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Changes in localization of cytochrome P450 cholesterol side-chain cleavage (P450scc) in Japanese eel testis and ovary during gonadal development

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Abstract

In this study, we generated and characterized a polyclonal antiserum against eel P450 cholesterol side-chain cleavage (P450scc) using a recombinant protein as the antigen. We examined the localization and abundance of P450scc by immunohistochemistry in Japanese eel testes and ovaries during artificially induced gonadal development. P450scc mRNA localization was also examined by in situ hybridization. In male eels, testicular development was induced by a single injection of human chorionic gonadotropin (HCG). In females, ovarian development was induced by weekly injections of salmon pituitary homogenate (SPH). Before HCG injection, the testis contained germ cells that were primarily type A spermatogonia. Additionally, several clusters of immunoreactive cells for P450scc were localized in the interstitial Leydig cells, but no P450scc mRNA signals were detected. This suggests that P450scc is either a relatively stable protein or it is produced by a mRNA that is present at too low a level to detect. Shortly after a single injection of HCG, expression of P450scc mRNA was stimulated and the number of immunoreactive clusters and their staining intensity were both increased. P450scc mRNA fell to an undetectable level 3 days after hormonal stimulation. Although the P450scc protein also decreased at the same time as the mRNA, it remained at a detectable level throughout this period. P450scc mRNA, but not the P450scc protein, was also detected in the spermatids and spermatozoa. The biological significance of P450scc mRNA expression at this stage is unknown. Prior to experimentation, the ovary contained oocytes that were developed to the oil-droplet stage, with several clusters of immunoreactive cells localized in the thecal layer and ovigerous lamella epithelium. Expression of P450scc mRNA was also stimulated by SPH injections in the ovary. In contrast to the testis, P450scc mRNA was continuously detected in the thecal cell layer throughout artificially induced maturation, possibly due to a repeated stimulus by the SPH injection every week. Clusters of immunoreactive cells in the thecal cell layer increased in number as ovarian development progressed. This increase in P450scc mRNA and protein may explain, at least in part, the increase in serum steroid hormones in female eels. The P450scc antiserum clearly immunostained interrenal steroidogenic cells in the head kidney of not only eel but also goldfish, indicating that this antibody could also be used in other teleost species.

Keywords: Japanese eel; *Anguilla japonica*; Goldfish; Cytochrome P450 cholesterol side-chain cleavage; cyp11al; Localization; Immunohistochemistry; In situ hybridization; Steroidogenesis; Ovary; Testis; Interrenal

1. Introduction

Gonadal sexual maturation is controlled directly by steroid hormones that are produced in the gonad. Steroid hormones are synthesized from cholesterol and the

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conversion of cholesterol to pregnenolone is the first and rate-limiting step for the production of these hormones. This conversion is catalyzed by the cytochrome P450 cholesterol side-chain cleavage enzyme (P450scc, CYP11A1), which is located on the matrix side of the inner mitochondrial membrane (Miller, 1988). Therefore, expression of P450scc in steroidogenic tissues is of fundamental importance for determining the levels of production of steroid hormones.

Japanese eels (*Anguilla japonica*) have immature gonads that do not achieve sexual maturation unless the eels are given injections of gonadotropic preparations. A single injection of human chorionic gonadotropin (HCG) induces spermatogenesis and spermiogenesis by stimulating production of 11-ketotestosterone in the testis (Miura et al., 1991a,b). In the female, weekly injections of salmon pituitary homogenate (SPH) induce the vitellogenic growth of eel oocytes. These injections induce maturation of eel oocytes to the migratory nucleus stage. However, the hormonal control of gonadal development in the eel during induced maturation is not understood. To provide a greater understanding of the regulation of steroid hormone production in the eel gonad during artificial maturation, we have isolated cDNAs encoding steroidogenic enzymes, such as P450c17 (Kazeto et al., 2000a), P450arom (Ijiri et al., 2003), 3β-HSD (Kazeto et al., 2003), 17β-HSD (Kazeto et al., 2000b), and P450scc (Kazeto et al., in preparation). In the female eel, changes in the steroidogenic ability of ovarian follicles (Ijiri et al., 1995) and mRNA levels in ovaries during artificial maturation have been quantitatively analyzed (Matsubara et al., 2003). These studies demonstrated that the fundamental capacity for steroid hormone production increased following induction of ovarian development by SPH injections. They also suggested a correlated change in the levels of P450scc mRNA was present (Adachi et al., 2003). These finding indicate that P450scc has an important role in regulating the capacity for steroid hormone production. However, the expression pattern and the localization of the P450scc protein have not yet been investigated. On the other hand, interrenal tissue is also well known as a steroidogenic organ that produces corticoid. In teleost including the eel, however, interrenal tissue is not present as an independent organ. Interrenal cells are localized towards the anterior portion of the kidney, which is generally termed the head kidney. In addition, localization of the interrenal cells that express P450scc in the head kidney has not been demonstrated. To investigate these issues, we generated an antibody against recombinant eel P450scc protein and used this antibody to investigate changes in the localization of P450scc. Additionally, we also examined the distribution of P450scc mRNA in the eel gonad during induced gonadal development.

2. Materials and methods

2.1. Experimental animals and induction of sexual maturation

Glass eels from a commercial eel supplier were feminized by feeding them estradiol-17β (10 mg/kg pellet) for 1 month. They were then cultivated for an additional two and a half years until used for experimentation (Ijiri et al., 1998). The eels were gradually acclimatized to seawater 2 weeks before the beginning of the induction of sexual maturation. Prior to induction of sexual maturation, most eels have an ovary whose oocytes are developed to the oil-droplet stage. Ovarian vitellogenic growth was initiated by weekly injection of salmon pituitary homogenate (SPH) suspended in eel Ringer solution (NaCl 3.0 mM, KCl 3.0 mM, $MgCl_2$ 3.5 mM, $CaCl_2$ 5.0 mM, and Hepes 10 mM, pH 7.4) at a dose of 40 mg/kg body weight. On average, after 11 weeks of treatment, approximately 70% of the eels were induced to enter the final maturational stage and contained oocytes in the migratory nucleus stage (Ijiri et al., 1998).

Male eels, that are 1-year old, were purchased from a commercial eel supplier and were acclimatized to seawater in the same manner as above for females. The most mature germ cells present in the testes of these eels were type A spermatogonia. Sexual maturation was induced by a single injection of human chorionic gonadotropin (HCG; 5 IU/g BW), and the first spermatozoa were observed 18 days later (Miura et al., 1991a). Female and male eels were sampled before and throughout the experimental period. Small pieces of ovary and testes were taken for analysis.

2.2. Synthesis of recombinant eel P450scc protein and production of a polyclonal antiserum

A cDNA encoding the open reading frame of the Japanese eel P450scc sequence from amino acid residues 271–521 (GenBank Accession No. "AY654741") was ligated into the pQE-30 vector (Qiagen, Chatsworth, CA) with a six-histidine residue tag at the amino-terminus. The recombinant protein was purified on nickel–nitrilotriacetic acid resin (Qiagen) according to the manufacturer's protocol. A male rabbit was immunized with purified recombinant protein emulsified in Freund's complete adjuvant (0.5 mg first injection). After 2 weeks, the rabbit was then immunized weekly with the same material but at a lower dose (0.2 mg per injection). One month after the first injection, blood was collected every 2 days for 3 weeks and immunoreactivity checked on each occasion. During the 3 weeks blood was collected, the rabbit was immunized continuously with the lower injection dose. Serum was purified by passing through a diethylaminoethyl (DEAE) cellulose column; the fraction that