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Testicular Expression of Steroidogenic Enzyme Genes Is Related to a Transient Increase in Serum 19-nortestosterone during Neonatal Development in Pigs

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ABSTRACT : Cytochrome P450 aromatase is responsible for the biosynthesis of estrogen. It is also responsible for the endogenous production of 19-nortestosterone (nandrolone), an anabolic androgen unique to pigs. Plasma concentrations of 19-nortestosterone are highest between two and four weeks after birth in male pigs. In the present study, the physiology of 19-nortestosterone was investigated by measuring the mRNA levels of steroidogenic enzymes, estrogen receptors and androgen receptor in the tissues of growing pigs. The expression of aromatase, 17α-hydroxylase and 3β-hydroxysteroid dehydrogenase in the testes of male piglets increased between birth and two weeks of age, and then decreased progressively. Similar developmental expressional patterns were observed for 17α-hydroxylase and 3β-hydroxysteroid dehydrogenase in the ovaries of female piglets, but without significant aromatase expression. The major form of aromatase expressed in the testes of piglets was identified as type I. Expression of estrogen receptor-α and -β and androgen receptor genes was also detected in both testes and ovaries. A transient elevation of androgen receptor mRNA in male piglets at two weeks of age was also observed in testes. Significant expression of the androgen receptor gene, but not of estrogen receptor-α and -β genes, was also demonstrated in adipose tissue and muscle. We conclude that the observed increase in the testicular expression of aromatase in male pigs could account for the production of large amounts of 19-nortestosterone at between two and four weeks of age in males. Androgen receptor and 19-nortestosterone appeared to be important for testicular development and might contribute to sexual dimorphism in body composition and muscle development in juvenile pigs. (**Key Words :** Cytochrome P450 Aromatase, Pig Testis, 19-Nortestosterone)

INTRODUCTION

Estradiol, testosterone and other endogenous estrogens and androgens play critical roles in the functional developments and activities of gonads and other tissues (Oh et al., 2005; Wierman, 2007). Moreover, these hormones and their synthetic pharmaceutical analogs have well described anabolic activity (Kuhn, 2002). The cytochrome P450 enzyme, aromatase, catalyzes the conversion of C18 estrogens to C19 androgens (Choi et al., 1996). Of the endogenous C19 sex steroids, 19-nortestosterone (17β-hydroxy-19-nor-4-androsten-3-one, also called nandrolone) has the highest ratio of anabolic to androgenic activity. Moreover, as compared to other mammalian species, the male pig produces extremely large amounts of gonadal steroids. Likewise, 19-nortestosterone is abundant in pigs and is readily detectable in blood plasma, and highest concentrations of 19-nortestosterone in male pig plasma are observed between two and four weeks after birth when 19-nortestosterone concentrations are equivalent to adult boar plasma levels of dehydroepiandrosterone sulphate, the most abundant androgen in the male pig (Schwarzenberger et al., 1993).

The pig is also unusual because it expresses aromatase from three distinct genes as compared with one gene in the other mammalian species (Gaucher et al., 2004). Porcine aromatase genes have homologous nucleotide sequences,

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Table 1. PCR primers used for real-time RT-PCR

	Size (bp)	Genbank	Tm (°C)	Nucleotide sequence	
GAPDH	135	AF017079	55	forward	5′- ATGCCTCCTGTACCACCAAC -3′
				reverse	5′- GTCTTCTGGGTGGCAGTGAT -3′
17α-Hydroxylase	152	M63507	53	forward	5′- CTGATAGATGGGGCACGATT -3′
				reverse	5′- CACTGTTGCGGACATCTTTG -3′
3β-HSD	112	NM0010040491	57	forward	5′- CTGCTGGAGGCCTGTGT -3′
				reverse	5′- TCTTCTTCGCAGGCGTTCTG -3′
Aromatase	356	U37312	55	forward	5′- GTCCTGGCTATTTTCTGGGAATTGG -3′
				reverse	5′- TGGAATCGGCACAGACGGTCACCAT-3′
AR	237	AF202775	53	forward	5′- ATGCGTTTGGAACCTACCAG-3′
				reverse	5′- GGCGACAAGATGGACAATTT-3′
ER-α	217	Z37167	57	forward	5′- AAGAGGGTGCCAGGATTTTT-3′
				reverse	5′- CGAGATGATGTAGCCAGCAA-3′
ER-β	213	AF164957	57	forward	5′- GTGATCACACAACCCGAGTG-3′
				reverse	5′- ATGAAGCCCGGAATTTTCTT-3′

The expected sizes (bp) of primer amplification products, Genbank accession numbers, melting temperatures (Tm) and the nucleotide sequences of each primer are listed.

but their expressions are tissue-dependent and they differ functionally (Conley et al., 1996; Haeussler et al., 2007). Type I porcine aromatase expression was originally identified in ovary, whereas type II is most highly expressed in placenta, and type III is most abundant in embryonic blastocysts (Corbin et al., 1995; Choi et al., 1997). For example, using radiolabeled testosterone as an *in vitro* substrate for recombinant porcine enzyme, porcine aromatases were found to produce 17β-estradiol and 19-nortestosterone as principal products, but in different ratios. Similar studies on the type I isoform of porcine aromatase using microsomal membrane fractions of ovary showed far greater production of 19-nortestosterone than estradiol from radiolabeled testosterone (Corbin et al., 1995; Kao et al., 2000). Commercially, anabolic steroids are chemically synthesized and are similar to androgen in chemical structure. Androgen has dual physiological functions in males, i.e., an androgenic effect and anabolic effect, and these are implicated in the developments of male sex organs and muscle, respectively. The name "anabolic steroid" derives from its relatively high anabolic effect with respect to the development of muscle via increased protein synthesis, as compared with its parent molecule. Thus, it has been illicitly used by some athletes to increase muscle bulk (Kuhn, 2002) and by some farmers to increase day yield in farm animals (Johnson et al., 1996; Lee et al., 2007). Moreover, it is known that most mammals, except the pig and horse, do not endogenously synthesize detectable amounts of anabolic steroid. Previous research has demonstrated that porcine aromatase, an enzyme responsible for estrogen biosynthesis, is also able to catalyze the production of anabolic steroid (nandrolone or otherwise known as 19-nortestosterone) (Kao et al., 2000). Therefore, in an attempt to understand the molecular mechanism underlying the temporal secretion of 19-nortestosterone in male piglets and its eventual physiological role in this species, we investigated the expressional patterns of genes coding for steroidogenic enzymes, including cytochrome P450 aromatase, 17α-hydroxylase, 3β-hydroxysteroid dehydrogenase, and the steroid receptors of estrogen receptors (ER-α and-β form), and androgen receptors in different tissues.

MATERIALS AND METHODS

RNA extraction from tissue

New-born Landrace×Yorksire×Duroc cross-bred piglets were grown at a local swine production facility until slaughter. Tissue and testis samples were collected weekly after birth by slaughtering and surgical castration, respectively, and stored at -80°C until required. Adipose tissues were taken from back fat and muscle tissues were taken from semimembranosus muscles at various growth stages immediately after sacrifice. Tissues were frozen until required for analysis. Four to six ovary and testis tissues obtained weekly were pooled for RNA extraction. Total RNA was extracted from testis and ovary tissues using Trizol® reagent (Invitrogen Co., Carlsbad, USA), and extracted RNA was dissolved in RNAase free water. The RNA concentrations were quantified by determining optical densities at 260 nm and the RNA was stored at -80°C prior to use.

cDNA synthesis

Reverse transcription was set up from mRNA by adding 1 μl Oligo-dT primer (Bioneer, Daejeon, Korea), 1 μl of 10 mM dNTP (Bioneer), 1 μl of DNA free total RNA (1 μg/μl) and 9 μl dH_2O to a total volume of 12 μl. Sample mixtures were preheated at 65°C for 5 minutes and centrifuged for 10 seconds at 4°C. Four μl of 5× First strand buffer, 2 μl 0.1 M DTT (Invitrogen, Carlsbad, USA) and 1 μl of RNase free

Recombinant RNase inhibitor (40 units/μl; Takara, Shiga, Japan) were then added into the sample mixtures and centrifuged. After adding of 1 μl Superscript-II reverse transcriptase (Invitrogen), samples were maintained at 42°C for 50 minutes and subsequently incubated at 70°C for 15 minutes to inactivate the reverse transcription reaction. After chilling, products were centrifuged and stored at -80°C until required for PCR analysis.

Real-time PCR analysis

Each cDNA was amplified in a total reaction volume of 20 μl, which included 5.5 μl of distilled water, 5 μl of dNTPs (2.5 mM), 5 μl of 4× Master mix (100 mM Tris-HCl, 400 mM KCl, 15 mM $MgCl_2$ at pH 9.0), and 10 picomoles of each forward and reverse primer for 3 μl of template. The nucleotide sequences of primers used for PCR are listed in Table 1. PCR was performed in an Exicycler (Bioneer, Daejeon, Korea) over 40 amplification cycles (30 seconds at 94°C (denaturation), 30 seconds at 94°C (annealing), 72°C for 30 seconds and scanning) after heating reaction mixtures at 95°C for 10 minutes to activated the enzymes. Amplification product identities were confirmed by checking melting points and the sizes of each PCR product by electrophoresis in 1% agarose gel.

DHPLC analysis

Denatured High Performance Liquid Chromatography (DHPLC) analysis was performed using a WAVE nucleic acid fragment analysis system (Transgenomic, USA). Heteroduplex formation was induced by denaturating PCR products at 95°C for 5 minutes followed by gradual re-annealing from 95°C to 23°C (room temperature) prior to analysis. Briefly, an aliquot (5 μl) of PCR product was injected into a temperature-equilibrated DNA separation Column (Transgenomic Inc., USA). Buffer A (0.1 M triethylammonium acetate, pH 7.0) and buffer B (0.1 M triethylammonium acetate containing 25% acetonitrile at pH 7.0) were used as gradient mobile phases for the sample separation. The flow rate of mobile phase was 0.9 ml/min. Generally, the analysis of an individual sample took 8.8 minutes, which including the regeneration and re-equilibration steps. The optimal temperature used was

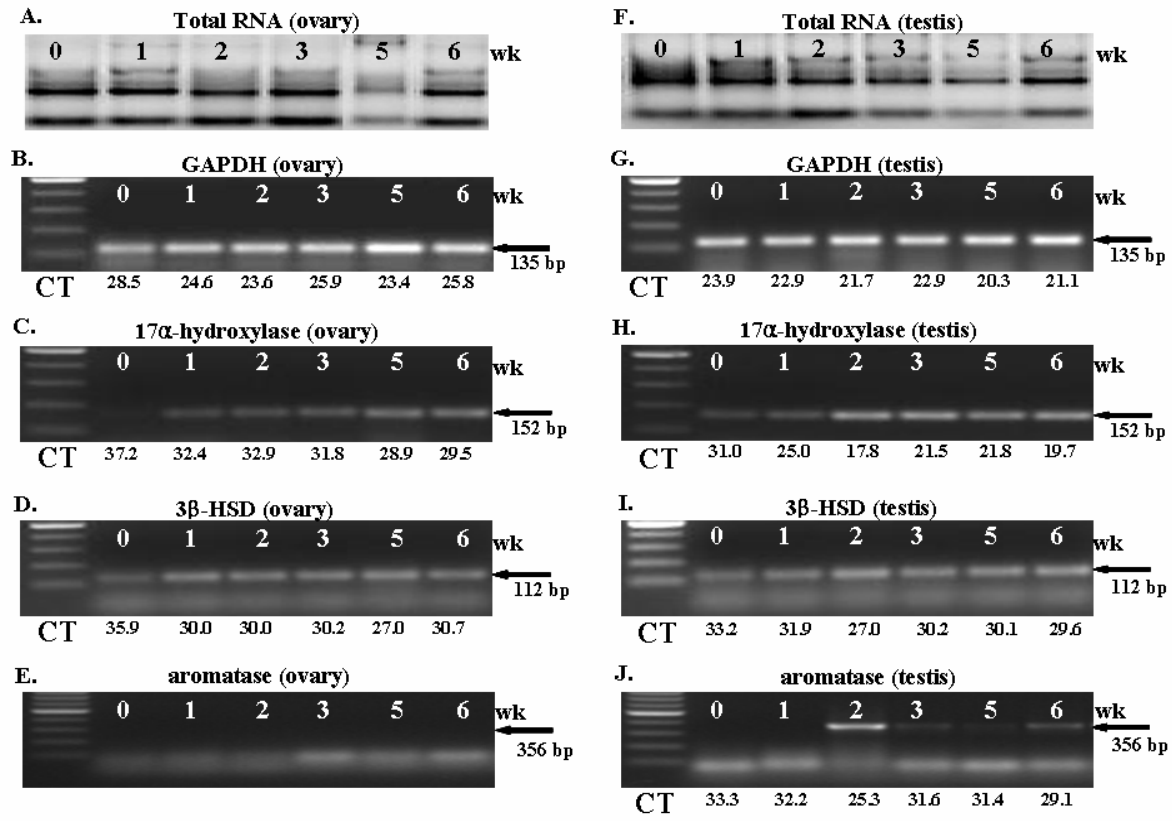

Figure 1. mRNA expressions of genes encoding steroidogenic enzymes. Two micrograms samples of total RNA were isolated weekly from piglet tissue after birth and separated on 1% agarose gel to determine RNA qualities (A and F). Note that the bands produced by RNA isolated from 5-week piglet ovaries are in the top lane above. PCR amplification products (40 cycles of real-time RT-PCR) of weekly samples obtained using gene-specific primers were separated in agarose gel with a DNA size marker (B-E and G-J). Numbers indicated above and below lanes indicate postnatal weeks, and fluorescence threshold cycles (CT) were calculated for each sample. Expected PCR product sizes for each primer are indicated by arrows (←).

58.9°C, which determined empirically for mutation detection. The differences in the nucleotide sequences of each PCR product were identified based on the appearances of chromatographic peaks.

GC/TOF/MS analysis

Internal standards (19-nortestostrone and d_4-19-norandrostreone) were purchased from the National Analytical Reference Laboratory (Pymble NSW, Australia), and N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA), ammonium iodide (NH_4I), and dithioerythritol (DTE) were obtained from Sigma Chemical Co. (MO, USA). Hydrogen chloride was supplied by Merck (Darmstadt, Germany). Acetonitrile and N-hexane were of HPLC grade and from J.T. Baker (Phillipsburg, NJ, USA). The GC/TOF/MS (GC/time-of-flight mass spectrometry) was a Pegasus unit from LECO Co. (St. Joseph, MI, USA) and consisted of an Agilent 6890N gas chromatograph with a split-splitless injector, a 7683 Series autosampler, and time-of-flight mass spectrometer LECO Pegasus II. ChromaTOF software was used to process collected data. A Lauda (Lauda-Ko¨nigshofen, Germany) Ecoline RE112 freezer was used to freeze aqueous layers, and a Turbovap®LV evaporator supplied by Zymark Co. (Hopkinton, MA, USA) was used to evaporate off organic solvents. A 7400 Rubigen shaker (Edmund Buchler) was used to shake the mixtures. The GC oven was started at 180°C with a 1 min initial hold, and programmed at 10°C/min to 220°C followed by 6°C/min to 260°C where it was held for 1 min, finally the temperature was increased at 30°C/min to 310°C and held for a further 2 min. The capillary column was an ultra 2 (crossed-linked 5% phenylmethylpolysiloxane) with a length of 25 m, 0.2 mm i.d., and 0.11 um film thickness. Treated samples (2 µl) were injected in split mode (split ratio 5:1). Helium was used as the carrier gas at a flow rate 1.0 ml/min. TOF/MS was operated at an acquisition rate of 10 spectra/sec in the mass range *m*/*z* 50-650 (ion source temperature: 230°C; transfer line temperature: 280°C; detector voltage: -1,850 V).

Plasma extraction

Plasma samples (1 ml) spiked with d_4-19-norandrostreone 10 µl (10 ng/ml in plasma, the internal standard) were hydrolyzed with 100 µl of 1 M HCl at 25°C for 30 min with shaking. Solutions were adjusted to pH 8-9 with 100 mg of potassium carbonate, 5 ml n-hexane was added, and the resulting mixes were shaken for 10 min, centrifuged at 2,100 g for 5 min, and frozen at -30°C. Organic layers were evaporated to dryness at 40°C under a gentle stream of nitrogen (Figure 1).

Tissue extraction

Two grams of tissue samples were spiked with d_4-19-norandrosterone 10 µl (5 ng/g tissue, internal standard), hydrolyzed with 100 µl of 6 M HCl at 25°C for 30 min with

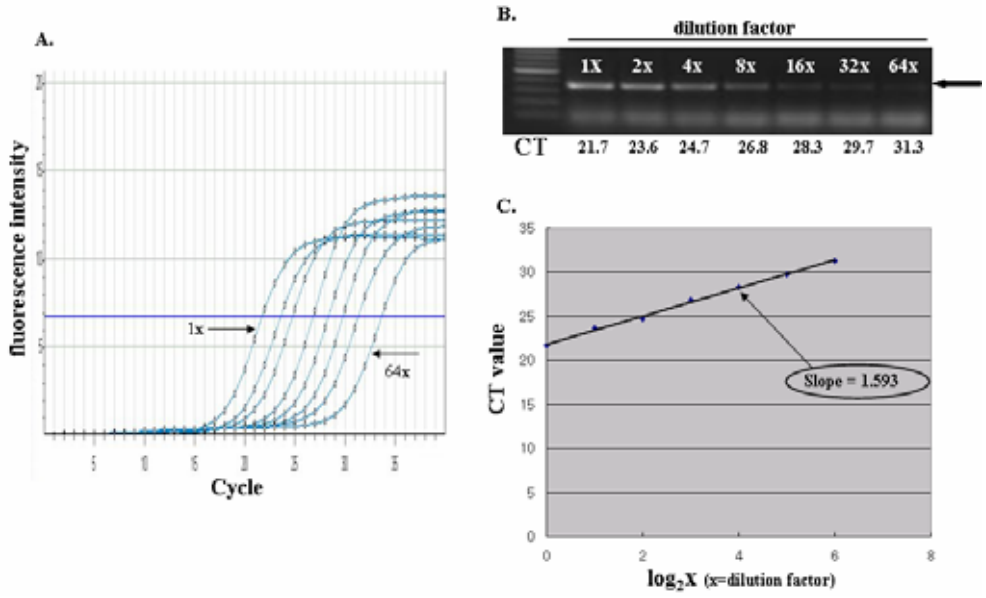

Figure 2. Efficiency of aromatase primers used for real-time RT-PCR. The figure shows representative RT-PCR plots of serially diluted templates (1× through 64×) of the 1st strand cDNA synthesized from testis RNA of 2-week piglets; the aromatase-specific primer is shown (A). The X and Y axes denote fluorescent intensity and PCR cycle numbers, respectively. The horizontal line used to determine threshold cycle number is shown, and the curves obtained by PCR amplification of sequentially diluted samples are indicated in order from left to right (only the 1× and 64× dilutions are indicated by arrows). PCR amplification products of diluted 1st strand cDNAs were electrophoresed on agarose gel; their CT values are indicated at the bottom of each lane (B). Primer efficiency was estimated from the slope of CT value at threshold line (y-axis) versus log dilution factor (x-axis) (C).

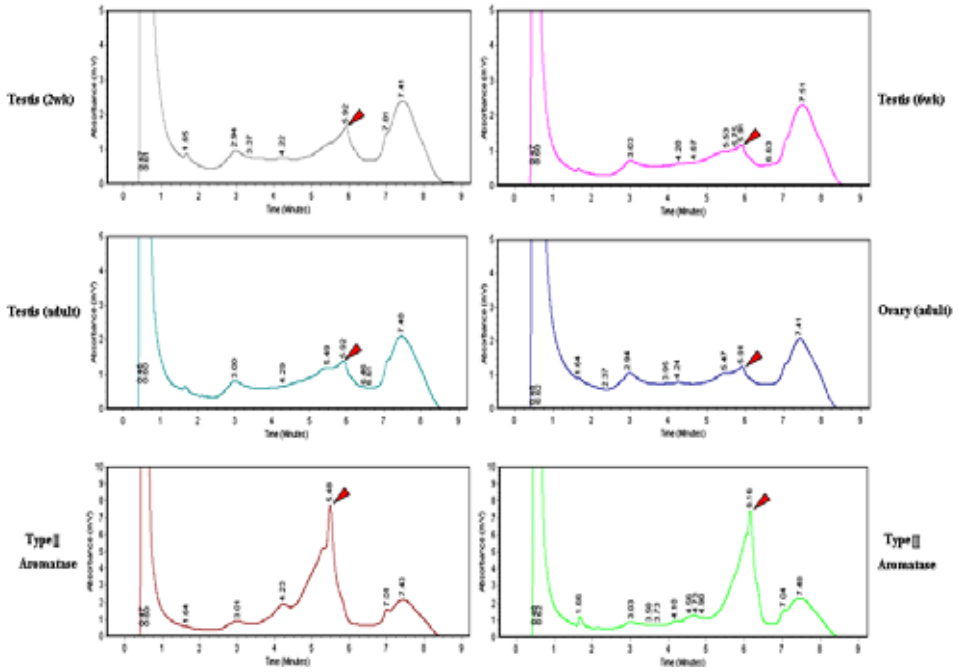

Figure 3. DHPLC analysis of aromatase RT-PCR product. The figures show the DHPLC elution profiles of DNA samples at optimum temperature (58.9°C). The major elution times for individual samples are indicated by black arrows. PCR amplification products obtained after 35 cycles of PCR using aromatase gene specific-primer and 1st strand cDNA synthesized from each RNA (2 wk testis, 6 wk testis, adult testis and adult ovary), and type II and type III aromatase cDNA templates were used for analysis. Equal amounts of PCR products (8 μl) were assayed.

shaking, adjusted to pH 8-9 with potassium carbonate (ca. 100 mg). After adding 5 ml of acetonitrile and n-hexane mix, mixtures were shaken for 10 min, centrifuged at 2,100 g for 5 min, and frozen at -30°C. Organic layers were then evaporated to dryness at 40°C under a gentle stream of nitrogen.

Derivatization

Residues were completely dried in vacuum desiccators over P_2O_5/KOH for 30 min before derivatization. To obtain the TMS ethers, dried residues were dissolved in 50 μl MSTFA/NH_4I/DTE (500:4:2, w/w/w) and then heated at 60°C for 15 min. Finally, 2 μl aliquots of this solution were analyzed by GC/TOF/MS.

Adipose cell culture and proliferation assay

Preadipocytes were isolated from the backfat of new-born pigs by collagenase digestion and grown as described previously (Suryawan et al., 1997). Cultured cells were treated with steroids for the first 4 days of culture. Cell numbers were counted using a WST kit (Roche, Mannheim, Germany). After the treatment period total RNA was extracted.

RESULTS AND DISCUSSION

The expressions of genes encoding the key enzymes required for steroid biosynthesis, including 17α-hydroxylase, 3β-HSD (3β-hydroxysteroid dehydrogenase), and aromatase, were investigated in piglet testes and ovaries during neonatal development by real-time RT-PCR (Figure 1). Total RNA samples isolated from four to six pooled ovaries (A) or testes (F) at each week were run in agarose gel to check their quality. The GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) gene was amplified as an internal control for normalization purposes. Expressions of 17α-hydroxylase and 3β-HSD mRNAs in ovaries were slightly elevated after birth and remained this level until the 6th post-natal week (C and D). No transcription of aromatase mRNA was observed in ovaries during this period (E). The expression of 17α-hydroxylase, 3β-HSD, and aromatase mRNAs were transiently enhanced after birth and peaked at 2 weeks in testis. To evaluate the efficiency of the aromatase-specific PCR primers used, PCR was carried out with serially diluted cDNAs synthesized from the RNAs of 2 week-old piglets (Figure 2). It was found that the efficiency of the aromatase-specific primer was about 79.65 (slope of CT value at threshold line versus log dilution factor equaled 1.593). About a six-fold difference was observed between post-natal weeks 0 and 2 in terms of aromatase mRNA expression (normalized versus GAPDH), indicating 16-fold elevation of aromatase gene expression in 2 week testes. Because the expected DNA sizes of the amplification products produced by other PCR

Table 2. Concentrations of steroids in the blood and tissues of male piglets

	Week	19-nor T	19-nor AND	E1	E2	T	Epi-T
Blood	0 (♂)	1.82	11.11	-	-	-	-
	0 (♀)	-	0.86	-	-	-	-
	1 (♂)	2.94	12.31	-	-	-	-
	1 (♀)	-	-	-	-	-	-
	1 (♂)	-	-	-	-	-	-
	2 (♂)	2.5	7.56	-	-	-	-
	2 (♀)	-	-	-	-	-	-
	2 (♂)	-	-	-	-	-	-
	3 (♂)	13.17	5.7	-	-	-	-
	3 (♀)	-	-	-	-	-	-
	3 (♂)	-	-	-	-	-	-
	4 (♂)	5.82	20.18	-	-	-	-
	4 (♀)	-	-	-	-	-	-
	4 (♂)	-	-	-	-	-	-
	5 (♂)	1.81	6.36	-	-	-	-
	5 (♀)	-	-	-	-	-	-
	5 (♂)	-	-	-	-	-	-
	6 (♂)	5.93	21.15	-	-	-	-
	6 (♀)	-	-	-	-	-	-
	6 (♂)	-	-	-	-	-	-
Testis	1.4	29.91	15.77	-	-	13.95	20.28
	2	13.84	6.57	-	-	3.21	4.15
	Adult	2.26	3.32	-	-	0.42	2.74
Ovary	Adult	-	3.03	-	-	-	-
Back fat	0 (♂)	-	2.57	-	-	-	-
	0 (♀)	-	-	-	-	-	-
	2 (♂)	-	-	-	-	-	-
	2 (♀)	-	-	-	-	-	-
	2 (♂)	-	-	-	-	-	-
Muscle	0 (♂)	-	2.66	-	-	-	-
	0 (♀)	-	-	-	-	-	-
	2 (♂)	-	-	-	-	-	-
	2 (♀)	-	-	-	-	-	-
	2 (♂)	-	-	-	-	-	-
Liver	0	-	-	-	-	-	-
	2	5.49	5.82	-	-	-	-

Blood concentrations of 19-nortestosterone and 19-norandrostenedione were found to peak in 3-week (47.9 nM) and 6-week piglet (77.6 nM), respectively by GC. 19-nortestosterone (50.4 nM) and 19-norandrostenedione (24.1 nM) concentrations were found to be elevated in the testes of 2-week piglets.

primers were smaller than that of aromatase (356 bp; Table 1), it is likely that the efficiencies of these primers were greater than that of the aromatase specific primer. Although isoforms of aromatase mRNA have been reported in some invertebrates (Callard et al., 1997; Chiang et al., 2001), there have only been positively identified in the pig. Multiple aromatase genes have been identified in at least three different pig chromosomes (Choi et al., 1996; Choi et al., 1997). Moreover, the analysis of the tissue-specific expressions of aromatase genes in the pig has been limited by the high homology shown by the nucleotide sequences the porcine aromatase genes. DHPLC (Denatured High Performance Liquid Chromatography) has been described as a highly effective means of detecting nucleotide sequence difference in PCR-amplified DNA fragments (Martin et al., 2002). Thus, DHPLC was used to identify the tissue-specific expressions of the three porcine aromatase isoforms (Figure 3). First-strand cDNA synthesized from total RNA extracted from testes (at 2 and 6 week postnatally and from adult) and ovaries were subjected to real-time PCR. Porcine aromatase-specific PCR primer, which had a nucleotide sequence matching that of all three isoforms, was designed as described above. Moreover, the retention times of the type III (6.16 min.) and type II (5.48 min) gene products on aromatase were found to differ. Ovary and testis showed similar retention times but the retention times of type II and III differed, which implies that type I aromatase is the major transcript expressed in ovary and testis. A previous report demonstrated that 19-norandrostenedione is the major form of steroid in porcine follicular fluid (Khalil and Walton, 1985), and high amounts of 19-nortestosterone (Raeside et al., 1989;

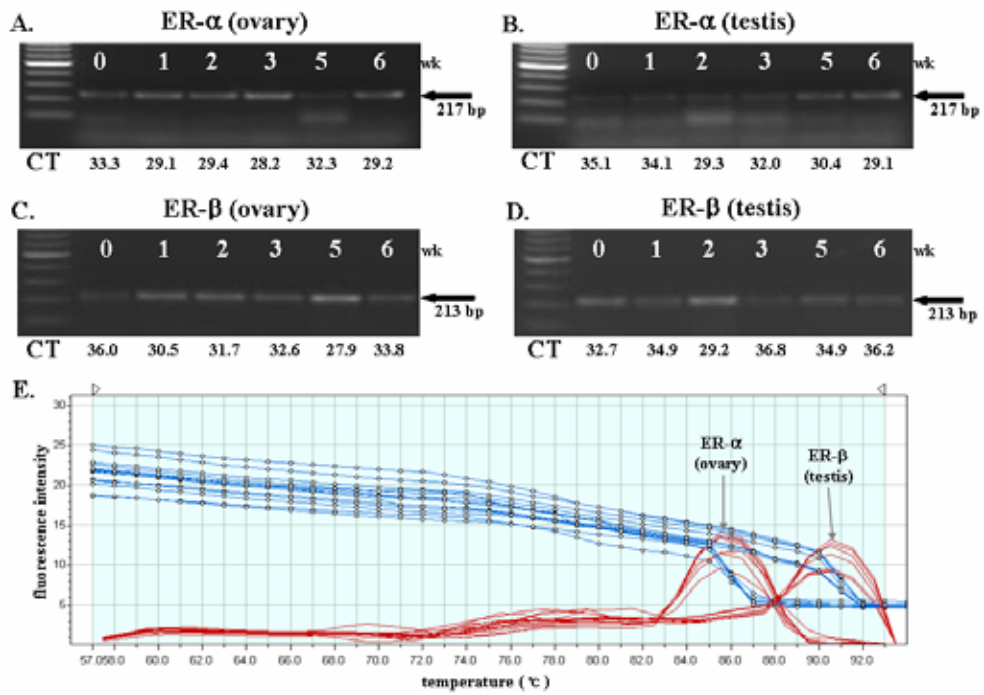

Figure 4. ER-α and -β gene expressions in testis and ovary. PCR amplification products (after 40 cycles of RT-PCR) of testis and ovary RNA samples from piglets of increasing age were obtained using ER-α or ER-β gene-specific PCR primers and separated by agarose gel electrophoresis; the DNA size marker is shown in the extreme left-hand lane (A through D). Numbers above and below lanes indicate age in postnatal weeks. Fluorescence threshold cycle (CT) was calculated for each sample. The expected sizes of the amplified cDNAs obtained using each primer are indicated by arrows (←). Specificity of amplification was confirmed by checking final product melting points (E). The X and Y axes represent temperature and fluorescence intensity, respectively. Melting temperatures of the ER-α and ER-β PCR products were 85°C and 91°C, respectively.

Schwarzenberger et al., 1993) have been reported in pig blood. Since both testes and ovaries express the same isoform of aromatase (type I), it is unclear how a single enzyme can synthesize these two different steroids.

The amounts of steroids present in blood and some piglet tissues (i.e., testis, ovary, backfat, muscle, liver) were estimated by GC/TOF/Mass (Table 2). 19-nortestosterone was detected in blood, testis and liver only in male piglets. 19-nortestosterone was not detected in castrated male or female piglets, which suggests that the testes are the major source of 19-nortestosterone. Serum 19-nortestosterone levels in male piglets increased after birth until 3 weeks postnatally to peak at 13.17 ng/ml and then gradually decreased. Piglet testes contained much higher amounts of 19-nortestosterone than those of adult boars. Thus, it is likely that a transient elevated production of 19-nortestosterone during the early postnatal period in male piglets is correlated with elevated aromatase expression, which is supported by our previous finding that porcine aromatase can convert androgen to 19-nortestosterone (Kao et al., 2000). It remains to be determined whether the 19-nortestosterone detected in the liver of 2 wk piglets is synthesized in liver or testes. 19-norandrostenedione was also detected in male piglets (mainly in blood and testes) but not in castrated piglets. Relatively small amounts of 19-norandrostenedione were detected only in new born male backfat and muscle (0 week), but significant amounts were observed in 2-week male piglet liver. On the other hand, adult ovaries contained high level of 19-norandrostenedione but no detectable 19-nortestosterone. This result is in good agreement with previous findings that the pig ovary endogenously produces 19-norandrostenedione and that this is the major steroid in follicular fluid (Khalil et al., 1988). No significant amounts of E_1 (estrone) or E_2 (17β-estradiol) were found in blood or tissue samples of any of the animals tested in the present study, and testosterone and epitestosterone were only detectable in testes. It should be noted that the majority of steroids, except the 19-norsteroids, are conjugated with sulfate in the pig and free steroid levels are low pig (Schwarzenberger et al., 1993), which is probably why we rarely detected these steroids in samples.

ER-α and ER-β mRNAs were also detected in piglet ovaries and testes (Figure 4), and as was observed for steroidogenic enzyme mRNAs, no marked changes in ER-α and ER-β mRNA expression were observed during the neonatal period in ovary. However, these two genes were upregulated in 2-week testes. Melting temperature analysis conducted on the two PCR products amplified using

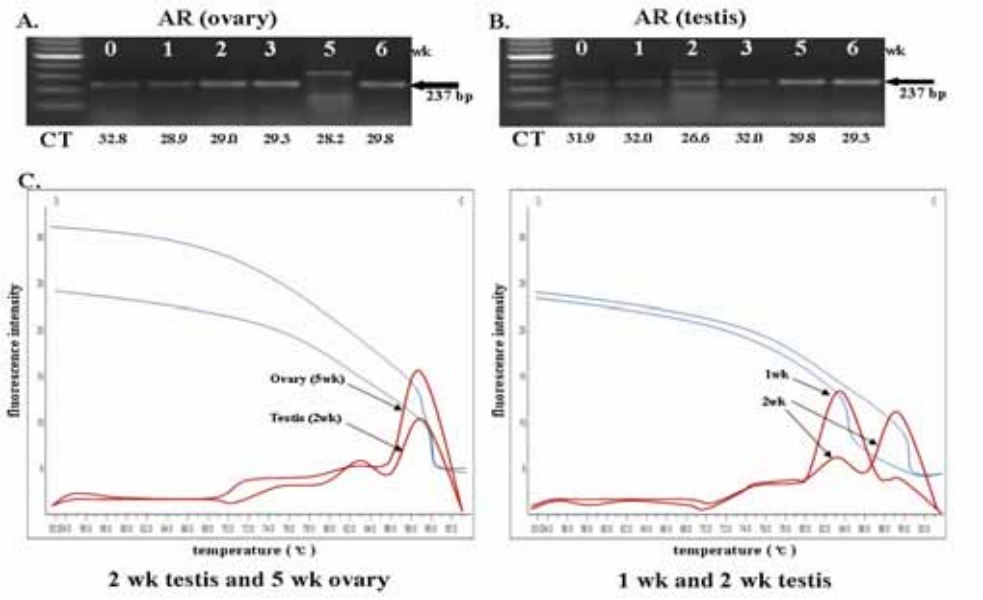

Figure 5. AR mRNA expression in testes and ovaries. PCR amplification products of RNA samples of piglet testes and ovaries obtained using AR gene-specific PCR primer were separated in agarose gel (the DNA size marker is shown in the left-hand lane) after 40 cycles of real-time RT-PCR (A and B). C. The melting points of the PCR products of 5- and 2-week piglet (left), and 1- and 2-week piglet (right) testis total RNAs (the melting point of the PCR product of 5-week piglet ovary total RNA is also shown).

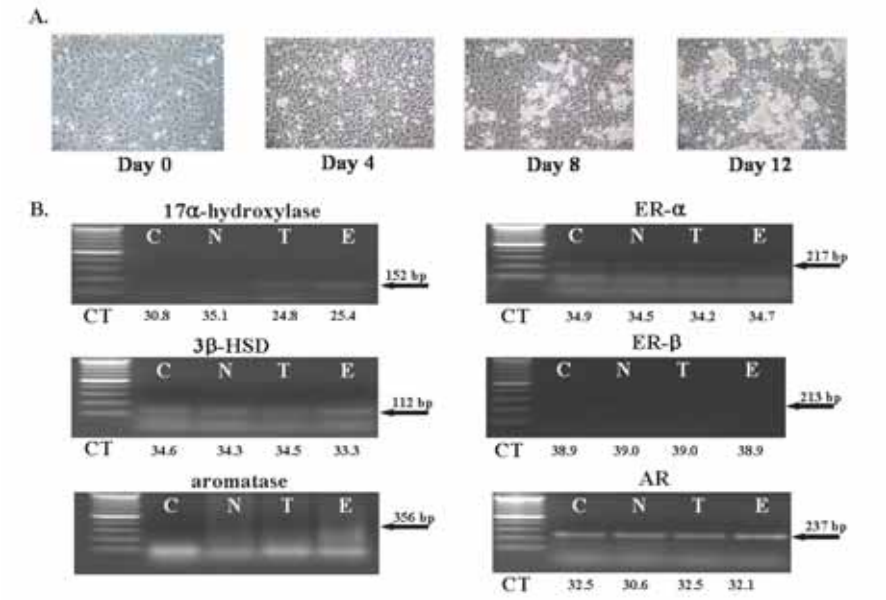

Figure 6. Expressions of steroidogenic enzyme and steroid receptor genes in adipose cells. Adipose cells were allowed to differentiate for different days (0, 4, 8, and 12) in culture (A). Lipid droplets were observed from day 4. PCR amplification products obtained using gene-specific primers and total RNA isolated from the adipose cells after 40 cycles of real-time RT-PCR were separated in agarose gel with a DNA size marker (B). Numbers at the bottoms of lanes indicate fluorescence threshold cycle (CT) calculated for each sample. The expected sizes of the amplification products for each primer are indicated by arrows (←).

specific PCR primers for the ER-α or ER-β genes resulted in two distinct peaks (E), which indicates that the PCR primers used were gene specific. Further analysis by cloning followed by DNA sequencing of each PCR product proved that these products contained the specific nucleotide sequences of the ER-α and ER-β genes. Androgen receptor (AR) mRNA was also detected in piglet ovaries and testes (Figure 5). However, no significant change in AR mRNA expression was observed in ovaries during the postnatal weeks. However, a slight increase in AR mRNA was observed at 2 weeks postnatally in testes. It was interesting to find that the sizes of ovary PCR products amplified at 5 weeks were slightly larger than those isolated at different times (A). In addition, the same size of extra band observed

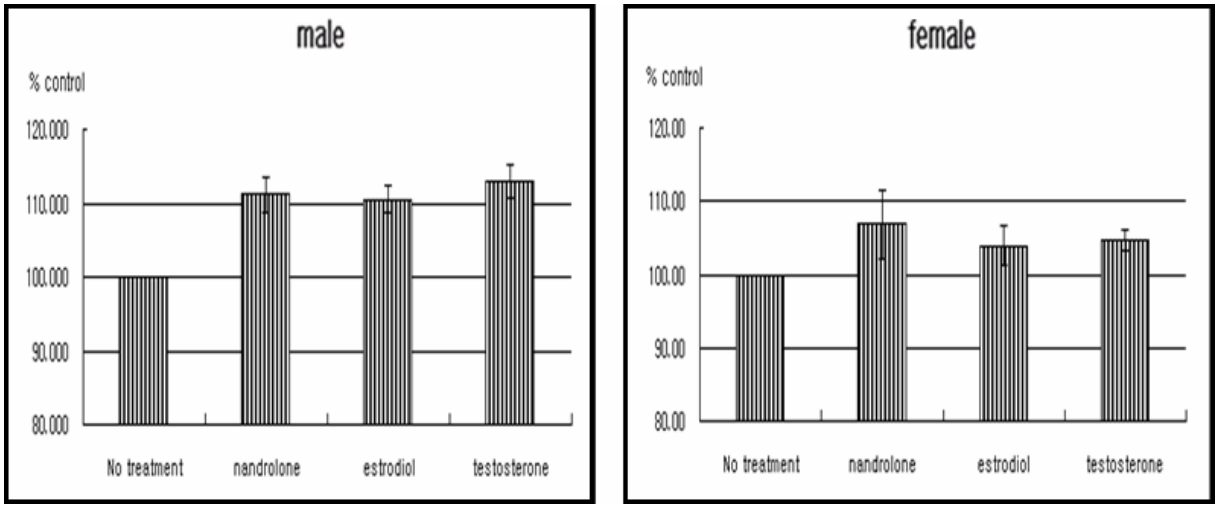

Figure 7. Steroid effects on adipose cell proliferation. Cell proliferation assays were conducted using adipose cells obtained from male and female piglets treated with 19-nortestosterone (1×10^{-8} M), 17β-estradiol (1×10^{-8} M), or testosterone (1×10^{-8} M). The values shown are means of six independent experiments.

from 5-week ovaries with the expected size was also detected in testes at 2 week postnatally (B). Melting temperature analysis demonstrated that the PCR products of 5-week ovaries and the additional product observed from 2-week testis had the same melting points. The two PCR products obtained using AR gene specific PCR primers from 2-week testes also had distinct melting temperatures, implying that the two fragments contain different nucleotide sequences (C). Although direct functions of ER-α, ER-β, and AR in testis (Isomma et al., 1987; Mutembei et al., 2005) have not been clarified, it is likely that estrogen and 19-nortestosterone, which are highly secreted by testis may act through their receptors to stimulate sex organ growth and development in the boar (Raeside et al., 1997). However, the physiologic roles of AR in the pig ovary (Slomczynska and Tabarowski, 2001) are not known.

The mRNA expressions of genes encoding steroidogenic enzymes (17α-hydroxylase, 3β-HSD and aromatase) and for steroid receptors (ER-α, ER-β and AR) were investigated in porcine adipocytes (Figure 6). Porcine preadipocyte cells differentiated *in vitro* into mature adipose cells containing lipid droplets (A). 17α-hydroxylase and 3β-HSD mRNA expressions were barely detectable and no aromatase mRNA expression was detected in adipocytes. Moreover, ER-α mRNA was weakly expressed and ER-β mRNA was not detected. Interestingly, porcine adipocytes showed high AR mRNA expression. Except for slight inductions of 17α-hydroxylase mRNA by testosterone (T) and by 17β-estradiol (E), no significant changes in gene expression were induced by steroid. Moreover, since 19-nortestosterone is known to act through AR like testosterone in adipose and muscle cells (Doumit et al., 1996; Singh et al., 2003), we examined the effect of this hormone on porcine adipocyte cells. It was found that testosterone and 19-nortestosterone enhanced in adipose cell proliferation (Figure 7). Moreover, the ablation of AR function by AR gene knock-out caused obesity in mice (Yeh et al., 2002), which suggests another important role for AR in adipose cells.

Table 3. Sexual dimorphism and AR gene expression

	Adipose tissue		Muscle	
	♂	♀	♂	♀
0 wk	23	22		
2 wk	20	21	21	19
3 wk	16	22	15	21
5 wk	23	23	18	22

The mRNA expressions of androgen receptor gene were measured by real-time RT-PCR in piglet adipose and muscle tissues weekly after birth (n = 4 for each gender and each week). Values represent average CT values of 4 piglets.

Because high amounts of 19-norsteroids were transiently produced during the neonatal period in piglets, we also investigated AR mRNA expressions in adipose tissues, as they are a known major target of this steroid (Kuhn, 2002). Interestingly, only male piglets showed higher AR mRNA expressions at 3 week postnatally in adipose and muscle tissues (Table 3). These findings encourage us to speculate that transiently enhanced 19-nortestosterone in 3-week-old male piglets, but not in female piglets, up-regulates its own receptor to modulate the genes responsible for cell growth or differentiation in these tissues (Doumit et al., 1996). It is also possible that this transient 19-nortestosterone production determines sexual behavior during early development in the pig.

Interestingly, boars have estrogen (female sex hormone) blood levels than sows (Claus and Hoffman, 1980), and higher levels of 19-nortestosterone, rather than testosterone.

Higher aromatase mRNA expression in testes may account for the higher blood estrogen and 19-nortestosterone concentrations observed in this species. Two key questions remain to be answered, i.e., how boars maintain male sexual characteristics given these higher concentrations of estrogen, and what is the real physiological role of 19-nortestosterone in this species. Reductions in estrogen induced by aromatase inhibitor administration to boars was found to reduce the serum concentration of IGF-I and the IGFBP-2 to -5 ratio (Hilleson-Gayne and Clapper, 2005) and to delay testicular maturation (At-Taras et al., 2006a). Based on the findings of our previous study and the present study, it is seems that estrogen and 19-nortestosterone are synthesized by the same aromatase in the boar. It is tempting to speculate that these results are not due to estrogen synthesis inhibition by aromatase inhibitor but rather to the inhibition of 19-nortestosterone synthesis. However, no effect on gonadotropin secretion was observed after reducing estrogen production using an aromatase inhibitor in the developing boar (At-Taras et al., 2006b), although an alteration in the development of uterine function was caused by early exposure of female neonatal piglets to estrogen (Tarleton et al., 2003) account for unique physiological features of this species developed during evolution. In conclusion, the dramatic increase observed in aromatase gene expression and in those of other steroidogenic enzyme genes in 2-week pig testes coincide with the temporal production of large amounts of 19-nortestosterone in the 2-4 week testis, which suggests that aromatase is responsible for 19-nortestosterone biosynthesis. Our analysis of the RT-PCR products of the aromatase gene by DHPLC demonstrates that the major form of aromatase expressed in testis and ovary is type I. Moreover, the estrogen and 19-nortestosterone, which are both produced at high levels in the neonatal testis, appear to act via ER-α, ER-β, and AR to modulate the genes involved in male sex organ maturation and to control adipose and muscle cell in the pig.

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