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学位論文題目　Carbonyl Reductase-Like 20β-Hydroxysteroid Dehydrogenases in the Ovarian Follicle of a Teleost Fish, the Rainbow Trout (*Oncorhynchus mykiss*):Their Structures and Reproductive Functions in Oocyte Maturation

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論文内容の要旨

Meiotic maturation of the oocyte (oocyte maturation) occurs prior to ovulation and is a prerequisite for successful fertilization. Oocyte maturation has been studied in various animal species, but its endocrine regulation has been investigated most extensively in fishes. It is now established that three major mediators, gonadotropin (GTH), in particular luteinizing hormone (LH, GTH-II in fish), maturation-inducing hormone (MIH), and maturation-promoting factor (MPF) are involved in the induction of oocyte maturation. 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP) was identified as the MIH of several teleost fishes including salmonid fishes. The interaction of two ovarian follicle cell layers, the thecal and granulosa cell layers, is required for the synthesis of 17α,20β-DP. The thecal layer produces 17α-hydroxyprogesterone (17α-HP) that is converted to 17α,20β-DP in granulosa cells by the action of 20β-hydroxysteroid dehydrogenase (20β-HSD). In this study, as a first step to investigate the molecular mechanism of GTH-regulated MIH production by fish ovarian follicles, 20β-HSD cDNAs were cloned from postvitellogenic ovarian follicles of a salmonid fish, the rainbow trout, *Oncorhynchus mykiss* (Chapter I). A series of studies employing site-directed mutagenesis were conducted to characterize the coenzyme NADPH-binding of rainbow trout 20β-HSD (Chapter II). Finally, dynamic changes in mRNA levels of 20β-HSD in ovarian follicles during oocyte growth and maturation were determined and compared with those of 3β-hydroxysteroid dehydrogenase (3β-HSD) and cytochrome P450 aromatase (P450arom) with a particular emphasis on GTH regulation of 20β-HSD gene expression in ovarian granulosa cells (Chapter III).

Two closely related 20β-HSD cDNAs were cloned from rainbow trout ovarian follicles. Both cDNAs belong to the short-chain dehydrogenase/reductase (SDR) family, with approximately 60% homology to mammalian carbonyl reductases (CRs) and termed rainbow trout CR/20β-HSD cDNA type A and type B. Type A and type B share high homology of 99% at the nucleotide level and 98.7% at the amino acid level within their open reading frames. Using the type B cDNA fragment, two clones, termed CR/20β-HSD genes I and II, were obtained from the λDASH genomic library of rainbow trout. The sequences of the cDNAs deduced from CR/20β-HSD genes I and II matched with CR/20β-HSD cDNA type A and type B, respectively. Genomic DNA analysis showed that the two CR/20β-HSD cDNAs are derived from two different genes. Both rainbow trout CR/20β-HSD genes consist of four exons. The structural organization of the genes is very similar, with the introns interrupting the genes at the same locations. Genes I and II sequences share 53% identity in the 5' upstream regions up to −700 bp from the initiation site. Comparison of the amino acid sequences of rainbow trout CR/20β-HSD with mammalian CRs reveals that the Rossmann fold, GlyXXXGlyXGly, which is the co-factor binding site, is well conserved.

Although the functional significance of the two genes remains unresolved, these results clearly demonstrate the presence of two distinct CR/20β-HSD transcripts in the trout ovary.

Recombinant CR/20β-HSD proteins produced in *E. coli* were incubated with [^{3}H]-17α-HP in the presence of NADPH and resulting steroids were separated by thin layer chromatograpy. A band which comigrated with authentic 17α,20β-DP was obtained from incubations of CR/20β-HSD type A, indicating that CR/20β-HSD type A cDNA encodes a protein with 20β-HSD activity. Recombinant CR/20β-HSD type A also catalyzed the reduction of a number of characteristic substrates of CR with efficient catalyzation of the reduction of quinones, or menadione, whereas prostaglandins and steroids including 17α-HP were reduced at lower rates. Recombinant protein derived from CR/20β-HSD type B cDNA did not recognize any of these substrates.

Northern blot analysis demonstrated that trout CR/20β-HSDs are expressed in various tissues, of greatest abundance in liver and gill, followed by brain, ovary and testis, adipose tissue, and kidney. Results of RT-PCR employing primers specific for CR/20β-HSD cDNA A and B was consistent with that of Northern blot, showing that CR20β-HSD cDNAs type A and type B were expressed in most tissues, but only the expression of CR/20β-HSD type A could be detected in liver.

As described above, trout CR/20β-HSD type B does not possess either CR or 20β-HSD activity. Among their three distinct amino acids, Ile15 in trout CR/20β-HSD type A was found to be well converted among many CRs. It is substituted to Thr in CR/20β-HSD type B. To test if this mutation is responsible for abolishing the stability of enzyme and co-enzyme complex, several mutations of CR/20β-HSD were created by site-directed mutagenesis and the enzyme activity of their recombinants expressed in *E. coli* were determined. Mutation of I15T in type A abolished enzyme activity with different substrates, and mutation of T15I in type B resulted in the acquisition of enzyme activity. Furthermore, by fluorescence titration assay, it was found that I15 is crucial in permitting the formation of the CR/20β-HSD – co-enzyme (NADPH) complex. Mutation of A/I15T abolished the ability of the trout CR/20β-HSD type A enzyme to bind with NADPH and further caused the enzyme to lose its activity. Taken together, these data provide evidence that Ile is critical in the GlyXXXGlyXGly co-factor binding structure.

Northern blotting revealed that 3β-HSD mRNA levels steadily increased during the vitellogenic stage, was further enhanced at oocyte maturation stage, and kept in high levels in postovulatory follicles. P450arom mRNA levels were high during active vitellogenesis, but rapidly decreased before oocyte maturation with undetectable levels at oocyte maturation and in postovulatory follicles. In contrast to the change in P450arom gene expression, CR/20β-HSD mRNA levels were low in follicles during active vitellogenesis, but markedly increased during oocyte maturation and remained

high during the postovulatory stage.

A major finding in this study is that CR/20β-HSD gene expression was markedly enhanced when granulosa cells isolated from postvitellogenic follicles were incubated with GTH. Furthermore, there was an increase in CR/20β-HSD enzyme content after GTH stimulation. It is also of importance to note that CR/20β-HSD type A gene, but not type B gene, is inducible in granulosa cells by GTH stimulation. Since CR/20β-HSD type A exhibits 20β-HSD activity, the enhanced expression of CR/20β-HSD type A gene increases the conversion of 17α-HP to 17α,20β-DP. These data provide information on dynamic molecular changes during oocyte growth and maturation, and also demonstrate that the expression of 20β-HSD gene is controlled by GTH. The effects of GTH on the promoter regions of CR/20β-HSD genes will provide further understanding of steroid and protein hormone modulation of steroidogenic enzymes.

論文の審査結果の要旨

卵成熟は、卵に減数分裂を促し、受精能を獲得させる重要な過程である。卵成熟は、さまざまな動物を用いて研究されてきたが、内分泌調節については主に魚類を用いた詳細な研究が進められ、哺乳動物の黄体形成ホルモン（LH）に相同な GTHII、卵成熟誘導因子（maturation-inducing hormone, MIH)、卵成熟促進因子（maturation-promoting factor, MPF）が、卵成熟の開始と進行に重要な役割を演じていることが知られている。とくに、サケ科を含む魚類では、17α,20β-dihydroxyl-4-pregnen-3-one (17α,20β-DP) が MIH であることが明らかにされている。17α,20β-DP が合成されるためには、卵巣濾胞細胞層の夾膜細胞と顆粒膜細胞との相互作用が必要であるといわれており、夾膜細胞で合成された 17α-hydroxyprogesterone (17α-HP) が顆粒膜細胞で 20β-hydroxysteroid dehydrogenese (20β-HSD)の働きによって、17α,20β-DP に変換されることがわかっている。申請者の関　桂君は、魚類卵胞での GTH による 17α,20β-DP の産生調節機構を明らかにするために、ニジマス（*Oncorhynchus mykiss*）から 17α,20β-HSD cDNA をクローニングし、その遺伝子構造や構造活性相関、GTH による発現調節機構を解析した。

ニジマス濾胞細胞の cDNA ライブラリーより、17α,20β-HSD type A および type B、２種の cDNA を単離し、両者のアミノ酸配列が極めてよく似ていることを示した。また、染色体遺伝子ライブラリーをスクリーニングすることにより、type A および type B それぞれの独立した遺伝子が存在することを示した。また、それらの上流域の構造を含めた遺伝子構造を明らかにし、両者のイントロン/エキソン構成は保存されているものの、type B には type A に比べて長いイントロンが挿入されていることなどを示した。17α,20β-HSD type A と type B の３つのアミノ酸の違いのうち、ひとつは酵素活性に必須の補酵素結合サイト中の Ile から Thr へのアミノ酸置換であった。両者タンパク質を大腸菌で発現させることにより、type A は 20β-HSD を有するが、type B にはその活性がないことを示した。さらに、type B の補酵素結合サイトに変異を導入し、type A 同様に Ile へ置換することによって、NADPH の結合能とともに、20β-HSD 活性を獲得することを明らかにした。

また、20β-HSD 遺伝子の発現調節機構を明らかにするために、顆粒膜細胞を用いた培養実験を行い、GTH によって 20β-HSD 活性の増加が認められること、その際に type A だけが発現誘導されていることを示した。本研究は、魚類における 20β-HSD の遺伝子構造を明らかにしたばかりでなく、その構造活性相関および発現調節機構にまでおよぶ重要な研究であり、魚類卵ばかりでなく卵成熟機構一般の研究に大きく貢献すると判断され、審査委員会は本論文に対して合格の判定を下した。

学位論文として提出された研究結果については、口頭発表させた後、審査委員が論文内容について試問した。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、口頭試問により審査した。これらの試問に対する申請者の応答は、いずれも的確であった。また、提出された学位論文は英語で書かれており、英語の能力についても適正であると考えられた。さらに、論文の一部は国際誌である Biochem. Biophys. Res. Commun.に受理されている。これらの結果をもとに、審査委員会は申請者のもつ研究能力および学力は、学位取得に値するものと判定した。