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# The role of porcine cytochrome b5A and cytochrome b5B in the regulation of cytochrome P45017A1 activities

M.J. Billen, E.J. Squires*

*Department of Animal Poultry Science, University of Guelph, Guelph, Ontario, Canada N1G 2W1*

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ABSTRACT

Male pigs are routinely castrated to prevent the accumulation of testicular 16-androstene steroids, in particular 5α-androst-16-en-3-one (5α-androstenone), which contribute to an off-odour and off-flavour known as boar taint. Cytochrome P450C17 (CYP17A1) catalyses the key regulatory step in the formation of the 16-androstene steroids from pregnenolone by the andien-β synthase reaction or the synthesis of the glucocorticoid and sex steroids via 17α-hydroxylase and C17,20 lyase pathways respectively. We have expressed CYP17A1, along with cytochrome P450 reductase (POR), cytochrome b5 reductase (CYB5R3) and cytochrome b5 (CYB5) in HEK-293FT cells to investigate the importance of the two forms of porcine CYB5, CYB5A and CYB5B, in both the andien-β synthase as well as the 17α-hydroxylase and C17,20 lyase reactions. Increasing the ratio of CYB5A to CYP17A1 caused a decrease in 17α-hydroxylase ($p < 0.013$), a transient increase in C17,20 lyase, and an increase in andien-β synthase activity ($p < 0.0001$). Increasing the ratio of CYB5B to CYP17A1 also decreased 17α-hydroxylase, but did not affect the andien-β synthase activity; however, the C17,20 lyase, was significantly increased. These results demonstrate the differential effects of two forms of CYB5 on the three activities of porcine CYP17A1 and show that CYB5B does not stimulate the andien-β synthase activity of CYP17A1.



## 1. Introduction

Androstenone is a C19 16-androstene steroid produced as a phermonal hormone by leydig cells in porcine testis from C21 precursors, pregnenolone and progesterone [1]. Androstenone is transported via the blood stream to the submaxillary salivary glands [2], where it binds to a specific binding protein which concentrates the steroid in this area [3]. Androstenone is also highly lipophilic and accumulates in the adipose tissue [4] leading to the disagreeable boar taint odour and flavour of meat from uncastrated male pigs.

The primary step in the synthesis of androstenone from pregnenolone is the formation of 5,16-androstadien-3β-ol (ANβ), which is catalyzed by the andien-β synthase system in a cytochrome P450C17 (CYP17A1) dependent reaction [2,5]. Alternatively, pregnenolone can be converted to 17α-hydroxypregnenolone (17OHP) via the 17α-hydroxylase reaction and then via the C17,20-lyase reaction to the androgen, dehydroepiandrosterone (DHEA). CYP17A1 is thus a key enzyme that regulates the production of androgens versus 16-androstene steroids.

The involvement of CYP17A1, microsomal cytochrome b5 (CYB5A, Type I), NADPH cytochrome P450 reductase (POR) and cytochrome b5 reductase (CYB5R3) in the andien-β synthase system has been shown previously [6,7]. Ogishima et al. [8] demonstrated that the outer mitochondrial membrane cytochrome b5 (CYB5B, Type II) is also involved in steroid hormone metabolism in rats and guinea pigs. The potential role of CYB5B in ANβ synthesis in pigs has not yet been investigated.

CYB5 has many roles such as: (a) transfer of electrons from NADH to desaturase [9], (b) NADH-dependent reduction of methemoglobin to regenerate hemoglobin [10], and (c) stimulation of cytochrome P450 dependent oxygenation [8]. The exact mechanism of CYB5 is not yet completely understood; however, POR is the primary electron donor because the ferric state of P450 is a lower redox potential than CYB5 [8]. Experiments with apo-CYB5, which lacks the heme moiety, and holo-CYB5 suggest that CYB5 is not responsible for direct electron transfer but exerts a saturable, allosteric effect on the CYP17A1–POR complex [11]. The POR functions by catalyzing electron transfer from NADPH to cytochrome P450 during catalysis [12] and is also involved in electron transfer from NADPH to heme oxygenase [13] and CYB5 [14].

NADH cytochrome b5 reductase (CYB5R3) works in a similar manner as shown below:

$$\mathrm{NADH} + \mathrm{H^+} + 2\mathrm{ferricytochromeCY}B5 \rightarrow \mathrm{NAD^+} + 2\mathrm{ferrocytochromeCY}B5$$

* Corresponding author. Tel.: +1 519 824 4120; fax: +1 519 836 9873.
*E-mail address:* jsquires@uoguelph.ca (E.J. Squires).

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CYB5R3 is a flavoprotein that catalyzes the reduction of CYB5, using FAD as a prosthetic group. Two forms of CYB5R3 are known, a membrane-bound form in somatic cells and a soluble form in erythrocytes [15]. The membrane-bound form consists of both the membrane binding domain and the catalytic domain, where as the soluble form only consists of the catalytic domain [16,17]. The role of CYB5R3 on the 17α-hydroxylase, C17,20 lyase and andien-β synthase activity of CYP17A1 has previously been investigated; CYB5R3 is not necessary for the production of 17OHP or DHEA, but CYB5R3 significantly increases andien-β synthase activity [6].

Among the several systems developed to characterize enzymatic activity, transfection of expression constructs into intact mammalian cells provides the opportunity to study the activity of the expressed proteins in the native microsomal environment and to study various combinations of enzymes, redox partners and substrates used [18,19]. In this report, we studied the effects of CYB5A and CYB5 B on the activities of CYP17A1 by transient transfection of human embryonic kidney (HEK-293FT) cells with expression constructs for CYP17A1, POR, CYB5R3 and either CYB5A or CYB5B. With this system we have the ability to over-express the proteins of interest, and to vary the relative amounts of these proteins to produce a well defined and active system with minimal interference from endogenous proteins that would occur with hepatocytes or microsomal preparations.

Our objective was to determine how CYB5A and CYB5B modulate the three activities of porcine CYP17A1: 17α-hydroxylase, C17,20 lyase and andien-β synthase activity. We were especially interested in systems that would maintain the normal production of sex steroids (through the 17α-hydroxylase and C17,20 lyase reactions) while decreasing the production of the 16-androstene steroids (through the andien-β synthase reaction), since this might lead to methods for controlling boar taint.

## 2. Materials and methods

### 2.1. Construction of expression vectors for POR, CYP17A1, CYB5R3, CYB5A and CYB5B

The entire coding regions of porcine POR, CYP17A1, CYB5R3, CYB5A and CYB5B were amplified from porcine testis cDNA by PCR using platinum Pfx DNA polymerase (Invitrogen) and primers listed in Table 1. The amplified segments were then cloned into pcDNA3.1/V5-His TOPO (Invitrogen) to produce expression vectors. Expression vectors for V5-His tagged proteins were generated so that the expressed proteins could be detected by Western blotting using anti-V5-HRP antibody; vectors expressing the untagged protein were also generated to determine if the V5-His tag adversely affected the activity of the protein. The PCR primers for porcine NADPH cytochrome P450 reductase (POR) were based on Genbank accession number L33893 and for porcine CYP17A1 were based on accession number NM 214428. The primers for porcine CYB5A and CYB5B were based on accession numbers NM 001001770 and AY609739 respectively. The sequence of porcine CYB5R3 was assembled from pig ESTs retrieved by BLAST searching the NCBI database using human CYB5R3 (accession number NM 000398) as a template. This sequence was used to design primers to amplify and clone porcine CYB5R3. The identity of all clones was confirmed by sequencing.

### 2.2. Transient expression in human embryonic kidney (HEK-293) cells

Human embryonic kidney cells (HEK-293FT; Invitrogen) were plated at $7 \times 10^5$ cells per well in Falcon 6-well culture tissue plates (BD Biosciences) and grown at 37 °C in Dulbecco's modified eagle's medium (GIBCO) supplemented with 10% fetal calf serum (PAA Laboratories, Etobicoke, ON), 1% geneticin, penicillin/streptomycin and L-glutamine, non-essential amino acids and sodium pyruvate. Once the cells were 90–95% confluent, vectors expressing CYP17A1, POR, CYB5R3, and CYB5A or CYB5B were transfected into HEK-293 cells using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions. The amounts of the plasmids for expression of CYP17A1, POR and CYB5R3 used in the transfections were adjusted to give approximately equal levels of expression for each protein. Variable amounts of the expression plasmids for CYB5A and CYB5B were used, with empty pcDNA3.1 vector added to bring the total amount of DNA to 4 μg for each transfection.

### 2.3. Western analysis of protein expression

Protein expression of HEK-293 cells transfected with pcDNA3.1/CYP17A1–V5-His, pcDNA3.1/POR–V5-His, pcDNA3.1/CYB5R3–V5-His and pcDNA3.1/CYB5A–V5-His or pcDNA3.2/CYB5B–V5-His was analyzed by Western blotting. The cells were harvested and lysed by sonication (three times for 30 s) in 300 μL RIPA lysis solution (1% Nonidet P-40, 0.1%, SDS and 0.5% sodium deoxycholate) including Complete protease inhibitor cocktail tablets (Roche). Samples containing 20 μg of protein were separated by electrophoresis on sodium dodecyl sulfate–16% polyacrylamide gels and transferred by semi-dry transblot to PVDF membranes. The blots were incubated overnight in 5% (w/v) dried skim milk in PBS with 0.1% Tween 20 and then incubated with a 1:5000 dilution of anti-V5-HRP antibody (Invitrogen) in PBS containing 0.1% Tween 20 and 5% skim milk powder. Antibody antigen complexes were identified by incubating with 50 μL of 68 mM p-coumaric acid (Sigma) in DMSO followed by 15 μL of 3% hydrogen peroxide added into 5 mL of 1.25 mM luminal (Sigma) in 0.1 M Tris pH 8.5. After 1 min the blot was exposed to film (Kodak) for 5–10 min and developed. The densities of the bands on the Western blots were analyzed by Northern Eclipse software (Empix Imaging).

### 2.4. Assay of enzymatic activity

The metabolism of pregnenolone was measured in HEK-293 cells transiently transfected with vectors expressing CYP17A1, POR, CYB5R3, and CYB5A or CYB5B. At 48 h after transfection

**Table 1**
Primers used to amplify sequences of tagged and untagged CYP17A1, POR, CYB5R3, CYB5A and CYB5B and the expression plasmids produced.

| Protein produced | Forward primer (5′ → 3′) | Reverse primer (5′ → 3′) | Plasmid name |
|---|---|---|---|
| CYP17A1 | gccatgtgggtgctcttggtttt | cagggtggagtcaggaggta | pcDNA3.1/CYP17A1 |
| CYP17A1–V5-His | gccatgtgggtgctcttggtttt | ggaggtactcccctcagtgt | pcDNA3.1/CYP17A1–V5-His |
| POR | gccatgggggactccaacgtgg | ggctggcagaagacagtgag | pcDNA3.1/POR |
| POR–V5-His | gccatgggggactccaacgtgg | gctccacacgtccagggagt | pcDNA3.1/POR–V5-His |
| CYB5R3 | gccatgggggcccagctgagca | ccatcagaaggcgaagcag | pcDNA3.1/CYB5R3 |
| CYB5R3–V5-His | gccatgggggcccagctgagca | gaaggcgaagcagcgctcct | pcDNA3.1/CYB5R3–V5-His |
| CYB5A | gccatggccgaacagtccgac | tttcttccattggcttctgc | pcDNA3.1/CYB5A |
| CYB5A–V5-His | gccatggccgaacagtccgac | gtttccgatgtgtagaagt | pcDNA3.1/CYB5A–V5-His |
| CYB5B | gccatggcgactgtggaagcca | tcaggaggatttgctctccg | pcDNA3.1/CYB5B |
| CYB5B–V5-His | gccatggcgactgtggaagcca | ggaggatttgctctccgccg | pcDNA3.1/CYB5B–V5-His |

[7-$^3$H(N)]-pregnenolone (10 μM, specific activity = 33 μCi μmol$^{-1}$) was added in fresh media to the 6-well culture plates. After incubation for 16 h, the media was collected and the cells were harvested. Cell debris was separated at 15,000 × *g* for 15 min. The media and cell debris were extracted twice with 4 mL ether and the organic phases were pooled and evaporated to dryness under nitrogen. The extracts were dissolved in 85% acetonitrile:15% $H_2O$ and the radioactive steroids were separated by HPLC on a Luna 5 μ 250 mm × 4.60 mm reverse phase C-18 column (Phenomenex, Torrance, CA). The equipment consisted of a Spectra-Physics model SP8880 autosampler, a Spectra-Physics model SP8800 Ternary HPLC Pump (Spectra-Physics, San Jose, CA) and a β-Ram model 2 radioactivity detector (IN/US Systems, Tampa, FL). The 16-androstene steroid product (ANβ) was separated from the pregnenolone substrate and other products (17OHP and DHEA) using a mobile phase of 85% acetonitrile delivered at 1 mL/min [20]. DHEA and 17OHP were separated using a 50% acetonitrile mobile phase [21]. Substrates and metabolites were identified by comparison with the retention time of reference steroids (Sigma).

### 2.5. Statistical analysis

All statistical analyses were made using SAS/STAT version 9.1 (SAS Institute Inc.). Treatments were compared using the general linear model procedure and a *t*-test was used to compare different means. Dunnets test was used to compare transfection levels of CYB5A/CYB5B to control at a confidence level of 95%. The Pearson correlation procedure was used to check for positive or negative correlations between the amount of CYB5A or CYB5B used in the transfections and production of ANβ, 17OHP and DHEA.

## 3. Results

### 3.1. Cloning of porcine CYB5R3 and optimization of the expression system

The nucleotide and deduced amino acid sequence of porcine CYB5R3 compared with human CYB5R3 is shown in Fig. 1. The

(A)

```
human   61   AGTCTGCTCATGAAGCTGTTCCAGCGCTCCACGCCAGCCATCACCCTCGAGAGCCCGGAC  120
             AG CTGCTCATGAAGCTGTTCCAGCG TCCAC CC GCCATCACCCTCGAGA CCC GAC
Pig     61   AGCCTGCTCATGAAGCTGTTCCAGCGTTCCACCCCGGCCATCACCCTCGAGAACCCAGAC  120

human  121   ATCAAGTACCCGCTGCGGCTCATCGACCGGGAGATCATCAGCCATGACACCCGGCGCTTC  180
             ATCAAGTA CCGCTG GGCTCAT GAC  GGAG TC TCA CCATGACACCCGGCG TTC
Pig    121   ATCAAGTATCCGCTGAGGCTCATTGACAAGGAGGTCGTCAACCATGACACCCGGCGGTTC  180

human  181   CGCTTTGCCCTGCCGTCACCCCAGCACATCCTGGGCCTCCCTGTCGGCCAGCACATCTAC  240
             CGCTTTGCCCTGCCGTC CCCCAGCACATCCTGGGCCTCCCTGT GGCCAGCACATCTAC
Pig    181   CGCTTTGCCCTGCCGTCGCCCCAGCACATCCTGGGCCTCCCTGTGGGCCAGCACATCTAC  240

human  241   CTCTCGGCTCGAATTGATGGAAACCTGGTCGTCCGGCCCTATACACCCATCTCCAGCGAT  300
             CTCTCGGCTCG ATTGATGG AA CTGGTC T CGGCCCTA AC CCC TCTCCAG GAT
Pig    241   CTCTCGGCTCGGATTGATGGGAATCTGGTCATTCGGCCCTACACGCCCGTCTCCAGTGAT  300

human  301   GATGACAAGGGCTTCGTGGACCTGGTCATCAAGGTTTACTTCAAGGACACCCATCCCAAG  360
             GA GACAAGGGCTT GTGGACCTGGTCATCAAGGT TACTTCAA GACACCCA CCCAAG
Pig    301   GACGACAAGGGCTTTGTGGACCTGGTCATCAAGGTGTACTTCAAAGACACCCACCCCAAG  360

human  361   TTTCCCGCTGGAGGGAAGATGTCTCAGTACCTGGAGAGCATGCAGATTGGAGACACCATT  420
             TTTCCCGC GGAGGGAAGATGTC CAGTACCTGGAGAGCATG AGAT GGAGACACCAT
Pig    361   TTTCCCGCCGGAGGGAAGATGTCCCAGTACCTGGAGAGCATGAAGATCGGAGACACCATC  420

human  421   GAGTTCCGGGGCCCCAGTGGGCTGCTGGTCTACCAGGGCAAAGGGAAGTTCGCCATCCGA  480
             GAGTTCCGGGGCCCCA  GGGCTGCTGGTCTACCAGGGCAAAGG AAGTT GCCATCCG
Pig    421   GAGTTCCGGGGCCCCAACGGGCTGCTGGTCTACCAGGGCAAAGGAAAGTTTGCCATCCGC  480

human  481   CCTGACAAAAAGTCCAACCCTATCATCAGGACAGTGAAGTCTGTGGGCATGATCGCGGGA  540
             CC GACAA AA TCCA  CC  TCATCA GAC GTGAAGTCTGT GGCATGATCGCGGGA
Pig    481   CCGGACAAGAAATCCAGTCCCGTCATCAAGACGGTGAAGTCTGTTGGCATGATCGCGGGA  540

human  541   GGGACAGGCATCACCCCGATGCTGCAGGTGATCCGCGCCATCATGAAGGACCCTGATGAC  600
             GG AC GGCATCACCCC ATGCTGCAGGTGATCCG GCCATCATGAAGGACCC GATGAC
Pig    541   GGAACCGGCATCACCCCAATGCTGCAGGTGATCCGAGCCATCATGAAGGACCCGGATGAC  600

human  601   CACACTGTGTGCCACCTGCTCTTTGCCAACCAGACCGAGAAGGACATCCTGCTGCGACCT  660
             CACAC GTGTGCCACCTGCTCTTTGCCAACCAGACCGAGAAGGACATCCTGCTGCG CC
Pig    601   CACACCGTGTGCCACCTGCTCTTTGCCAACCAGACCGAGAAGGACATCCTGCTGCGGCCC  660

Human  661   GAGCTGGAGGAACTCAGGAACAAACATTCTGCACGCTTCAAGCTCTGGTACACGCTGGAC  720
             GAGCTGGAGGAACT AGGAA  AACATTCTGC CGCTTCAAGCTCTGGTACACG TGGAC
Pig    661   GAGCTGGAGGAACTGAGGAATGAACATTCTGCGCGCTTCAAGCTCTGGTACACGGTGGAC  720

human  721   AGAGCCCCTGAAGCCTGGGACTACGGCCAGGGCTTCGTGAATGAGGAGATGATCCGGGAC  780
             AGAGCCCC GAAGCCTGGGACTAC GCCAGGGCTTCGTGAA GAGGAGATGATCCGGGAC
Pig    721   AGAGCCCCAGAAGCCTGGGACTACAGCCAGGGCTTCGTGAACGAGGAGATGATCCGGGAC  780

human  781   CACCTTCCACCCCCAGAGGAGGAGCCGCTGGTGCTGATGTGTGGCCCCCCACCCATGATC  840
             CACCTTCC CCCCC GAGGAGGAGCCGCTGGTGCTGATGTG GG CCCCC CCCATGATC
Pig    781   CACCTTCCGCCCCCGGAGGAGGAGCCGCTGGTGCTGATGTGCGGGCCCCCGCCCATGATC  840

human  841   CAGTACGCCTGCCTTCCCAACCTGGACCACGTGGGCCACCCCACGGAGCGCTGCTTCGTC  900
             CAGTACGCCTGCCT CCCAACCTGGA C CGTGGGCCACCCCA GGAGCGCTGCTTCG C
Pig    841   CAGTACGCCTGCCTGCCCAACCTGGAGCGCGTGGGCCACCCCAAGGAGCGCTGCTTCGCC  900

human  901   TTCTGA  906
             TTCTGA
Pig    901   TTCTGA  906
```

**Fig. 1.** The nucleotide (A) and deduced amino acid (B) sequence of porcine CYB5R3 compared to human CYB5R3. The homologous sequences between porcine and human are highlighted.

(B)

```
human   1    MGAQLSTLGHMVLFPVWFLYSLLMKLFQRSTPAITLESPDIKYPLRLIDREIISHDTRRF  60
             MGAQLSTLGH+VL PVWFLYSLLMKLFQRSTPAITLE+PDIKYPLRLID+E+++HDTRRF
Pig     1    MGAQLSTLGHVVLSPVWFLYSLLMKLFQRSTPAITLENPDIKYPLRLIDKEVVNHDTRRF  60

human   61   RFALPSPQHILGLPVGQHIYLSARIDGNLVVRPYTPISSDDDKGFVDLVIKVYFKDTHPK  120
             RFALPSPQHILGLPVGQHIYLSARIDGNLV+RPYTP+SSDDDKGFVDLVIKVYFKDTHPK
Pig     61   RFALPSPQHILGLPVGQHIYLSARIDGNLVIRPYTPVSSDDDKGFVDLVIKVYFKDTHPK  120

human   121  FPAGGKMSQYLESMQIGDTIEFRGPSGLLVYQGKGKFAIRPDKKSNPIIRTVKSVGMIAG  180
             FPAGGKMSQYLESM+IGDTIEFRGP+GLLVYQGKGKFAIRPDKKS+P+I+TVKSVGMIAG
Pig     121  FPAGGKMSQYLESMKIGDTIEFRGPNGLLVYQGKGKFAIRPDKKSSPVIKTVKSVGMIAG  180

human   181  GTGITPMLQVIRAIMKDPDDHTVCHLLFANQTEKDILLRPELEELRNKHSARFKLWYTLD  240
             GTGITPMLQVIRAIMKDPDDHTVCHLLFANQTEKDILLRPELEELRN+HSARFKLWYT+D
Pig     181  GTGITPMLQVIRAIMKDPDDHTVCHLLFANQTEKDILLRPELEELRNEHSARFKLWYTVD  240

human   241  RAPEAWDYGQGFVNEEMIRDHLPPPEEEPLVLMCGPPPMIQYACLPNLDHVGHPTERCFV  300
             RAPEAWDY QGFVNEEMIRDHLPPPEEEPLVLMCGPPPMIQYACLPNL+ VGHP ERCF
Pig     241  RAPEAWDYSQGFVNEEMIRDHLPPPEEEPLVLMCGPPPMIQYACLPNLERVGHPKERCFA  300

human   301  F  301
             F
Pig     301  F  301
```

**Fig. 1.** (*Continued*).

homology between porcine and human was 90% and 93% at the nucleotide and amino acid level respectively.

We first optimized the HEK-293 system to obtain similar levels of protein expression and activity and to determine the optimum time for protein expression following transfection. Fig. 2A displays a Western blot of cells expressing V5-tagged CYP17A1, POR, CYB5R3, CYB5A and CYB5B at 24, 48, 72 and 96 h after transfection. The densities of the bands on the Western blots were analyzed by Northern Eclipse software (Empix Imaging) and the percent change in band densities are plotted in Fig. 2B. The expression of the different proteins varied over time. The expressions of CYP17A1 and CYB5A were maximal at 48 h and then declined thereafter, while the expressions of POR, CYB5B and CYB5R3 continued to increase up to 96 h. The 48 h time point was subsequently chosen as the optimal time for expression and assay of enzyme activity.

The production of the pregnenolone metabolites, ANβ, and DHEA + 17OHP was determined next over time (Fig. 3). HEK-293 cells were transfected with expression vectors for CYP17A1, POR, CYB5R3 and CYB5A and 48 h after transfection, [7-$^3$H(N)]-pregnenolone substrate was added in fresh media. Cells and media were collected after 2, 4, 8, 16 and 24 h of incubation with substrate and the metabolites were extracted and analyzed by HPLC with a mobile phase of 85% acetonitrile:15% $H_2O$. The production of ANβ and DHEA + 17OHP was linear up to 16 h. The activity of the proteins in the expression system was similar for both the untagged and V5-His tagged proteins (data not shown).

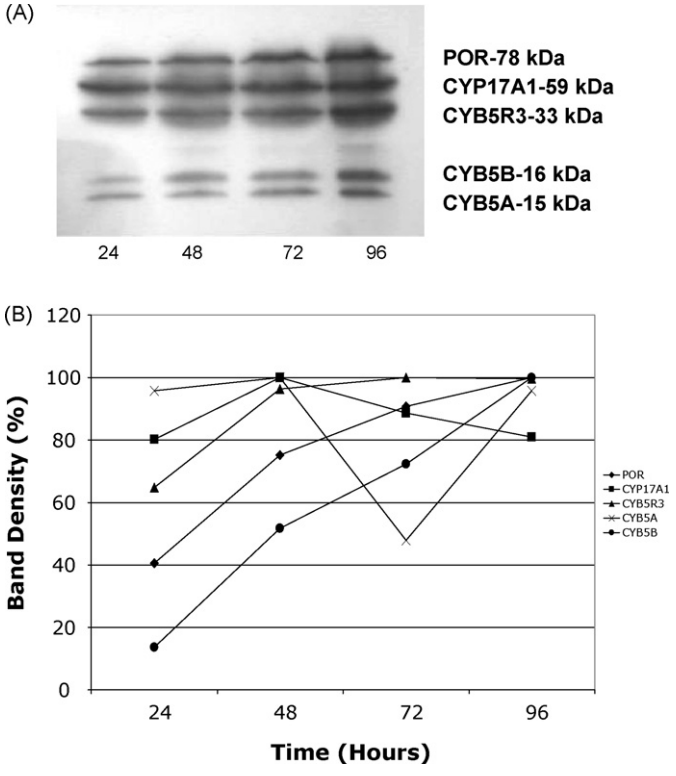


**Fig. 2.** (A) Western blot showing the expression of CYP17A1, POR, CYB5R3, CYB5A and CYB5B at 24, 48, 72 and 96 h after transfection. Proteins were separated on 16% SDS-PAGE, blotted and anti-V5 HRP antibody was used for detection. CYP17A1 (59 kDa), POR (78 kDa), CYB5R3 (33 kDa), CYB5A (15 kDa) and CYB5B (16 kDa). (B) Band densities were determined by scanning the Western blots and the results are plotted as percent of maximum.

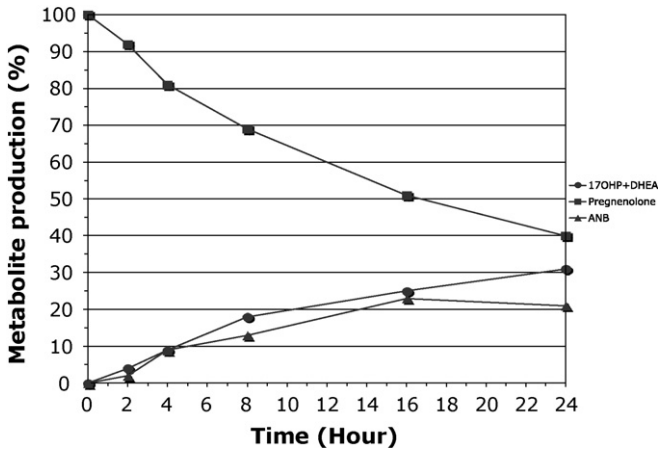


**Fig. 3.** The production of pregnenolone metabolites ANβ, DHEA + 17OHP over time. HEK-293 cells were transfected with expression vectors for POR (0.35 μg), CYP17A1 (0.25 μg), CYB5R3 (0.25 μg) and CYB5A (1 μg). At 48 h after transfection, cells were incubated with $^3$H-pregnenolone substrate and products were analyzed by HPLC with a mobile phase of 85% acetonitrile.

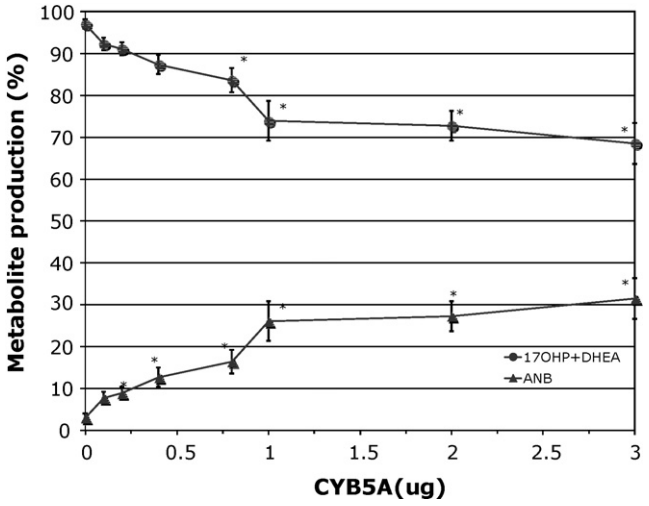


**Fig. 4.** The effect of increased expression of CYB5A on the production of DHEA + 17OHP and ANβ. CYP17A1, POR, CYB5R3 and CYB5A were transfected into HEK-293 cells and products were analyzed by HPLC with a mobile phase of 85% acetonitrile. The results are expressed as the mean ± S.E. of five replicate experiments with duplicate transfections in each experiment. *Significantly different ($p < 0.05$) compared to the control (CYB5A = 0 μg).

### 3.2. Effect of CYB5 isoforms on C17α-hydroxylase/C17,20 lyase activities versus andien-β synthase activity

Transfections of HEK cells were performed with 0.25 μg pcDNA3.1/CYP17A1, 0.35 μg pcDNA3.1/POR, 0.25 μg pcDNA3.1/CYB5R3 and from 0 to 3 μg pcDNA3.1/CYB5A or pcDNA3.1/CYB5B, with empty pcDNA 3.1 vector added to maintain a consistent level of 4 μg of plasmid DNA in each transfection. The transfected cells were incubated with $^{3}$H-pregnenolone and the products of the combined 17α-hydroxylase/C17,20 lyase reactions (17OHP + DHEA) and andien-β synthase (ANβ) reactions were analyzed via HPLC with a mobile phase of 85% acetonitrile. Increasing the expression of CYB5A (Fig. 4) resulted in a significant decrease in the combined 17α-hydroxylase/C17,20 lyase activities ($p < 0.013$) from 97% of total products without CYB5A, to 69% of total products formed with 3 μg of CYB5A transfected. However, increasing the expression of CYB5B had no significant effect on combined 17α-hydroxylase/C17,20 lyase activities (Fig. 5).

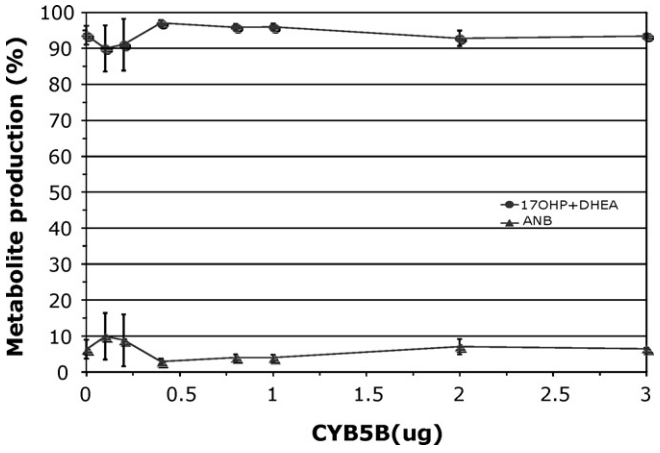


**Fig. 5.** The effect on increased expression of CYB5B on the production of DHEA + 17OHP and ANβ. CYP17A1, POR, CYB5R3 and CYB5B were transfected into HEK-293 cells and products were analyzed by HPLC with a mobile phase of 85% acetonitrile. The results are expressed as the mean ± S.E. of five replicate experiments with duplicate transfections in each experiment. *Significantly different ($p < 0.05$) compared to the control (CYB5B = 0 μg).

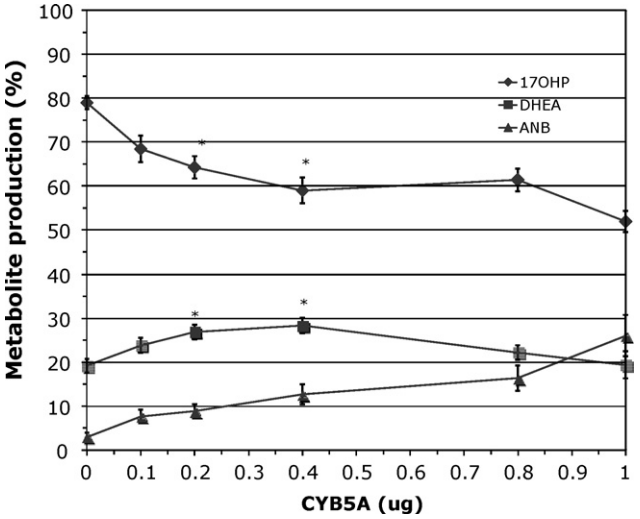


**Fig. 6.** The effect of increased expression of CYB5A on the production of 17OHP and DHEA. CYP17A1, POR, CYB5R3 and CYB5A were transfected into HEK-293 cells and products were analyzed by HPLC with a mobile phase of 50% acetonitrile. The results are expressed as the mean ± S.E. of five replicate experiments with duplicate transfections in each experiment. *Significantly different ($p < 0.05$) compared to the control (CYB5A = 0 μg).

There was a significant positive correlation ($r = 0.503$) between the amounts of CYB5A plasmid transfected and ANβ production ($p < 0.0001$), to a maximum of 31% of total products at 3 μg of CYB5A plasmid transfected (Fig. 4). However, increased expression of CYB5B did not affect the production of ANβ, with maximal production of ANβ not exceeding 10% of overall products formed (Fig. 5).

### 3.3. Effects of CYB5 isoforms on 17α-hydroxylase versus C17,20 lyase activity

The effect of the CYB5 isoforms on 17α-hydroxylase versus C17,20 lyase activities was analyzed by separating 17OHP and DHEA production for each individual transfection by HPLC using a mobile phase of 50% acetonitrile. The production of 17OHP was negatively correlated ($r = -0.779$) with increasing amounts of

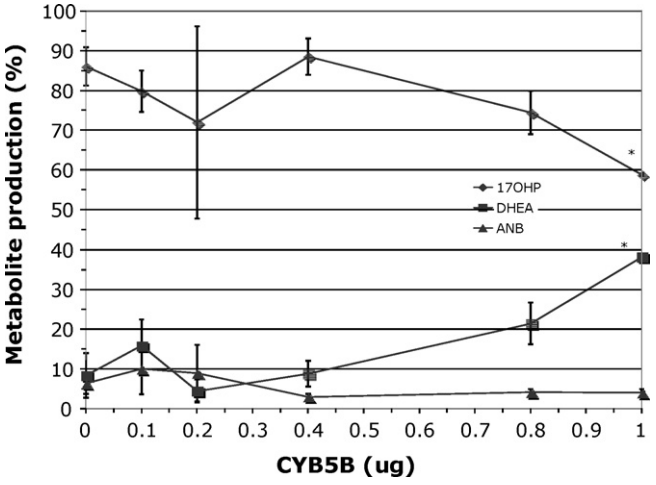


**Fig. 7.** The effect of increased expression of CYB5B on the production of 17OHP and DHEA. CYP17A1, POR, CYB5R3 and CYB5B were transfected into HEK-293 cells and products were analyzed by HPLC with a mobile phase of 50% acetonitrile. The results are expressed as the mean ± S.E. of five replicate experiments with duplicate transfections in each experiment. *Significantly different ($p < 0.05$) compared to the control (CYB5B = 0 μg).

CYB5A ($p < 0.0001$, Fig. 6). The production of DHEA was transiently increased at the 0.2 and 0.4 μg levels of CYB5A transfection and then decreased at higher transfection levels of CYB5A. The production of 17OHP was also negatively correlated ($r = -0.564$) with increasing CYB5B transfection levels ($p < 0.004$, Fig. 7) while DHEA production was significantly positively correlated ($r = 0.565$) with increasing expression of CYB5B ($p < 0.004$).

## 4. Discussion

The primary purpose of this study was to investigate the effect of CYB5A versus CYB5B on the activity of porcine CYP17A1 in catalyzing the synthesis of ANβ. This is the initial step in the synthesis of 16-androstene steroids, which are a major component of boar taint in entire male pigs. A more complete understanding of this process may lead to targets for selective breeding or other methods for decreasing the production of 16-androstene steroids and thereby reduce boar taint.

It has been demonstrated previously that CYB5A stimulates the synthesis of ANβ by porcine CYP17A1, as well as decreasing 17OHP production and increasing DHEA production [6,7]. CYB5B has been shown to stimulate the 17α-hydroxylase and C17,20 lyase reactions of CYP17 from rat and guinea pig [8], but the potential role of CYB5B on synthesis of ANβ in pigs has not previously been investigated. We postulated that CYB5B may have the potential to stimulate the production of 17OHP and DHEA by porcine CYP17A1 at a level similar to CYB5A, while significantly reducing the production of 16-androstene steroids. Thus, increasing the expression of CYB5B and decreasing the expression of CYB5A would maintain the production of androgens, while decreasing the production of 16-androstene steroids. This would allow entire male pigs to be produced that have the improved lean growth and feed efficiency due to the anabolic effects of testicular steroids, but do not have boar taint.

In this experiment, we investigated the interactions between CYP17A1, POR, CYB5R3 and CYB5A/CYB5B by over-expressing various amounts of these proteins in HEK-293 cells. CYP17A1 catalyzes two types of reactions: hydroxylation and the cleavage process also referred to as the fragmentation path by Lee-Robichaud et al. [22]. These authors demonstrated that when porcine CYP17A1 is challenged with a substrate analogue in which the C-20 position contains a highly electrophilic aldehyde group, the iron peroxide is trapped to alter the entire course of enzymatic reactions towards the exclusive formation of 16-androstene steroids [22].

CYP17A1 catalyzes the production of 17OHP and DHEA from pregnenolone via the 17α-hydroxylase and C17,20 lyase reactions [23], as well as the production of ANβ by the andien-β synthase pathway. Consistent with our results, it has been previously observed in porcine microsomes that the production of ANβ required the presence of CYB5A [6,7,22]. Our results show that the increased expression of CYB5A is negatively correlated with the production of 17OHP, while concurrently positively correlated with the production of ANβ. CYB5A thus switches the activity of CYP17A1 from 17α-hydroxylation of pregnenolone to removal of the carbon side chain and formation of the delta 16 double bond.

The potential role of the outer mitochondrial CYB5B in 16-androstene steroid production has not been previously investigated; however, the ability of CYB5B to modulate 17α-hydroxylase and C17,20 lyase activity has been investigated in humans [24]. Our present results show that porcine CYB5A and CYB5B increase the production of DHEA by CYP17A1, while decreasing the production of 17OHP. However, CYB5B had no effect on the production of ANβ by CYP17A1. Thus, CYB5A stimulates both the C17,20 lyase and andien-β synthase pathways, while CYB5B only stimulates the C17,20 lyase pathway and has no effect on the andien-β synthase pathway.

The role of CYB5A as a primary electron donor was investigated with "humanized" yeast strains and it was observed that when the amount of POR was low, maximal stimulation of C17,20 lyase activity occurred at a CYB5:CYP17A1 ratio of 3:1, with higher ratios inhibitory [25]. These results were consistent with our observations of an inhibitory effect on C17,20 lyase activity at higher ratios of CYB5A but not CYB5B. This suggests that CYB5 may not function as an electron donor, but perhaps facilitates the transfer of electrons from POR to CYP17A1. Our results also suggest that CYB5A and CYB5B work by different mechanisms and likely interact differently with POR and CYP17A1. The stimulatory effect of CYB5A on androgen and 16-androstene steroid production was previously investigated and it was hypothesized that (a) CYB5 is involved in the second electron transfer, since participation in the first electron transfer is unlikely because the redox potential of the ferric state of POR is lower than that of the ferrous state of CYB5 or (b) POR undergoes conformational changes through complex formation between POR and CYB5 [8].

We have shown that 17OHP production was negatively correlated with increasing expression of CYB5A and CYB5B, while DHEA production was positively correlated with expression of CYB5B. The decrease in 17OHP production and the concurrent increase in DHEA production suggests that 17OHP may be a substrate for the C17,20 lyase activity. Previous studies by Soucy and Luu-The [26] also suggest that the reactions catalyzed by human CYP17A1 follow two distinct steps; with a dissociation of the intermediate from the active site, the increase in DHEA would not necessarily correspond with a decreased accumulation of 17OHP.

The homology of the amino acid sequences between porcine CYB5A and CYB5B is 48%, with CYB5A and CYB5B consisting of 134 and 144 amino acids respectively. Perhaps the lack of an increase in ANβ production at higher expression of CYB5B can be explained by conformational differences between CYB5 isoforms A and B. Lee-Robichaud et al. [22,27] concluded in a study of human CYP17A1 that the interaction between CYP17A1 and CYB5 is governed by the presence of a membrane insertable hydrophobic region on CYB5, but also by the defined spatial orientation of the exposed globular domain at the C-terminus. Residues 1–92 are folded to create a compact globular domain, whereas the C-terminal sequence residues from 93-onwards form a hydrophobic tail, which protrudes through the membrane bilayer [27]. Perhaps the extra 10 amino acids on CYB5B change the interaction of the globular domain with CYP17A1. This conformational difference may allosterically alter the conformational change of POR once bound to CYP17A1and favour the C17,20 lyase activity over the andien-β synthase pathway, reducing the production of ANβ. This may be related to conformational changes that allow the 17α-hydroxylase intermediate to remain bound to the active site of CYP17A1 more than when CYB5A is present, thus increasing DHEA synthesis.

In summary, we have found that CYB5B and CYB5A interact with porcine CYP17A1 to produce increased amounts of DHEA, but CYB5B does not stimulate the production of ANβ. Increased expression of CYB5A first increases and then decreases the production of DHEA, but this was not seen with CYB5B. It is likely that the mechanisms by which CYB5A and CYB5B interact with the CYP17A1–POR complex are different. These results point to the different isoforms of CYB5 as selective targets for producing entire male pigs with a normal production of sex steroids and decreased production of the 16-androstene steroids that contribute to boar taint.

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