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The sulfoconjugation of androstenone and dehydroepiandrosterone by human and porcine sulfotransferase enzymes

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ABSTRACT

Porcine sulfotransferase 2A1 (pSULT2A1) is a key enzyme involved in the testicular and hepatic sulfoconjugation of steroids such as dehydroepiandrosterone (DHEA) and potentially androstenone. This latter steroid is a major cause of boar taint, which is an unpleasant off-odour and off-flavour in pork from male pigs. Sulfotransferase 2B1 (pSULT2B1) may also be important, although no direct evidence exists for its involvement in sulfoconjugation of steroids. The purpose of this study was to investigate the sulfoconjugation activity of human and porcine sulfotransferases towards DHEA and androstenone. pcDNA 3.1 vectors expressing porcine (p) SULT2A1, pSULT2B1, human (h) SULT2A1, hSULT2B1a, and hSULT2B1b enzymes were transfected into human embryonic kidney cells. Transfected cells were then incubated with either androstenone or dehydroepiandrosterone (DHEA) in both time-course and enzyme kinetics studies. The production of sulfonates of androstenone metabolites and DHEA sulfonate increased over time for all enzymes with the exception of pSULT2B1. Enzyme kinetics analysis showed that androstenone and DHEA were poor substrates for the human orthologs, hSULT2B1a and hSULT2B1b. Human and porcine SULT2A1 showed substantially different substrate affinities for androstenone (K_m 5.8 ± 0.6 μM and 74.1 ± 15.9 μM, respectively) and DHEA (K_m 9.4 ± 2.5 μM and 3.3 ± 1.9 μM, respectively). However, these enzymes did show relatively similar sulfonation efficiencies for DHEA (V_{max}/K_m 50.5 and 72.9 for hSULT2A1 and pSULT2A1, respectively). These results highlight the species differences in sulfonation activity and provide direct evidence, for the first time, suggesting that pSULT2B1 is not involved in sulfonation of either androstenone metabolites or DHEA.

1. Introduction

Sulfoconjugation, or sulfonation, of hormones, neurotransmitters, and xenobiotic compounds involves the transfer of a sulfonate group (SO3) from 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to the hydroxyl group of an acceptor molecule to form a sulfonate conjugate [1–3]. These reactions are mediated by a superfamily of cytosolic sulfotransferase (SULT) enzymes [2]. While six cytosolic SULT gene families have been identified in mammals, only the SULT1 and SULT2 families are capable of sulfonating steroids [3–5]. In the male pig, this is an important metabolic process, as the sulfonation of 16-androstene steroids will decrease their accumulation in adipose tissue [6], which is a major cause of boar taint, an unpleasant odor and flavor from heated pork products.

The 16-androstene steroids (androstenone and its metabolites 3α-androstenol and 3β-androstenol) exist in blood primarily as sulfoconjugates, reaching levels of up to 72% relative to their unconjugated form in the peripheral and testicular vein plasma of mature boars [6]. Porcine (p) SULT2A1 has been implicated as a key enzyme involved in the testicular and hepatic sulfonation of 16-androstene steroids [7,8]. This enzyme is capable of sulfonating a number of different steroids, although it is most reactive towards dehydroepiandrosterone (DHEA), an important precursor steroid in androgen and estrogen synthesis [9]. Furthermore, pSULT2B1 may play a role in androstenone metabolism, as its expression in testis has been negatively correlated to androstenone concentration in fat [8] and the human ortholog has selectivity for 3β-hydroxysteroids [10,11]. However, direct evidence for the involvement of this enzyme in androstenone metabolism has yet to be generated. In pigs, only one SULT2B1 enzyme is thought to exist (NCBI Reference Sequence: NP_001230626.1); however, in humans this gene encodes two isoforms, SULT2B1a and SULT2B1b, which are generated by alternate splicing of the first exon

Abbreviations: DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfonate; DMSO, dimethyl sulfoxide; HEK, human embryonic kidney cells; HRP, horseradish peroxidase; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; PCR, polymerase chain reaction; PVDF, polyvinylidene fluoride; RIPA, radio-immunoprecipitation assay; SDS-PAGE, sodium dodecyl sulfate – polyacrylamide gel electrophoresis; SULT, cytosolic sulfotransferase

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[10]. These two isoforms have been shown to preferentially sulfonate pregnenolone and cholesterol, while DHEA was observed to be a poor substrate [12,13].

Pigs share many similar anatomical and physiological characteristics with humans, making them a valuable experimental model for biomedical research applications and toxicology testing [14]. Consequently, similarities and differences between human and porcine enzymes are important, particularly in preclinical toxicological testing of pharmaceuticals, as many SULTs are known to conjugate drugs and in some cases, are required to activate them [14,15].

In this study we hypothesized that the sulfonation of the 16-androstene steroids was facilitated by pSULT2A1 and pSULT2B1. Therefore, our objective was to express human and porcine SULT2A and SULT2B enzymes in cell culture and characterize and compare the sulfonation activity of human and porcine sulfotransferase enzymes towards androstenone and dehydroepiandrosterone (DHEA).

2. Materials and methods

2.1. Materials

Human embryonic kidney cells (HEK293FT) were purchased from Invitrogen (Carlsbad, CA, USA). The pMK-RQ vector containing cDNA encoding porcine SULT2B1 was custom synthesized by Life Technologies Inc. (Burlington, ON, Canada). The pKK233-2 vector containing cDNA encoding human SULT2A1 was kindly provided by Dr. Charles Falany (University of Alabama at Birmingham, Birmingham, AL, USA) while pCR3.1 vectors containing cDNA encoding human SULT2B1a and human SULT2B1b were generously donated by Dr. Richard Weinshilboum (Mayo Clinic, Rochester, MN, USA). Radiolabelled [^{3}H]-androstenone (10 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA, USA) and radiolabelled [1,2,6,7-^{3}H(N)]-DHEA (70.5 Ci/mmol) was purchased from Perkin-Elmer (Boston, MA, USA). Nonradioactive steroids were purchased from Steraloids Inc. (Newport, RI). Organic solvents of analytical grade were obtained from Fisher Scientific (Toronto, ON, Canada). All other chemicals used for the incubation of HEK293FT cells were of analytical grade and were purchased from Sigma–Aldrich Canada, Ltd. (Oakville, ON, Canada) or Fisher Scientific, unless specified otherwise.

2.2. Plasmid constructs

The pSULT2A1 coding sequence was amplified from porcine liver cDNA and the pSULT2B1, hSULT2B1a, and hSULT2B1b coding sequences were amplified from their respective vectors by polymerase chain reaction (PCR) using platinum Taq DNA polymerase and gene-specific primers (Table 1). These primers were designed based on sequences available from the National Center for Biotechnology Information (NCBI). The PCR procedure consisted of heating at 94 °C for 2 min, 34 cycles of 94 °C for 30 s, 60 °C for 30 s, and 68 °C for 1 min, and finally incubating at 68 °C for 10 min. Amplified DNA fragments containing appropriate start codon and Kozak sequence (GCC) were gel-purified using a PureLink quick gel extraction kit (Invitrogen) and were subcloned into mammalian expression vector pcDNA3.1/V5-His-TOPO using a TA cloning kit (Invitrogen). Expression vectors for V5-His tagged proteins were generated in order to detect protein expression by Western blot using anti-V5-horseradish peroxidase (HRP) antibody [16]. Sequences of all constructs were confirmed by DNA sequence analysis.

2.3. Cell culture transfection, enzyme expression and incubations with substrates

HEK293FT cells were routinely maintained as monolayer cultures in 75 cm^2 cell culture flasks (Greiner Bio-One North America Inc., Monroe, NC, USA) and grown in complete growth medium (Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% penicillin/streptomycin, 1% non-essential amino acids, 1% sodium pyruvate, and 1% geneticin) in a humidified atmosphere at 37 °C and 5% CO_2 in air. Cells were plated in triplicate at 7×10^5 cells per well in 6-well cell culture plates (Greiner Bio-One) with 2 ml/well of complete growth medium and allowed 24 h for adherence. Vectors expressing sulfotransferases were then transfected into cells using Nano Juice core transfection reagent (1:2, w/v of DNA:reagent) and Nano Juice transfection booster (1:3.2, w/v of DNA:reagent) (Novagen, Etobicoke, ON, Canada) with 0.08 μg pSULT2A1, 1.25 μg pSULT2B1, 1.25 μg hSULT2A1, 0.045 μg hSULT2B1a, and 0.045 μg hSULT2B1b expression vectors. Vector amounts were varied in an attempt to obtain similar levels of protein expression assessed by Western blotting without compromising activity. Empty pcDNA 3.1 vector was added, when appropriate, so that the total plasmid DNA level was 1.25 μg/well. Empty pcDNA 3.1 vector was also used to transfect control samples. After 48 h, transfected cells were treated with radiolabelled [^{3}H]-androstenone (20 μM, 31.2 μCi/μmol) or [1,2,6,7-^{3}H(N)]-DHEA (20 μM, 15.5 μCi/μmol) dissolved in ethanol (final concentration of ethanol 0.1%) for 4, 8, 16, and 24 h in a time-course study. Kinetic parameters were also obtained in three separate experiments by incubating cells for 24 h with varying concentrations of androstenone or DHEA prepared by serial dilution. In order to confirm that pSULT2B1 was an active enzyme, cells transfected with expression vectors for pSULT2B1 and hSULT2B1b were incubated with 100 μM 25-hydroxycholesterol for 24 h. Media was subsequently removed and diluted 1:1 (v/v) with acetonitrile, centrifuged for 10 min at room temperature to precipitate protein, and analyzed by HPLC. Cells were harvested and lysed by sonication in 300 μl of radio-immunoprecipitation assay (RIPA) buffer (0.5% sodium deoxycholate, 1% Nonidet P-40, and 0.1% SDS) with 1% protease inhibitor cocktail (EMD Millipore Corp., San Diego, CA, USA) and used for Western blotting analysis. Protein concentrations were determined using the Bradford protein assay (Bio-Rad).

2.4. High-performance liquid chromatography (HPLC)

Media samples diluted with acetonitrile were analyzed using reversed phase HPLC by injecting a 100 μl aliquot onto a Luna 5μ C18 HPLC column (250 × 4.60 mm, from Phenomenex, Torrance, CA, USA). Elution of radiolabelled steroids was monitored by a β-RAM model 2 isotope detector (IN/US Systems, Tampa, FL, USA). The HPLC mobile phase system for samples incubated with radiolabelled [^{3}H]-androstenone consisted of an isocratic flow with acetonitrile/water (33:67, v/v) for 8 min, a linear gradient from 33 to 60% acetonitrile for the next 17 min, an isocratic flow with 100% acetonitrile for the next 5 min, and

Table 1
Gene-specific primers used for PCR of human and porcine SULTs.

Gene	Forward Primer (5′ → 3′)	Reverse Primer (5′ → 3′)	Accession Number
pSULT2A1	GCCATGACAGAAGAGGAGG	TTGCCATGGGAACAGCTCTT	NM 001037150.1
pSULT2B1	GCCATGGATGGGCCTGCCG	CGGGTGGGGGACCTCG	NM 001243697.1
hSULT2A1	GCCATGTCGGACGATTTCT	TTCCCATGGGAACAGC	NM 003167.3
hSULT2B1a	GCCATGGCGTCTCCCCCAC	TGAGGGTCGTGGGTGC	NM 004605.2
hSULT2B1b	GCCATGGACGGGCCCGCCG	TGAGGGTCGTGGGTGC	NM 177973.1