenic (PV) female shortfinned eels were captured during early summer and eight early vitellogenic (EV) females during early autumn using fyke nets set overnight in Lake Ellesmere, South Island, New Zealand [19,21]. Eels were euthanized in benzocaine overdose and blood and ovarian tissues collected for steroid assay (Section 2.2) and analysis by qPCR (Section 2.3). Some aspects of the ovarian physiology of these same fish (lipoprotein lipase [21] and growth differentiation factor-9 [22]) have been published previously.

2.1.2. Plasma steroid levels and ovarian StAR mRNA levels in artificially maturing eels

Wild-caught EV eels were purchased from a commercial eel processor (Gould Aquafarms, Leeston, New Zealand) and acclimated to laboratory conditions during a 2-week period. As oogenesis in captive eels does not proceed beyond this stage, fish were induced to mature artificially using established protocols; these have previously been described in detail for the same animals [21,22]. Briefly, fish were injected intramuscularly once every 2 weeks with 10 mg/kg of salmon pituitary homogenates (SPH). Control fish were injected with equivalent volumes of eel ringer [24] only. At most sampling points (Time 0, 2, 4 and 12 weeks), three SPH and three control fish ($n = 2$ at 12 weeks) were euthanized and blood and ovarian tissues collected for measurement of plasma steroid hormone levels (Section 2.2) and quantification of StAR mRNA levels (Section 2.3).

2.1.3. Regulation of StAR mRNA levels by gonadotropins in vitro

PV female eels (600–900 g) were sourced through Aquahaven Ltd., Leeston, New Zealand. Eels were chilled, transported by courier in polystyrene boxes and placed in tanks with recirculating fresh water on arrival. They were held under these conditions, whilst fasting, for up to 3 days until required for in vitro assay. On days of culture, eels were netted from the tanks, euthanized in anaesthetic overdose and drained of blood by removal of the tail. Fish were subsequently surface-sterilized in an ethanol bath and dissected in a sterile hood before isolation of ovarian tissue. Ovarian tissue was incubated in vitro, as outlined in detail below, and subsequently retrieved, snap-frozen in liquid nitrogen and stored at −80 °C until assay for StAR mRNA levels by qPCR (Section 2.3).

Trial I: Ovarian explants containing perinucleolar or early oil droplet stage-oocytes (50–100 μm in diameter) from 6 female PV eels were assigned to wells that did or did not contain 100 ng/mL recombinant eel FSH or LH. Recombinant gonadotropins, kindly gifted by Dr Yukinori Kazeto (National Research Institute of Aquaculture, Mie, Japan), had been expressed in *Drosophila* S2 cells and purified, as described previously [23]. Cultures were carried out at 15 °C for either 24 h or for 4 days in Leibovitz-15 media containing amino acid supplements, 0.5% bovine serum albumin, 10 mM Hepes, bovine insulin (1 mg/L), as outlined in Miura et al. [24] and Lokman et al. [16]. Levels of antibiotics were purposefully kept low (1 mg/L streptomycin; 1000 U/L penicillin) to minimize potential suboptimal effects. All incubations were done with submerged fragments and under conditions of continuous shaking.

Trial II: Around 40 mg of ovarian tissue was retrieved from each of 6 PV eels whose ovaries were in the perinucleolus stage (40–60 μm in diameter). Tissue was finely cut and subsequently divided between 4 equal portions by eye; two portions were used for incubation in eel ringer [24] with or without 10 ng/mL recombinant eel FSH at 15 °C for 8 h on a shaking platform. The remaining