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論文内容の要旨

Estradiol-17β, a major estrogen, is essential for normal ovarian development in vertebrates. In fishes, as in other oviparous vertebrates, estradiol-17β is thought to be the main vitellogenic estrogen and to stimulate hepatic production of vitellogenic, the precursor of egg proteins. Plasma levels of estradiol-17β are high during active vitellogenic growth of oocytes, but low as the oocytes approach maturity. Likewise, the ability of ovarian follicles to produce estrogens is high in follicles during active vitellogenesis and low in those collected during postvitellogenesis and final oocyte maturation. Another important role of estrogens in fish female reproduction is their involvement in ovarian differentiation.

The cytochrome P450 aromatase is an important steroid-metabolizing enzyme responsible for the conversion of androgens to estrogens. The enzyme localizes in the membranes of the agranular endoplasmic reticulum of several steroidogenic cells including the ovary and testis. Other steroidogenic enzymes required for estradiol-17β production are cholesterol side-chain cleavage(P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), cytochrome P450 17α-hydroxylase/C17,20-lyase(P450c17), and 17β-hydroxysteroid dehydrogenase (17β-HSD).

Tilapia (*Oreochromis niloticus*) were reared under natural light in laboratory glass aquaria with aerated water at 24℃ until use. Tilapia ovaries contain a large number of ovarian follicles at different stages of development. In the Chapter I, he isolated, sequenced, and inserted a cDNA encoding tilapia ovarian P450arom into a eukaryotic expression vector. This vector system was used to transfect non-steroidogenic COS-7 cells. Furthermore, he developed a polyclonal antibody against tilapia P450arom. These tilapia P450arom cDNA and antibody were used to compare, for the first time in any fish species, the contents of P450arom mRNA and protein with enzyme activity in tilapia ovarian follicles during oogenesis.

A cDNA clone encoding the complete tilapia P450arom was isolated from an ovarian follicle cDNA library. The deduced amino acid sequence (522 amino acid residues) had 72.2% and 59.5% homology with rainbow trout and catfish P-450 arom, respectively, and about 50% homology with mammalian and avian P-450arom. Expression of this cDNA in COS-7 cells produced a protein that converted exogenous testosterone to estrogens. Northern blots using a tilapia P-450arom cDNA fragment and Western blots using an antiserum against a tilapia P-450arom polypeptide fragment revealed a single P-450arom mRNA (2.6 kb) and a single protein (59 kDa) in tilapia ovarian tissue, respectively. These analyses also revealed that the levels of both P-450arom mRNA and protein were low in early

vitellogenic follicles (0.8-1 mm in diameter), increased in midvitellogenic follicles (1.5-1.8 mm), and declined to non-detectable levels in post-vitellogenic follicles (2.0-2.1 mm). Changes in the ability of follicles to convert exogenous testosterone to estrogens (aromatase activity) were similar to those of P-450arom mRNA and protein. These observations indicated that the capacity of tilapia ovarian follicles to synthesize estradiol-17β is closely related to the contents of P-450arom mRNA and protein within them.

In the Chapter II, he investigated the immunocytochemical localization of four kinds of steroidogenic enzymes (P450scc, 3β-HSD, P450c17, and P450arom) in the tilapia ovary and testis throughout the entire process of gonadal differentiation, development and maturation. In addition to the P450arom antibody described above, polychronal antibodies were raised against the C-terminal peptides of rainbow trout P450scc, 3β-HSD, and P450c17. Immunoreaction for four steroidogenic enzymes initially appeared in gonads collected at 20 days after hatching (prior to sex differentiation). In gonads of tilapia fry at 23-26 days after hatching, ovarian differentiation became morphologically evident by the differentiation of stromal aggregations in the proximal and distal region of the gonad on the side facing the lateral wall. This represents the initial formation of the ovarian cavity. At the same time as ovarian differentiation, a few large cells appeared initially in the vicinity of blood vessels. These cells were intensely stained with all of the antibodies tested in this study. Thereafter, by 30-50 days after hatching, these immunoreactive cells increased gradually in number in the area enclosing the blood vessels of ovaries.

By 70-80 days after hatching, the number of immunoreactive in the area near blood vessel was increased, and the capillaries spread among the developing perinucleolar stage oocytes, and into the ovarian tunica. Clusters of immunoreactive cells also migrated into the interstitial region and into the tunica along with these capillaries. In the ovary 100 days after hatching, some immunoreactive cells could be found in the thecal layer enclosing vitellogenic oocytes. Moreover, masses of the cells could now be observed infiltrating the thecal layer of the oocyte. During these periods, there were no P450arom immunostaing cells in the testes. Furthermore, immunoreaction for P450scc, 3β-HSD, and P450c17 was also found to be rare.

In vitellogenic follicles, in addition to the P450arom immunolocalization in thecal cell layer, P450arom also was present in granulosa cells. The P450arom immunoreaction increased as vitellogenesis proceeded. Immunolocalization of P450scc, 3β-HSD, and P450c17 were still restricted to the thecal cell layer. Towards the end of vitellogenesis, the P450arom immunoreaction in both thecal and granulosa cell layers drastically declined.

In contrast, immunoreaction for other three enzymes were still very strong in the thecal layer. These results suggest that a distinct shift in steroidogenesis from estradiol-17β to maturation-inducing hormone (probably 17α, 20β-hydroxy-4-pregnen-3-one or its closely related steroid) occurs in ovarian follicle cells immediately prior to oocyte maturation. This suggestion was also confirmed by the immunocytochemical observations on young postovulatory follicles which had very strong immunoreaction for P450scc, 3β-HSD, and P450c17, but not P450arom.

A novel finding presented in this study is that all of the steroidogenic enzyme proteins, except for 17β-HSD, required for the production of estradiol-17β were already present in gonads several days prior to ovarian differentiation. This finding strongly suggests that estradiol-17β is responsible for ovarian differentiation in tilapia.

審査結果の要旨

エストラジオール-17βは、コレステロールより5種のステロイド代謝酵素（P450scc、3β-HSD、P450c17、17β-HSD、P450arom）の作用により合成される雌性ホルモンであり、卵巣の性分化や卵胞の成長、成熟に重要な役割を果たすと考えられている。しかし、このエストラジオール-17βが卵巣で時期・細胞特異的に合成される機構は不明な点が多い。本研究では、硬骨魚類のティラピアを用いて、（1）エストラジオール-17βの合成に関して特に重要な働きをするP450aromのcDNAのクローニングと発現、及び（2）卵巣の分化、成長、成熟時における4種のステロイド代謝酵素の局在部位をそれぞれの特異抗体を用いた免疫組織化学法で解析した。

クローニングしたcDNAをCOS-7細胞に発現させ、テストステロンを添加するとエストラジオール-17βが合成されたことから、このクローンがティラピアP450aromをコードすることを確認した。cDNAから予想されるティラピアP450aromは522個のアミノ酸からなり、哺乳類や鳥類とは約50%、他の魚類とは60-70%のホモロジーを示した。さらに、合成オリゴペプチドを抗原としてティラピアP450aromの特異的抗体を作成した。これらのcDNAと抗体、及び細胞培養法を用いて、卵形成の早期、中期（盛期）、終期の卵胞におけるP450aromのmRNA、蛋白、活性を調べた。その結果、これら三者は完全に一致し、卵形成早期にやや高く、中期（盛期）に最高となり、終期には急減した。このことより、ティラピア卵巣におけるエストラジオール-17βの時期特異的合成は、P450arom遺伝子の転写、及びその翻訳により制御されていると考えられる。

前述したP450arom抗体に加えて、P450scc、3β-HSD、P450c17の抗体を作成し、ティラピアの生殖腺の分化時、及び卵胞の成長と成熟時におけるこれらステロイド代謝酵素の局在部位を調べた。その結果、エストラジオール-17βの合成に必要な4種のステロイド代謝酵素は卵巣分化が起こる直前の孵化後20日の卵巣の血管周辺の大型細胞（ストローマ細胞）に認められた。一方、卵形成期の卵胞におけるエストラジオール-17βの合成は莢膜細胞と顆粒膜細胞にみられが、なかでも顆粒膜細胞は卵形成期に限りP450aromを有し、この時期におけるエストラジオール-17βの主要な合成部位と考えられた。

これらの結果は、脊椎動物の卵巣におけるエストラジオール-17βの時期・細胞特異的合成機構に関する研究に重要な知見を与え、学位論文として充分な内容を含むものであると判断された。

試験結果については、学位論文として提出された研究成果について口頭発表させた後、審査委員が論文内容について諮問した。さらに、申請者の関連研究分野の一般知識およびその背景となる基礎的知識についても口頭諮門により審査した。これらの諮門に対する申請者の応答はいずれも適切であった。また、提出された学位論文は英文で書かれており、英語の能力についても適正であると考えられた。これらの結果をもとに、審査委員会は申請者の持つ研究能力および学力は学位取得に値するものと判断した。