Molecular cloning and regulation of porcine *SULT2A1*: relationship between *SULT2A1* expression and sulfoconjugation of androstenone

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Abstract

Hydroxysteroid sulfotransferase (*SULT2A1*) is a key enzyme in the testicular and hepatic metabolism of 5 α -androstenone, which is a major component of the off-odor and off-flavor in pork known as boar taint. The goals of this study were to determine the role of testicular and hepatic *SULT2A1* activity on plasma 5 α -androstenone sulfate levels, the accumulation of 5 α -androstenone in adipose tissue, and to gain insight into the regulatory control of *SULT2A1*. Testicular *SULT2A1* activity was negatively correlated ($r = -0.57$; $P < 0.01$) with 5 α -androstenone concentrations in fat. The differences observed in *SULT2A1* activity warranted investigation into potential genetic variation within porcine *SULT2A1*. The cDNA sequence of porcine *Sult2A1* was determined to be >82% homologous to the human, mouse, and rat *Sult2A1* genes. A single nucleotide polymorphism was detected within the coding region of the *Sult2A1* from individual testes and liver samples; however, this did not affect the amino acid sequence of the enzyme. Western blot analysis determined that animals with high concentrations of 5 α -androstenone in fat and low *SULT2A1* activity had corresponding low levels of *SULT2A1* protein compared with animals with low levels of 5 α -androstenone in fat. Real-time PCR analysis indicated that *Sult2A1* mRNA was increased 2.8-fold in animals with high levels of the protein relative to animals with low levels of the protein. Furthermore, we demonstrated the positive role of the nuclear receptors constitutive androstane receptor and pregnane X receptor, as well as the possible role of farnesoid X receptor in the regulation of testicular *SULT2A1* activity. Together, the results of this study suggest that differences in *SULT2A1* expression can influence 5 α -androstenone accumulation in fat.

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Sulfotransferase enzymes are cytosolic proteins involved in catalyzing the conjugation of many steroids, bile acids, and xenobiotics. Sulfotransferases utilize the donor molecule 3'-phosphoadenosine 5'-phosphosulfate (PAPS) for the transfer of a sulfate radical (SO₃⁻) to a hydroxyl acceptor site. In terms of steroids, hydroxyl groups at positions 3, 21, and 17 of the steroid nucleus are the most common locations for sulfoconjugation (Strott 1996). With the addition of the sulfate group, the polarity of the steroid conjugate is greatly enhanced, causing an increase in water solubility (Jakoby *et al.* 1980). Therefore, sulfoconjugation of hydroxysteroids has been regarded as a major mechanism for their metabolism and excretion (Strott 2002).

Steroid sulfotransferase enzymes are located in many different organs such as the liver, adrenal gland, ovary, and testis (Gasparini *et al.* 1976, Hobkirk 1985). In the boar, the testis is the main organ responsible for steroid sulfate synthesis (Raeside & Renaud 1983, Hobkirk *et al.* 1989). One of the major steroid sulfotransferases is hydroxysteroid sulfotransferase, which has very wide substrate specificity. Its primary substrate is dehydroepiandrosterone (DHEA), and thus it was originally named

DHEA-sulfotransferase (Comer *et al.* 1993). In recent years DHEA-sulfotransferase has been further classified to the 2A family of human sulfotransferases, and is designated as *SULT2A1* by the HUGO Nomenclature Committee (www.gene.ucl.ac.uk/hugo/index.html).

Hydroxysteroid sulfotransferase is responsible for sulfoconjugating the 16-androstene steroids (Sinclair & Squires 2005), which have pheromonal properties and are the most abundant steroids produced by the boar testes. The accumulation of high levels of the 16-androstene steroid, 5 α -androstenone, in adipose tissue produces an unpleasant odor when the meat from intact males is cooked (Patterson 1968). Our previous studies have indicated that increased levels of sulfoconjugated 16-androstene steroids present in the systemic circulation are associated with a reduction in the accumulation of 5 α -androstenone in adipose tissue (Sinclair & Squires 2005). However, the relationship between *SULT2A1* activity in the testis and liver, and the concentration of sulfoconjugated 16-androstenes in the circulation has not been demonstrated.

In humans, *SULT2A1* activity varies up to five-fold among individuals (Weinshilboum & Aksoy 1994), suggesting that genetic polymorphisms may be involved

in regulating SULT2A1 activity. A number of studies reported single nucleotide polymorphisms (SNPs) within the human *Sult2A1* gene (Igaz *et al.* 2002), some of which have resulted in reduced levels of both enzyme activity and protein (Thomae *et al.* 2002). Furthermore, research in both human and rodent SULT2A1 regulation has revealed that this gene may be controlled by various nuclear receptors including the constitutive androstane receptor (CAR) (Saini *et al.* 2004), the pregnane X receptor (PXR) (Kliwer *et al.* 1998, Duanmu *et al.* 2002, Echchgadda *et al.* 2004), and the farnesoid X receptor (FXR) (Makishima *et al.* 1999, Song *et al.* 2001). However, the involvement of nuclear receptors in the regulation of porcine SULT2A1 has not been determined.

The initial goal of this study was to determine whether differences in testicular and hepatic SULT2A1 activity are related to levels of sulfoconjugated 16-androstenes in blood, and thus, the capacity for 5 α -androstenone to accumulate in adipose tissue. Because of the significant role of sulfoconjugation of the 16-androstene steroids in the development of boar taint, it was important to understand the molecular basis for individual variation in the expression and function of the SULT2A1 gene in market weight boars. Therefore, the porcine *Sult2A1* cDNA was cloned and sequenced, and genetic polymorphisms in the *Sult2A1* gene that may cause alterations in enzyme function were examined. In addition, the regulatory control of SULT2A1 activity by various nuclear receptor ligands was investigated.

Materials and methods

Reagents

Sep-Pak C₁₈ solid-phase chromatography cartridges were purchased from Waters Ltd (Mississauga, ON, Canada). HANKS balanced salt solution (HBSS) was obtained from Invitrogen Life Technologies (Burlington, ON, Canada). Radiolabeled [³H]DHEA and DHEA sulfate (DHEAS) (approximately 0.01–0.05 μ Ci/nmol) were obtained from ICN Diagnostics (Montreal, QC, Canada). 5 α -Androstenone was obtained from Steraloids Inc. (Newport, RI, USA) and 6-(4-chlorophenyl)imidazo [2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl) oxime (CITCO) was obtained from Biomol Research Laboratories Inc. (Plymouth Meeting, PA, USA). Pregnenolone 16 α -carbonitrile (PCN), 1,4-bis-[2-(3,5-dichloropyridyl-oxy)]benzene; 3,3',5,5'-tetrachloro-1,4-bis (pyridyl-oxy)benzene (TCPOBOP) and all other reagents were obtained from Sigma-Aldrich Ltd (Mississauga, ON, Canada).

Tissue samples

A total of 28 Yorkshire boars (group A) of 175 $\pm$ 6 days of age were obtained from the Arkell Swine Research

Station at the University of Guelph, Guelph, ON. Procedures were approved by the Animal Care Committee at the University of Guelph and animals were cared for according to the Canadian Council on Animal Care requirements. Blood samples were taken from the orbital sinus, centrifuged at 4 °C to collect plasma and stored at –20 °C until extraction and analysis for 5 α -androstenone concentrations.

The animals were slaughtered at an average live weight of 125 $\pm$ 13 kg. A backfat (adipose tissue) sample was removed from the midline on the point of the 11th rib and frozen at –20 °C until assayed for 5 α -androstenone. Samples of liver and testis tissue were taken immediately following exsanguination, frozen in liquid nitrogen and stored at –70 °C until needed.

An additional three mature Yorkshire boars (group B) of 175 $\pm$ 6 days of age were used for the isolation of Leydig cells. After slaughter, the testes were immediately removed and transported to the laboratory within 5 min. Testicular tissue was sliced into pieces of 1 cm² $\times$ 2–3 mm thickness and 100 g tissue samples were incubated for 20 min in a shaking water bath at 37 °C with 1 mg/ml collagenase (type 1A), 50 μ g/ml DNase and 50 μ g/ml trypsin inhibitor in 250 ml TC 199 Media containing 1 g/l bovine serum albumin and 0.1 g/l L-glutamine. Preparations of purified Leydig cells were obtained by layering the collagenase-dispersed testicular cells onto discontinuous Percoll gradients supplemented with HBSS. The preparations were then centrifuged at 1500 $\times$ g for 15 min at 4 °C and the cells present at the 40–60% interface were collected as outlined previously (Raeside & Renaud 1983). Cell viability was determined by trypan blue exclusion. The typical viability of Leydig cells after this procedure was greater than 90%.

Steroid extraction and analysis

Conjugated steroids were separated from unconjugated steroids using methanol-primed Sep-Pak C₁₈ solid-phase chromatography cartridges (Raeside & Christie 1997). Sulfoconjugated steroids were hydrolyzed by incubating the conjugate fraction overnight in trifluoroacetic acid/ethyl acetate (1/100 v/v) at 45 °C. The hydrolyzed steroids were then purified by Sep-Pak C₁₈ solid-phase chromatography.

Fat and extracted plasma samples were analyzed for 5 α -androstenone with an ELISA method, as described previously (Squires & Lundström 1997). Media extracts were measured for DHEAS by radioimmunoassay (Schwarzenberger *et al.* 1993).

Preparation of cytosol

Frozen testes and liver samples were partially thawed and a 20% (w/v) homogenate was prepared in 100 mM Tris–HCl, 10 μ M EDTA, and 250 mM sucrose at

pH 7.4. Homogenates were centrifuged for 15 min at $10\,000 \times g$ at 4 °C and the resulting supernatant was removed and centrifuged for 60 min at $105\,000 \times g$ at 4 °C. The supernatant (cytosol) was removed and stored at -70 °C. The protein content was determined using the Bradford protein assay (Bio-Rad Laboratories Ltd, Mississauga ON, Canada) using bovine serum albumin as a standard.

Cytosolic sulfotransferase activity assay

Sulfotransferase activity was assayed by the method of Matsui *et al.* (1993) using DHEA as a substrate. The incubation media contained 100 mM Tris/HCl, 100 µM EDTA, 100 µM PAPS, 10 mM MgCl₂ and 50 µM [³H]DHEA (approx. 0.01–0.05 µCi/nmol) in a final volume of 500 µl at pH 7.4. Incubations were carried out at 37 °C for 15 min with 25 µg total cytosolic protein. Blank values were obtained with incubations without PAPS. The reactions were terminated by the addition of 100 µl 0.1 M NaOH, followed by snap-freezing in liquid nitrogen. The samples were then immediately extracted by Sep-Pak C₁₈ solid-phase chromatography, as described above. After extraction, 100 µl aliquots of the unconjugated and conjugated fractions were subjected to liquid scintillation counting.

Screening of a porcine cDNA RACE library and sequence analysis

A rapid amplification of the 5' and 3' cDNA ends (RACE) library was constructed from 1 µg total RNA from liver with the use of the Smart RACE cDNA amplification kit (BD Biosciences, Mississauga, ON, Canada) as described previously (Lin *et al.* 2004). The cDNA library was used as a template in the subsequent PCR screening of porcine *Sult2A1*. The first fragment of porcine *Sult2A1* was amplified with primers designed from a porcine expressed sequence tag (Genbank Accession No. BI402591) related to reproductive function that was 82% homologous to human *Sult2A1*. To obtain the full-length porcine *Sult2A1* cDNA, forward and reverse primers were designed based on the sequence obtained from the 5' and 3' RACE and used to amplify the full-length porcine *Sult2A1* with either 5' or 3' RACE cDNA as a template. The nucleotide sequence of the forward primer was 5' CACGAGGCGCAAA GAAC T 3', and the reverse primer was 5' CATGT GCAAGGACAGGTGAG 3'. The PCR consisted of 35 cycles of denaturing for 1 min at 94 °C, annealing for 1 min at 63 °C, and extending for 1 min at 72 °C. A final extension step was performed for 10 min at 72 °C. Ten microlitres of the PCR product were analyzed by electrophoresis on a 1% agarose gel.

The PCR fragments were ligated into a pGEM-T Easy Vector System (Promega, Madison, WI, USA), and then transformed into competent DH5α cells. Plasmid DNAs were purified and sequenced.

Isolation of total RNA and reverse transcription-PCR

A total of 100 mg of testes and liver tissue was homogenized in 1 ml Tri-Reagent (Sigma-Aldrich Ltd) and total RNA was isolated according to the manufacturer's instructions. Approximately 0.5 µg total RNA was used to synthesize first strand cDNA using Superscript II RNase H⁻ Reverse Transcriptase (Invitrogen Life Technologies), following the manufacturer's instructions. The RT reaction was performed at 25 °C for 10 min, 42 °C for 50 min, followed by a 15 min incubation at 70 °C. First-strand cDNAs were stored at 4 °C until single-strand conformational polymorphism (SSCP) or RT-PCR analysis.

Following the reverse transcription reaction, 2.5 µl of the first strand cDNA were used as a template for PCR. The PCR mixture contained 100 mM Tris/HCl pH 8.3, 500 mM KCl, 11 mM MgCl₂, 0.1% gelatin, 0.2 mM dNTP, 2.5 U Red Taq polymerase (Sigma-Aldrich Ltd), and 0.4 mM of the porcine SULT2A1 forward and reverse primers. The PCR profile was the same as stated above.

SSCP analysis

The PCR products from both testicular and hepatic cDNAs were digested into fragments (230, 345 and 459 bp) with XmiI and SacI restriction endonucleases (MBI Fermentas, Burlington, ON, Canada) for 3 h at 37 °C. A total of 7 µl of the digested cDNA fragments was combined with 13 µl loading buffer (10% sucrose, 0.01% Bromophenol blue, 0.01% Xylene cyanol FF). The samples were then denatured at 100 °C for 5 min, followed by immediate cooling on ice. The samples were then loaded onto a 10% polyacrylamide gel. Electrophoresis was carried out for 17 h at 160 V using a 130 × 160 × 1 mm vertical unit (Bio-Rad Laboratories Ltd) maintained at 15 °C with a refrigerated circulating water bath. After electrophoresis, the gels were silver stained to resolve the individual banding patterns. Polymorphisms were verified by sequencing all of the samples.

Western blot analysis

A total of 15 µg and 50 µg of testicular and hepatic cytosolic proteins respectively were resolved on 12% polyacrylamide gels and transferred to Hybond-C nitrocellulose membranes (Amersham Biosciences, Baie d'Urfé, QC, Canada), according to the method outlined by Laemmli (1970). Immunoreactive porcine SULT2A1 protein was determined using a commercially available

polyclonal antibody to human SULT2A1 (MBL International Co., Watertown, MA, USA) diluted 1:5000. The secondary antibody was a 1:2000 dilution of donkey anti-rabbit IgG coupled to horseradish peroxidase (Amersham Biosciences). Equal loading of protein samples was verified following transfer by Coomassie Blue staining of the membranes. Blots were visualized by chemiluminescence detection and subsequently quantified by densitometry.

Real-time PCR

Real-time PCR amplification was performed using a Copeheid Smart Cyclor System (Fisher Scientific, Toronto, ON, Canada). Forward and reverse primers were designed based on the porcine *Sult2A1* sequence. The forward primer 5' CCATGCGAGACAAGGAG AAC 3', and reverse primer 5' CATGACCTGGAAG GAGCTGT 3' amplified a product of 155 bp in length at an annealing temperature of 60 °C. The QuantiTect SYBR Green PCR Amplification kit (Qiagen, Mississauga, ON, Canada) was used for the real-time quantification of the PCR products. The real-time PCR reaction consisted of 2.5 µl cDNA, and 20 µM of the forward and reverse primers, in a total volume of 25 µl. As an internal control, the hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene was amplified for each real-time PCR reaction. HPRT amplification included the forward primer 5' CTTTGCTGACCTG CTGGATT 3', and reverse primer 5' CTTGACCAA GGAAAGCAAGG 3', which amplified a product of 232 bp in length. Relative quantification of mRNA levels between animals with different levels of SULT2A1 protein was analyzed by the ΔCt method (Livak & Schmittgen 2001) using the average ΔCt of the low SULT2A1 protein group as the calibrator.

Identification of nuclear receptors involved in regulating SULT2A1

To further understand the molecular mechanisms that control SULT2A1 gene transcription, various nuclear receptor ligands were evaluated for their ability to induce SULT2A1 activity in Leydig cells. Aliquots of re-suspended Leydig cells (1×10^6 cells/ml) in TC 199 Media mixture were plated in 24-well plates. The Leydig cells were then treated with various nuclear receptor ligands as outlined in the figure legends in less than 0.5% DMSO vehicle and incubated at 37 °C in 95% air and 5% CO₂ for 24 h.

After the 24-h incubation period, the media were aspirated and approximately 10 000 c.p.m. [³H]DHEA (0.01–0.05 µCi/nmol) were added to each well in a final volume of 1 ml TC 199 Media. The cells were incubated for 4 h and the media were removed and immediately extracted by Sep-Pak C₁₈ solid-phase chromatography,

as described above. Sulfotransferase activity was assayed by measuring the conversion of [³H]DHEA (free) to [³H]DHEAS (conjugate) fractions by liquid scintillation counting. The results from the different Leydig cell preparations were normalized as a percentage of the average results from the control incubations from each cell preparation.

Statistical analyses

Pearson correlation coefficients were calculated for the following measures: (1) SULT2A1 activity vs plasma concentrations of sulfoconjugated 5 α -androstenone, (2) SULT2A1 activity vs fat concentrations of 5 α -androstenone (SAS 8.2, SAS Institute, Cary, NC, USA). Correlations were considered statistically significant at $P \leq 0.05$. Differences in SULT2A1 activity, protein and mRNA levels between high and low boar taint pigs were analyzed by Student's *t*-test (SAS 8.2, SAS Inst. Inc.). Differences in SULT2A1 activity following treatment with nuclear receptor ligands were analyzed by ANOVA using the Dunnett post-hoc test (SAS 8.2, SAS Inst. Inc.).

Results

Relationship between SULT2A1 activity and 5 α -androstenone in plasma

A strong positive correlation ($r=0.66$; $P<0.01$) was observed between the testicular activity of SULT2A1 and plasma concentrations of 5 α -androstenone in the sulfoconjugate fraction (Fig. 1A); however, this relationship was not as strong ($r=0.13$; $P<0.01$) with respect to hepatic SULT2A1 activity (Fig. 1B). In terms of testicular SULT2A1 activity, animals with the highest sulfotransferase activity had the highest concentrations of sulfated 5 α -androstenone in peripheral plasma, with concentrations as high as 57 ng/ml. In addition, these animals also had the lowest concentrations of unconjugated 5 α -androstenone, at levels below 9 ng/ml (data not shown).

There was a negative correlation ($r=-0.57$; $P<0.01$) between testicular SULT2A1 activity and the concentrations of 5 α -androstenone in fat (Fig. 2A). All of the animals with fat 5 α -androstenone concentrations less than 0.5 µg/g had correspondingly high SULT2A1 activities. This relationship was also observed to a lesser extent ($r=-0.31$; $P<0.05$) for hepatic SULT2A1 activity (data not shown). In order to examine further the relationship between SULT2A1 activity and 5 α -androstenone accumulation in fat, animals were separated into two groups based on fat 5 α -androstenone concentrations either above or below the limit of 0.5 µg/g. This limit has been determined to be relevant to consumer satisfaction in the detection of taint

Figure 1 Positive relationship between testicular and hepatic SULT2A1 activity and plasma levels of sulfoconjugated 5α -androstenone. Cytosolic testicular (A) and hepatic (B) SULT2A1 activities were compared with circulating plasma levels of sulfoconjugated 5α -androstenone. Analysis of 27 Yorkshire boars aged 175 ± 6 days showed a positive relationship ($r=0.66$ (A) and $r=0.13$ (B), $P<0.01$) to plasma sulfoconjugated 5α -androstenone.

(Bonneau *et al.* 2000). Both testicular and hepatic SULT2A1 activities (Fig. 2B) were significantly lower ($P<0.01$) in animals with fat 5α -androstenone concentrations higher than $0.5 \mu\text{g/g}$.

Isolation and sequence characterization of porcine *Sult2A1* cDNA

The nucleotide sequence of the porcine *Sult2A1* cDNA (Genbank Accession No. DQ172907) was 1019 bp long and contained a 855 bp long open reading frame (ORF), which encodes for 285 amino acids (Fig. 3). The porcine *Sult2A1* ORF displayed 82% homology to human *Sult2A1* and 84% and 85% homology to mouse and rat hydroxysteroid sulfotransferase respectively. The deduced amino acid sequence for porcine SULT2A1 had 81% homology to human SULT2A1, and 80%

Figure 2 Negative relationship between testicular and hepatic SULT2A1 activity and 5α -androstenone accumulation in fat. Cytosolic testicular (A) SULT2A1 activity was negatively related ($r=-0.57$, $P<0.01$) to fat levels of 5α -androstenone as determined in 175 ± 6 -day-old Yorkshire boars ($n=27$). (B) Boars were divided into low ($<0.5 \mu\text{g/g}$; open bars) or high ($>0.5 \mu\text{g/g}$; solid bars) fat 5α -androstenone levels for comparison with testes and liver SULT2A1 activity. Analysis showed a significantly ($*P<0.01$) lower level of SULT2A1 activity in boars with high fat 5α -androstenone in both testes and liver. Values are presented as means $\pm$ S.E. ($n=13$).

homology to both mouse and rat hydroxysteroid sulfotransferase. In humans, Gly248, Val249, Ser250, Gly251, Asp252, Trp253, and Lys254 are reported to be the PAPS binding site for SULT2A1. In the porcine sequence, Gly248, Gly251, Asp252, Trp253, and Lys254 are conserved (Fig. 4).

SULT2A1 genetic polymorphisms

SSCP was used to scan for genetic polymorphisms in the porcine *Sult2A1* coding region from both testicular and liver samples. Three different types of banding patterns were detected (Fig. 5). Sequence analysis showed that the observed SSCP banding patterns corresponded to a mutation from a cytosine to a thymine within the coding

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-36  tgaaacgctcccacgaggcgcaaagaactcagcatcatgacagaagaggaggtgcggttt 24
      M T E E E V R F
25  gaagggatatttttctaaacagattctctcgctgaaatgctgcaagaggtgagggag 84
      E G I F F P K Q I L S P E M L Q E V R E
85  gaatttacatttaaagaggaagatgttttaatactgacttttcccaatcaggaaccaac 144
      E F T F K E E D V L I L T F P K S G T N
145  tggatgattgaaatcatctgcctgattctctccaagggggataccaagtggatccagtct 204
      W M I E I I C L I L S K G D T K W I Q S
205  gtacccaactgggatcgctcgccctggctagagagtatctctgggtatgaaatctcaag 264
      V P N W D R S P W L E S I S G Y E N L K
265  aacaaggaaggcccacgcctcatcagttccacctcccatccaactcttccccaggct 324
      N K E G P R L I S S H L P I Q L F P K A
325  ttcttcaagtccaaggccaagatgatttacatcatcagaaatcccagagatattattgtg 384
      F F K S K A K M I Y I I R N P R D I I V
385  tctggttatttttctggaagtctacaaatttagttaagagaccagagtcactggaacaa 444
      S G Y F F W K S T N L V K R P E S L E Q
445  tattttgaatggttcatccaaggaaatgtgccctatggatcatggtttgaccacattcgt 504
      Y F E W F I Q G N V P Y G S W F D H I R
505  ggctggttgcccatgcgagacaaggagaacgtcctgatactcagctatgaagagctgaaa 564
      G W L P M R D K E N V L I L S Y E E L K
565  cgggacacgagaagcgccgtagagaaaatctgccagttcctggggaagaagctagaaccc 624
      R D T R S A V E K I C Q F L G K K L E P
625  gaggaactgagctcggtcgtcgaaaacagctccttccaggtcatgaaagaaaacaacatg 684
      E E L S S V V E N S S F Q V M K E N N M
685  tccaatttttccctcctgaagggttgcacatcttggggacactggatgtcttttgagaaaa 744
      S N F S L L K G L H L G D T G C L L R K
745  ggcaactcctggggactggaaaaactacttcacggtggcccaagccgaagcctttgataaa 804
      G T P G D W K N Y F T V A Q A E A F D K
805  ctattccaggagaagatggcagatcttctcctcaagagctgttcccatggcaataatgttca 864
      L F Q E K M A D L P Q E L F P W Q stop
865  caaacttcccaatcctctatggatattgatattcgctcacctgtccttgccacatgtgaat 924

925  gatgggggctaattacacaaaaacctcttgtttcatccttgaaaaaaaaaaaaaaaaaa 983

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Figure 3 Porcine SULT2A1 cDNA nucleotide and deduced amino acid sequences.

region at bp position 166. This mutation did not cause a change in amino acid sequence and is therefore a silent mutation that is not directly associated with SULT2A1 activity.

Comparison of SULT2A1 levels and 5 α -androstenone accumulation in fat

The lack of functional polymorphisms within the coding region warranted investigation into whether the observed variation in SULT2A1 activity was due to individual differences in the amount of SULT2A1 protein that is produced. Western blot analysis determined that the differences in SULT2A1 activity were due to differences in the levels of SULT2A1 protein; animals with low SULT2A1 activity had low levels of both testicular and hepatic SULT2A1 protein. Figure 6 demonstrates the positive correlation ($r=0.53$; $P<0.01$) between the testicular level of SULT2A1 protein and enzyme activity. A similar relationship was observed with hepatic levels of

SULT2A1 (data not shown); however, the relationship was not as strong ($r=0.22$; $P<0.05$). In addition, the concentration of 5 α -androstenone in the sulfoconjugate fraction of the plasma was positively correlated with testicular SULT2A1 protein ($r=0.42$; $P<0.01$) and hepatic SULT2A1 protein ($r=0.14$; $P<0.01$).

The levels of testicular and hepatic SULT2A1 protein as related to 5 α -androstenone accumulation in fat demonstrated a similar trend for SULT2A1 activity, with animals showing high levels of 5 α -androstenone in fat having significantly lower levels ($P<0.001$) of total SULT2A1 protein (Fig. 7).

Comparison of SULT2A1 protein and mRNA expression levels

In order to determine whether the difference in SULT2A1 protein expression is regulated at the transcriptional level, quantitative real-time PCR was performed to measure the levels of *Sult2A1* mRNA.

Figure 4 Alignment of the amino acid sequence of human and porcine SULT2A1. The alignment of the 285 amino acid sequence of human and porcine SULT2A1 is shown, demonstrating the 81% homology (shaded) and conservation of the PAPS binding site (boxed).

Relative quantification revealed that the expression of the *Sult2A1* gene was increased 2.8-fold ($P < 0.01$) in animals with 1.5 ± 0.2 - and 1.7 ± 0.2 -fold increases in testicular and liver SULT2A1 protein levels ($P < 0.05$) relative to animals with low levels of the protein.

Identification of nuclear receptors involved in regulating SULT2A1

To further understand the transcriptional regulation of testicular SULT2A1, primary isolated Leydig cells were incubated with various nuclear receptor ligands for 24 h prior to the determination of SULT2A1 activity. As seen in Fig. 8, SULT2A1 activity was increased by treatment with some of the ligands to CAR, PXR and FXR. Of the CAR inducers, only phenytoin resulted in a significant increase in SULT2A1 activity ($P < 0.05$). Rifampicin, a ligand of human and pig PXR (Moore *et al.* 2002), significantly increased ($P < 0.05$) SULT2A1 activity. The glucocorticoid receptor (GR) ligand, dexamethasone, did not affect SULT2A1 activity, whereas treatment with the FXR/PXR ligand, lithocholic acid, resulted in an increase in SULT2A1 activity ($P < 0.05$).

Discussion

The extent to which 5 α -androstenone accumulates in fat is influenced by the amount of unconjugated steroid that is present in the circulation. Differences in the ability to sulfoconjugate 5 α -androstenone will affect the level of unconjugated steroid that is available to accumulate in

fat. The levels of sulfoconjugated 16-androstene steroids present in the circulation are a result of the balance between the capacity for testicular steroidogenesis, sulfoconjugation, and metabolic clearance. The results of this study show that the concentration of sulfoconjugated 5 α -androstenone in the peripheral plasma is correlated with testicular SULT2A1 activity ($r = 0.66$) and to a lesser extent to hepatic SULT2A1 activity ($r = 0.13$). Furthermore, we show that treatment with ligands for the nuclear receptors CAR, PXR and possibly FXR can stimulate SULT2A1 activity in isolated Leydig cells. Together, these results further clarify the magnitude of testicular sulfoconjugation of androstenone and the role of SULT2A1, and suggest the possible control of porcine SULT2A1 expression by nuclear receptors.

High levels of steroid sulfotransferase enzymes are present in the boar testis, which are responsible for the large proportion of sulfoconjugated steroids that are secreted from this organ (Tan & Raeside 1980, Raeside & Renaud 1983, Raeside *et al.* 1989). The liver also sulfoconjugates the 16-androstene steroids, but to a lesser extent than the testis (Sinclair & Squires 2005, Sinclair *et al.* 2005). Our present results indicate that animals with high concentrations of 5 α -androstenone in fat had significantly lower SULT2A1 activity in both testis and liver; thus more of the unconjugated form of the steroid will be available to deposit into the adipose tissue to contribute to boar taint.

The relationship between SULT2A1 activity and the accumulation of 5 α -androstenone in fat warranted investigation at the molecular level, as genetic polymorphisms within the *Sult2A1* gene may account for the

Figure 5 Identification of a genetic polymorphism in the porcine *SULT2A1* gene. A representative silver stained SSCP gel (A) and corresponding sequence analysis (B) is shown demonstrating heterozygotes and homozygotes in both testicular (T) and liver (L) tissue samples for a T/C polymorphism at the 166 bp position.

differences observed in enzyme activity. In order to test this hypothesis, the cDNA sequence for porcine *Sult2A1* was determined and found to be highly homologous to its orthologous human, mouse and rat genes. These findings lead to the conclusion that the putative *Sult2A1* is in fact porcine *Sult2A1*.

Human *SULT2A1* has been mapped to chromosome 19q13.3 (Luu-The *et al.* 1995, Otterness *et al.* 1995), which is homologous with porcine chromosome 6q11, 21 and chromosome 7p12. The homology to porcine chromosome 7 is of interest, as a quantitative trait loci for fat 5 α -androstenone levels has been mapped to this location (Quintanilla *et al.* 2003, Tanaka *et al.* 2003).

SULT2A1 is involved in the biotransformation of many steroid hormones, neurotransmitters, bile acids, drugs and xenobiotics. Therefore, identification of genetic differences that cause changes in enzyme activity may be crucial in determining the individual response to

Figure 6 Positive relationship between testicular *SULT2A1* protein and activity levels. *SULT2A1* activity was determined in testis cytosol from 175 $\pm$ 6-day-old Yorkshire boars ($n=27$) and compared with the corresponding *SULT2A1* protein levels as determined by western blotting and densitometry. Analysis revealed a significant positive correlation ($r=0.53$, $P<0.01$) between protein and activity levels.

specific compounds. In humans, substantial efforts have been made to detect genetic polymorphisms in *SULT2A1* due to its involvement in cardiovascular diseases and cancer (LaCroix *et al.* 1992, Stahl *et al.* 1992, Shibutani *et al.* 1998). Polymorphisms within the human *Sult2A1* gene have been observed in a number of studies (Igaz *et al.* 2002), some of which have resulted in reductions in the levels of both enzyme activity and protein levels (Thomae *et al.* 2002). However, despite the

Figure 7 Increased levels of *SULT2A1* protein are associated with lower levels of 5 α -androstenone accumulation in fat. Boars were divided into low (<0.5 μ g/g; open bars) or high (>0.5 μ g/g; solid bars) fat 5 α -androstenone levels, and *SULT2A1* protein levels in testes and liver as determined by densitometric analysis of western blots were compared. Analysis showed a significantly ($*P<0.001$) higher level of *SULT2A1* protein levels in boars with low fat 5 α -androstenone in both testes and liver. Values are presented as means $\pm$ s.e. ($n=13$).

Figure 8 Effect of nuclear receptor ligands on SULT2A1 activity in porcine Leydig cells. Primary porcine Leydig cells were isolated from mature Yorkshire boars and the SULT2A1 activity was determined. Leydig cells were cultured in the presence of CITCO (1 μ M), TCPOBOP (250 nM), phenobarbital (2 mM), phenytoin (50 μ M), rifampicin (10 μ M), PCN (10 μ M), lithocholic acid (100 μ M), cholic acid (100 μ M), and dexamethasone (0.1 μ M) for 24 h. Treatments are grouped according to the nuclear receptor affected: constitutive androstane receptor (CAR), pregnane X receptor (PXR), farnesoid X receptor (FXR), and glucocorticoid receptor (GR). Values are presented as means $\pm$ S.E. of 3 independent experiments with significant differences indicated (* P < 0.05).

fact that this enzyme is highly polymorphic, the mutation may not be functionally correlated to the activity or level of the protein levels. In the present study, there were no significant functional polymorphisms detected within the ORF of the porcine *Sult2A1* gene. Therefore, differences in enzyme activity are not attributed to mutations within the coding region. After western blot analysis, it was determined that the differences observed in SULT2A1 activity were a result of differences in the amount of SULT2A1 protein that was expressed within the tissue. Animals with low SULT2A1 activity in testis and liver tissues had correspondingly low protein levels. This decrease in protein will therefore contribute to a decreased sulfoconjugation activity towards the 16-androstene steroids.

There are multiple regulatory mechanisms involved in controlling gene expression, many of which influence the level of functional protein. At the transcriptional level, alterations in receptor-dependent mechanisms or transcription factors could lead to differences in the amount of mRNA that is transcribed (Tsai & O'Malley 1994). Post-transcriptional mechanisms such as mRNA stabilization can also play a significant role in determining the

level of production of an end product (Day & Tuite 1998). In addition, there are multiple processing events that operate at the translational and post-translational levels that could have an equal impact on the amount or stability of functional product. The results from the quantitative real-time PCR analysis suggest that the level of SULT2A1 production is regulated at the transcriptional level, as animals with high testicular (1.5 ± 0.2 -fold) and liver (1.7 ± 0.2 -fold) levels of SULT2A1 protein expressed 2.8-fold more mRNA for *Sult2A1* than animals with low levels of the protein.

The molecular mechanisms that control SULT2A1 gene transcription have not been fully characterized. Chenodeoxycholic acid has been demonstrated to be a strong inducer of the rat SULT2A1 gene (Makishima *et al.* 1999). This inducing effect is controlled, in part, by the bile acid-activated FXR (Song *et al.* 2001). Similarly, human SULT2A1 has been shown to be induced in response to various ligands for the PXR (Kliwer *et al.* 1998, Duanmu *et al.* 2002, Echchgadda *et al.* 2004). More recently, it has been determined that the CAR is also potentially involved in rodent SULT2A1 regulation (Saini *et al.* 2004). The results of this study suggest that the recently identified human CAR activator, phenytoin

(Jackson *et al.* 2004), resulted in a significant increase in pig SULT2A1 activity, whereas CITCO (Maglich *et al.* 2003) and the CAR agonists TCBOPOP and phenobarbital did not affect SULT2A1 activity. While these classical inducers are known as potent rodent inducers, their influence on human gene expression is often absent or attenuated in comparison with rodents (Moore *et al.* 2002). Similarly, the treatment of Leydig cells with the PXR ligands rifampicin and PCN demonstrated that only rifampicin resulted in a significant increase in SULT2A1 activity. This observation is consistent with the results of Moore *et al.* (2002) who demonstrated in a reporter assay system that rifampicin is a potent activator of pig PXR. However, PCN, a PXR inducer in mouse, rabbit, and monkey, does not activate either human or pig PXR. Furthermore, dexamethasone, which can affect CAR and PXR both directly and indirectly (Pascucci *et al.* 2000a,b), did not increase SULT2A1 activity of Leydig cells at 10^{-7} M within the 24 h treatment period. In comparison with these exogenous ligands, lithocholic acid, a bile acid that acts through the FXR, induced pig SULT2A1 activity. However, Moore *et al.* (2002) demonstrated that lithocholic acid, but not cholic acid, may also activate porcine PXR. Together, these data suggest the importance of CAR, PXR, and potentially FXR in the regulation of SULT2A1 activity. This does not exclude the involvement of other orphan receptors in the regulation of pig SULT2A1 not investigated here. For instance, both steroidogenic factor 1 (SF1) and GATA-6 have been shown to regulate the transcription of human *Sult2A1* (Saner *et al.* 2005). Furthermore, this study also highlights the similarity in responsiveness of nuclear receptors of human and pig compared with rodent species. Differences in the level of gene transcription of *Sult2A1* may be due to individual differences in the activation of these receptors or differences in the proximal promoter region of the gene that contains the recognition sequences for these transcription factors. However, the 5'-proximal promoter region of porcine *Sult2A1* gene has not yet been characterized.

In summary, the accumulation of 5 α -androstenone in fat is influenced by the individual capacity for sulfoconjugation. SULT2A1 has been identified to be the key enzyme involved in the sulfoconjugation of the 16-androstene steroids. After isolation of the porcine *Sult2A1* cDNA, it was determined that differences in enzyme activity were not the result of genetic polymorphisms in the coding region. The proportion of sulfoconjugated 5 α -androstenone present in the circulation is dependent on the level of SULT2A1 protein that is expressed and thus its relative activity. The finding that animals with low levels of SULT2A1 have resulting higher levels of 5 α -androstenone concentrations in fat suggests that sulfoconjugation plays a vital role in regulating the level of unconjugated or 'free' steroid that

is capable of accumulating in adipose tissue. Based on these results, SULT2A1 is a potential candidate gene for the development of genetic markers for selecting animals with low boar taint. Further investigation into the transcriptional regulation of SULT2A1 is required to confirm this possible new and appealing application.

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