Detection of Tissue-specific Expression of Porcine Cytochrome P450 Aromatase Genes by Use of Denaturing High Performance Liquid Chromatography(DHPLC) Technique

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DHPLC 기술을 이용한 돼지 Cytochrome P450 Aromatase 유전자의 조직 – 특이적 발현양상 관찰

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적 요

본 연구는 돼지에서 특이적으로 만들어지는 것으로 알려진 19-nortestosterone(nandrolone) 및 여성호르몬(estrogen)의 합성에 관여하는 효소인 Cytochrome P450 aromatase에 대한 유전자의 발현 양상을 밝혀내기 위해 실시되었다. RT-PCR과 최근에 개발된 DHPLC(Denature High Performance Liquid Chromatography 또는 WAVE라고 함) 분석 장치를 이용하여 정소와 난소에서 어떤 isoform의 aromatase 유전자가 발현되는지에 관해 조사하였다. 이러한 방법을 통해 같은 양의 RNA 중에 존재하는 정소내 aromatase mRNA가 난소보다 상대적으로 많이 존재한다는 것이 밝혀졌으며, 이는 돼지의 경우 수컷이 암컷 보다 혈중 여성호르몬이 더 높게 나타난다는 이전의 연구 발표가 돼지에서 여성호르몬을 만드는 aromatase 유전자가 난소에 비해 정소에서 더 많이 만들어지기 때문이라는 사실을 입증하였다. 또한, 정소와 난소에서 발현되는 aromatase 유전자를 PCR를 이용하여 증폭한 후 DHPLC를 이용하여 분석한 결과 type Ⅱ Ⅲ ㅏ 다르다는 것을 확인하였다. RT-PCR에 의해 증폭된 aromatase DNA 단편을 plasmid vector에 cloning한 후에 그 염기서열을 분석한 결과, 정소 및 난소에서 발현되는 aromatase는 모두 type I(난소형)으로 밝혀졌다. 이는 어떻게 정소와 난소의 두 다른 조직에서 같은 aromatase 효소로부터 다른 steroid가 만들어 질 수 있는지에 대한 새로운 의문을 제시하는 연구결과이며, 현재 추가적인 연구가 진행 중이다. 또한, DHPLC 기술을 활용하여 염기서열이 매우 유사한 isoform 유전자들의 발현을 관찰할 수 있다는 사실이 증명되었다.

(**주요어** : Aromatase, DHPLC, Anabolic steroid, Testosterone, 19-Nortestosterone, Estrogen)

Ⅰ INTRODUCTION

Estrogens play very critical roles in reproduction and body growth in most of the mammalian species including the pig. Cytochrome P450 aromatase(simply, aromatase) is one of the steroidogenic enzymes converting androgens(male sex hormone; mainly, androstenedione and testosterone) into estrogens(female sex hormone; mainly, estrone and 17β-estradiol). The precise biochemical mechanism by which aromatase enzyme produces estrogens using androgen substrates has not yet

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been completely understood(Graham-Lorence et. al., 1995). Furthermore, despite very complicated biochemical reaction steps in biosynthesis of estrogens, it was reported that only a single aromatase enzyme is solely responsible for the synthesis of estrogens(Corbin et. al., 1988). Interestingly, to date, the pig is the only mammalian species proven to possess at least three isoforms of aromatase genes that reveal high similarity to each other in their nucleotide sequences(Choi et. al., 1996; Choi et. al., 1997b). Each isoform of the gene is expressed in tissue-specific fashion. Type I aromatase gene is highly expressed in ovary, while type Ⅱ Ⅲ genes have been initially detected in placenta and embryos at pre-implantation period, respectively(Corbin et. al., 1995; Choi et. al. 1997a).

Type Ⅱ aromatase enzyme(placenta type), which is a product of type Ⅱ aromatase gene, mainly synthesizes estrogens using androgen substrates, while type I(ovary type) and Ⅲ mbryo type) aromatases give rise to more 19-nortestosterone(or called nandrolone) than estrogen from the same androgen substrates(Kao et. al., 2000). 19-Nortestosterone, also known as “anabolic steroid” (Kuhn et. al., 2002), is a synthetic steroid illegally used to improve athletic performance in sports and animal growth in farm animals. This suggests that 19-nortestosterone may be involved in muscle and bone growth in pig, too. Since high amounts of 19-nortestosterone were detected in serum of boar and stallion through the secretion from the testis, but not in those of other mammals(Dintinger et. al., 1989 and Schwarzenberger et. al., 1993), it is likely that the 19-nortestosterone is the endogenous hormone, at least, present in these two species. However, the physiological role(s) of this hormone has not been clearly understood yet.

Thus, as an initial step toward understanding the gene regulatory mechanisms of the three lately-discovered porcine aromatase genes and their biochemical and physiological roles in these species, the current research has been designed to examine the expression pattern of each type of aromatase gene. Because it is very difficult to detect specific form of aromatase mRNA expression using either Northern or RT-PCR methods due to very high similarity of each aromatase gene in its nucleotide sequences, RT-PCR coupled with denaturing high performance liquid chromatography(DHPLC) analysis(Gross et al, 2001; Martin et al, 2002) was employed in this experiment.

Ⅱ MATERIALS AND METHODS

1. RNA extraction from tissue

The ovaries of the Landrace × Yorksire × Duroc cross-bred market gilts were obtained from the slaughter house. The testes were collected by surgical castration of 4～ day-old Landrace× Yorksire × Duroc cross-bred market piglets from the local farm. Total RNAs were extracted from the testis and ovary tissues. The tissues were homogenized in Trizol® reagent(Gibco, USA). The samples were incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. Further their purification were induced by the addition of 0.2mℓ chloroform per 1mℓ of Trizol reagent. After shaking vigorously, samples were incubated at room temperature for 3 minutes and centrifuged at 12,000rpm for 10 minutes at 4℃ for phase separation. The RNA in aqueous phase was precipitated by adding 500μℓ of Isopropyl alcohol per 1mℓ of Trizol reagent and subsequent incubation at room temperature for 10 minute. After incubation, sample were centrifuged at 12,000rpm for 10 minutes at 4℃ This step was followed by RNA washing with 75% ethanol and centrifuged at 7,500rpm for 5 minutes at 4℃ After washing, it was air dried for 10 minutes

and re-dissolved in RNase free water and incubated for 10 minutes at 60℃ The concentration of each RNA sample was quantitated by reading its optical density at 260 nm and samples were stored at −80℃ ırior to its use.

2. cDNA synthesis

The reverse transcription reaction was set up from the mRNA by the addition of 1μℓ Oligo dT(Invitrogen, USA), 1μℓ of 10mM dNTP(Bioneer, Korea), 1μℓ of DNA free total RNA(1μg/μℓ) and 9μℓ dH_2O with the total volume of 12μℓ. Sample mixture was preheated at 65℃ or 5 minutes and centrifuged for 10 second at 4℃ This step was followed by addition of 4μℓ of 5X First strand synthesis buffer, 2μℓ 0.1M DTT (Invitrogen, USA) and 1μℓ of RNase free Recombinant RNase inhibitor(40 units/μℓ; Takara, Japan) and centrifuged. After heating at 42℃ ˙or 2 minutes for enzyme activation, 1μℓ SuperScript II reverse transcriptase(Invitrogen, USA) was added. For further activation of reaction, sample was continuously heated at 42℃ for 50 minutes and subsequently incubated at 70℃ or 15 minutes to inactivate reverse transcription reaction. After chilling, the products were centrifuged and stored at −80℃ ıntil PCR analysis.

3. RT-PCR analysis

Amplification of each cDNA was achieved in 50μℓ total reaction volume including 35μℓ distilled water, 5μℓ of dNTPs(2.5mM), 5μℓ of 10X PCR buffer(100mM Tris-HCl, 400mM KCl, 15mM $MgCl_2$ and pH 9.0), 1unit of pfu Taq DNA Polymerase(SolGent, Korea) and 20 pico moles of each forward and reverse primer to 1μℓ of template. The nucleotide sequences of primers used for PCR were as follows: pAro-E3f(5'-GTCCTGGCTAATTTCTGGGAATTGG-3') and pAro-E5r(5'-TGGAATCGGCACAGACGGTCACCAT-3') with twenty-five base pairs. The PCR reactions were performed in a thermocycler for 25 and 30 cycles(25 and 30 cycles of 30 seconds at 95℃ 40 seconds at 64℃ 72℃ ˙or 40 seconds), after heating the reaction mixtures at 95℃ for 2 minutes to denature the DNA templates. All amplification were finished by a prolonged extension step for 5 minutes at 72℃ After the PCR, 10μℓ of each aliquot were electrophoresed on 1% agarose gel.

4. DHPLC analysis

Denature High Performance Liquid Chromatography(DHPLC) analysis was performed on WAVE Nucleic acid fragment analysis system(Transgenomic, USA). Heteroduplex formation was induced by heat denaturation of PCR products at 95℃ or 5 minutes followed by gradual re-annealing from 95℃ to 23℃ ɔom temperature) prior to its analysis. An aliquot(5μℓ) of the PCR product was injected into a temperature-equilibrated DNA separation Column(Transgenomic, USA). Buffer A (0.1M tri- ethylammonium acetate, pH 7.0) and buffer B(0.1M triethylammonium acetate with 25% acetonitrile, pH 7.0) were used as mobile phase for sample separation with gradient and the flow rate of the mobile phase was 0.9mℓ/min. WAVE Marker software determined the start and end points of the gradient and melting temperature where it also showed retention time versus temperature graph to yield a fragment specific curve. Generally, the analysis of an individual sample took 8.8 minutes including regeneration and re-equilibration step. The optimal temperature used was 58.9℃ which was empirically determined for the mutation detection. The results were identified on the basis of the appearance of peaks by visual inspection of the chromatograms.

Ⅲ RESULTS AND DISCUSSION

Estrogens are known as an important key player in reproduction in mammals, which act via estrogen receptor located in the nucleus of target cells. Estrogen receptor(ER) is a member of superfamily of nuclear receptors. Thus, estrogen and estrogen receptor complex affects cellular function through modulating the transcription of a variety of genes(Katzenellenbogen et. al., 2000; Choi et. al., 2000). Cytochrome P450 aromatase is the enzyme responsible for estrogen production from androgen. Although isoforms of aromatase mRNA were reported in some invertebrate species (Callard et. al., 1997; Chiang et. al., 2001), they have so far been found in only one mammalian species, pig. These multiple form of aromatase mRNAs have been identified from at least three different chromosomal genes of pig(Choi et. al., 1996; Choi et. al., 1997b). Thus, it was of great interest for us to investigate tissue-specific expression of aromatase genes and to understand biochemical and physiological functions of each enzyme. However, the analysis of tissue-specific

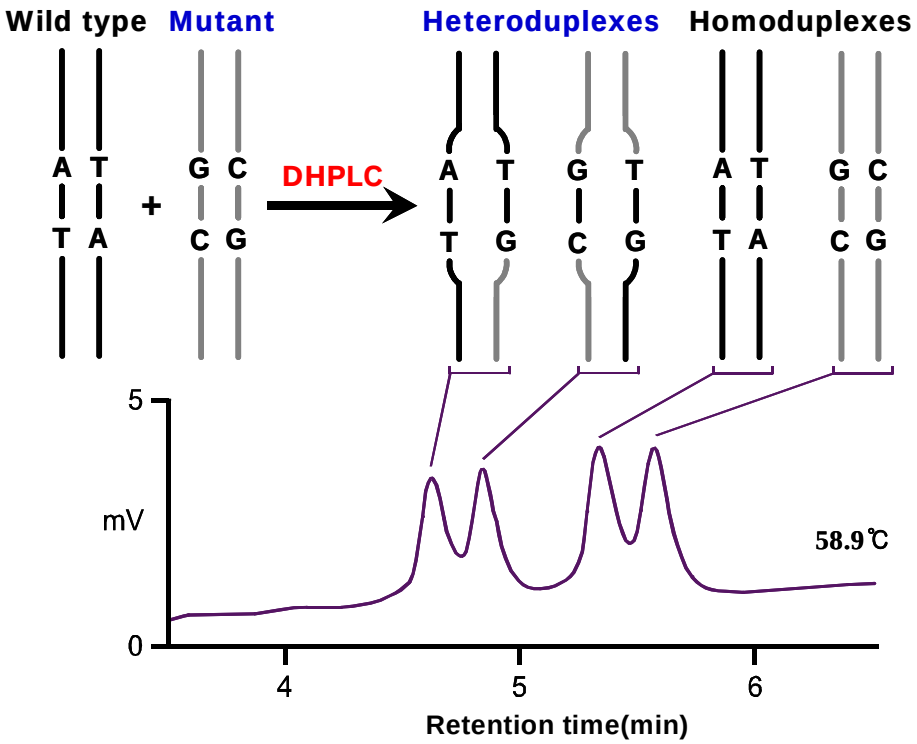

Fig. 1. Principle of WAVE(DHPLC) analysis. Illustrated fig. 1 is showing the heteroduplex formation for mutation detection.
The PCR products of wild type and mutant allele are denatured by heating and reannealed by slow cooling(room temperature) causes heteroduplex formation. The Chromatography identifies the mutations on the basis of the difference in the melting temperature of homo and heteroduplex. Mutation detection by Denature High Performance liquid chromatography (DHPLC) resolution of Hetero and homo duplex at 58.9℃ emperature is shown. Using DHPLC, the heteroduplex products (in the form of elution profiles) were eluted faster than homoduplex. In this case, any changes in the profile indicate that a mutation is present.

expression of aromatase genes in this species has been limited by the high homology of the nucleotide sequences among isoforms of porcine aromatase genes. Trials of RT-PCR analysis with isoform-specific PCR primers resulted in failure due to cross-amplification of aromatase genes by primers(data not shown).

Therefore, a recently-developed new technique, so-called, Denatured High Performance Liquid Chromatography(DHPLC or WAVE analysis system) was performed in order to identify tissue-specific expression of three(or more, if any) types of porcine aromatase isoform. DHPLC has been described as a very effective method to detect mutation of nuclear genes. The principle of DHPLC is shown in Fig. 1. DHPLC analysis was performed to screen nucleotide sequence difference in PCR-amplified DNA fragments. The amplified PCR products, after denaturation and reannealing at room temperature, were allowed to run through the DHPLC column. According to DNA structure and molecular weight, the chromatogram showed a single elution peak. PCR primers were designed at which the nucleotide sequences are identical but differ in the nucleotide sequences between the primers among

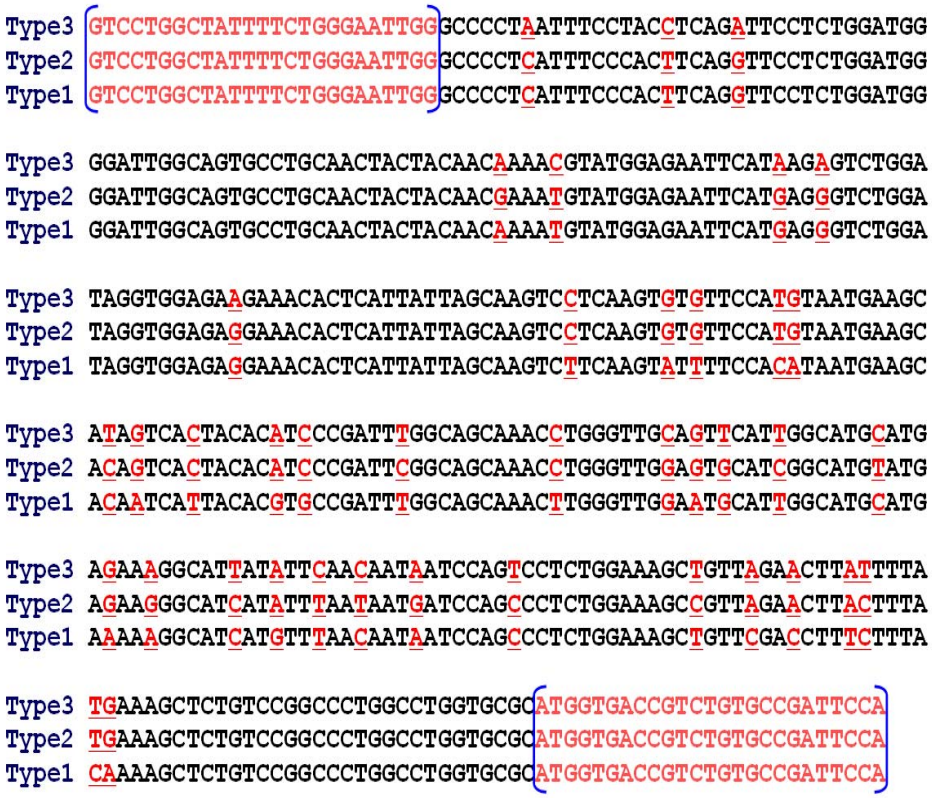

Fig. 2. Comparison of the nucleotide sequences in three types of porcine aromatase cDNAs:
Type I (ovary type, GenBank#U92246), Type II (placenta or P2 type, GenBank#U92245), Type III (embryo or 34B type, GenBank#U37311), where utilized for PCR amplification. The nucleotide sequences which were used to design PCR primers are highlighted by light letter in parentheses. Nucleotide sequences that differ in each aromatase are indicated by light underlined characters.

three isoforms(Fig. 2). A number of PCR products were injected on to the WAVE analysis system. And these injected products gave individual peaks. Based upon this principle, each aromatase isoform was evaluated.

Total RNAs of ovary and testis were extracted and used for the synthesis of first-strand cDNA. Using the same PCR primer for all three isoforms, polymerase chain reaction was carried out with the synthesized first-strand cDNAs of ovary and testis and the double-stranded DNAs of 34B (type Ⅲ Ⅱ as positive controls. As shown in Fig. 3, almost equal amounts of PCR amplification for two aromatase genes(type Ⅲ Ⅱ lane 4) were observed using the same PCR primers, implying that the PCR primers able to amplify the two types of aromatase genes with the same efficiency. The same size of PCR amplification products also detected from the ovary and testis(lane 7 and 8, respectively). It is likely that there are more PCR amplified-products in testis than in ovary. More clear difference was noticed after 25 cycles of PCR compared with 30 cycles. This result proved that the expression of aromatase gene is relatively higher in testis than in ovary. Interestingly, in pig, blood level of estrogen, female sex hormone, is higher in male than in female(Claus and Hoffman, 1980). Higher expression of aromatase gene may in part account for higher blood estrogen concentration in this species. However, since pooled tissue samples of testes from day 4～ piglets and ovaries from gilts were used to extract total RNAs, we cannot completely rule out the possibility of differential expression of aromatase gene depending on developmental stage or estrous cycles.

To test if the same type of aromatase gene(s) is expressed in testis and ovary, PCR products amplified by using the same primers above were used for WAVE analysis. This difference in nucleotide causes a variation in flow rate inside DHPLC column. Although the same size of PCR product was observed after agarose gel electro-

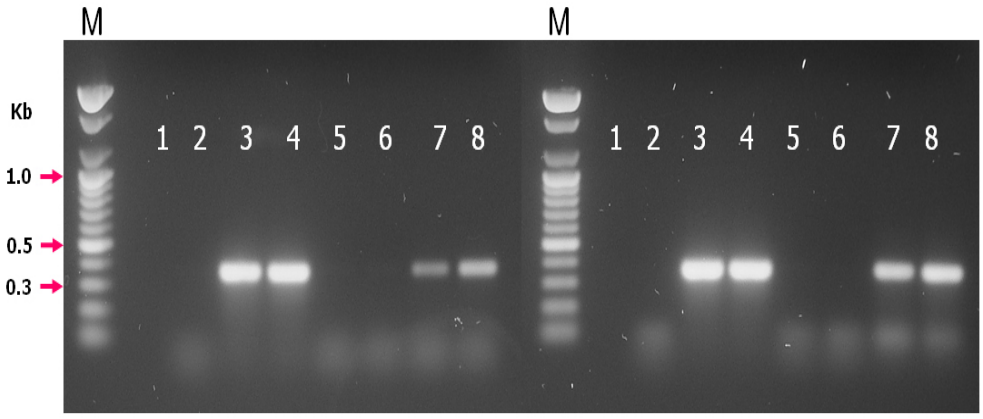

Fig. 3. Expression of cytochrome P450 aromatase mRNA in ovary and testis.
PCR amplifications(25cycles and 30 cycles) were performed on total RNAs of testis and ovary (pooled from 20 pigs) using the same primer set indicated in Fig. 2: lane 1, 2(negative control, without template); lane 3, 4 (positive control, type Ⅱ Ⅲ aromatase cDNA); lane 5, 6 total RNA of ovary and testis; lane 7, 8(first strand cDNA synthesized from total RNA of ovary and testis, respectively). Described in Materials and Methods. Expected size of each aromatase is 357 bp. The numbers on the left side indicate the sizes(Kilo base) of the DNA molecular weight marker (M).

phoresis(Fig. 3), the result from WAVE analysis revealed that each type of aromatase gene product has distinct retention time. Retention time of type Ⅲ gene product(34B; 4.53 min.) differ from

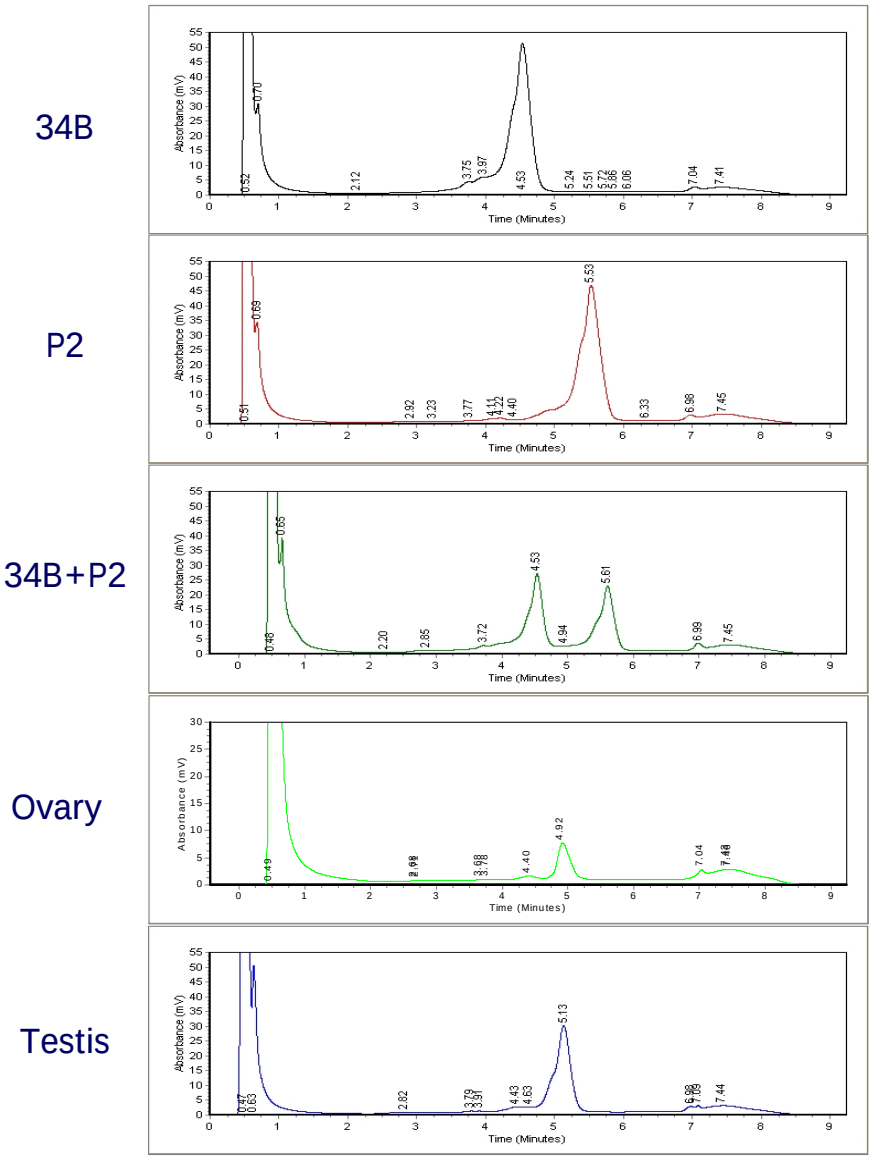

Fig. 4. DHPLC analysis of RT-PCR product of aromatase genes.
The DNA fragments of aromatase gene obtained from PCR or RT-PCR at 30 cycles(Fig. 3) were used for WAVE μl of PCR product, except for 34B+P2 sample(2.5μℓ were used to assay. The above figure showing the analysis of DHPLC elution profiles, P2(RT-PCR product of type Ⅱ Ⅲ aromatase), and ovary and testis profiles. Although the number of peaks and shape of heteroduplex profile changes depending upon the temperature, the DHPLC profile with optimum temperature(58.9℃ were applied to detect the potential mutation in this experiment. The Type Ⅱ Ⅲ were used as control amplicons.

that of Ⅱ '2; 5.53 min), and the mixture of type Ⅱ Ⅲ retained both peaks at very similar retention times(Fig. 4). The ovary(5.27 min) and the testis(5.13 min) showed trace difference in their retention time but quite different from those of type Ⅱ Ⅲ suggesting additional distinct type of aromatase isoform, neither type Ⅱ Ⅲ is transcribed in ovary and testis. To further identify what type of aromatase gene is expressed in the ovary and testis, RT-PCR products from the ovary and testis were subcloned into plasmid vector and arbitrarily selected clones were subjected to DNA sequencing. The nucleotide sequencing results demonstrated that all clones from ovary and testis contained the identical nucleotide sequence to that of previously found in ovary and designated as type I aromatase gene(Corbin et. al., 1995). Thus, the result of WAVE analysis proved that both ovary and testis expressed the same type of aromatase gene and type I aromatase is the major form of gene expressed in these tissues. Previous report demonstrated that high amounts of 19-norandrostenedione (secreted from ovary, Khalil and Walton, 1985) and 19-nortestosterone(secreted from testis, Raeside et. al., 1989; Schwarzenberger et. al., 1993) were detected in the blood of the pig. In addition, it was found that type I and Ⅲ aromatase retained high conversion rate of 19-nortestosterone compared with that of estrogen when testosterone was used as substrate(Kao et. al., 2000). These results indicate that type I aromatase is responsible for the production of 19-norandrogens in ovary and testis in the pig. It will be of interest to examine the expression profiles of aromatase gene in other tissues and the regulatory mechanisms for tissue-specific transcription of porcine aromatase isoform genes. In addition, the physiological role of 19-norandrogens uniquely secreted in the pig remains to be answered. Taken together, the present study proves that type I aromatase gene is the major form of aromatase expressed in both ovary and testis and DHPLC (WAVE) analysis is a powerful tool to investigate the expression profiles of the multiple isoforms of genes.

Ⅳ IMPLICATION

The expression pattern of the aromatase gene from the testis and ovary tissue was examined by using RT-PCR coupled with WAVE(DHPLC) analysis system. The result of RT-PCR against the aromatase gene using the testis and ovary RNA confirmed that the aromatase gene expression is more in testis than in ovary tissue. The results obtained from the WAVE analysis showed that all aromatase genes expressed in testis and ovary were corresponding to type I(ovary type). Since different 19-norandrogens were proven to be secreted from the ovary and the testis in the pig, it is likely that the same aromatase protein produces distinct 19-norandrogen product. Finally, DHPLC system(a new powerful technique) helped us to demonstrate the experiment to detect tissue-specific expression profiles of multiple isoforms of the aromatase genes.

Ⅴ ABSTRACT

Cytochrome P450 aromatase is the enzyme responsible for biosynthesis of female sex hormone(estrogen) and 19-nortestosterone(nandrolone), a unique steroid hormone endogenously synthesized in the pig. By use of RT-PCR coupled with DHPLC technique (WAVE alysis), expression pattern of isoforms of porcine cytochrome P450 aromatase gene was investigated. Relatively higher expression of aromatase mRNA was observed in testis than in ovary and this result accounted for the previous findings of higher blood estrogen level in male compared with female in this species. The result from the DHPLC demonstrated that PCR amplified DNA

fragments of ovary and testis tissues, using unique PCR primers for all three types of aromatase genes, were different from those of type Ⅱ and Ⅲ genes. Further nucleotide sequence analyses of the plasmid clones containing the PCR products revealed that nucleotide sequences of all clones were identical to type I aromatase gene(ovary type). Thus, the result from the present study indicates that the ovary and testis express the same type of aromatase gene. Therefore, the efficacy of DHPLC techniques used for this study helped us to analyze tissue-specific expression of isoform of genes containing the nucleotide sequences with high homology.

Key words : Aromatase, DHPLC, Anabolic steroid, Testosterone, 19-Nortestosterone, Estrogen.

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