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Effects of nuclear receptor transactivation on steroid hormone synthesis and gene expression in porcine Leydig cells

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ABSTRACT

Male pigs are routinely castrated at a young age to prevent the formation of androstenone, a 16-androstene testicular steroid that is a major component of boar taint. The practice of castration has been increasingly viewed as unfavorable, due to both economic considerations and animal welfare concerns. Other means of controlling boar taint, including reducing the synthesis of androstenone in the testes, would eliminate the need for castration. In this study, we determined the effects of transactivation of three nuclear receptors, the constitutive androstane receptor (CAR), pregnane X receptor (PXR), and farnesoid X receptor (FXR), on gene expression and steroid hormone metabolism in primary porcine Leydig cells. Primary cells were isolated from mature boars, and transcript expression levels were assayed using real-time PCR. The transcripts of interest included porcine orthologs of common phase I and phase II metabolic enzymes, enzymes involved in steroidogenesis, and transcripts previously shown to be differentially expressed in boars with high androstenone and boar taint levels. Transactivation of CAR, PXR, or FXR increased the expression of several genes involved in steroidogenesis, including *cytochrome B5A* (*CYB5A*) and *cytochrome B5 reductase 1* (*CYB5R1*), as well as *hydroxysteroid (17-beta) dehydrogenase 4* (*HSD17B4*) and *retinol dehydrogenase 12* (*RDH12*). Treatment with (6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl)oxime (CITCO), a CAR agonist, or rifampicin (RIF), a PXR agonist, resulted in significantly ($p < 0.05$) decreased sex steroid production and significantly ($p < 0.05$) increased production of 16-androstene steroids. Treatment with the FXR agonist chenodeoxycholic acid (CDCA) resulted in significantly ($p < 0.05$) decreased sex steroid production. These results indicate that transactivation of these nuclear receptors may lead to increased levels of 16-androstene steroids, likely by altering the activity of CYP17A1 through *CYB5A* and *CYB5R1* to the andien-β synthase reaction and away from the 17α-hydroxylase and C17, 20 lyase reactions.

1. Introduction

Nuclear receptors are a large family of related transcription factors found in metazoans; in humans, 49 individual nuclear receptors have been identified [1]. These transcription factors control a variety of physiological processes, including lipid metabolism and energy homeostasis, bile acid and xenobiotic metabolism, steroidogenesis, reproduction and development, and central nervous system, circadian, and basal metabolic functions [2]. Within the nuclear receptor super family there are several classes of nuclear receptors, defined by their method of dimerization and the response element structure they bind to [3]. The class II nuclear receptors form heterodimers with the retinoid X receptor (RXR) and bind to response elements arranged as either direct or symmetrical repeats. Included in class II are the nuclear receptors that are transactivated by non-steroidal ligands, such as retinoids or bile acids, or by xenobiotic ligands [3]. Two receptors that are primarily transactivated by xenobiotics are the constitutive androstane receptor (CAR) and pregnane X receptor (PXR). CAR was originally identified as a protein involved in the response to retinoic acid and its metabolites [4], although further research found that CAR is responsive to xenobiotics [5]. Like CAR, PXR was originally identified as an orphan nuclear receptor, which was responsive to pregnane and various endogenous and synthetic hormones [6]. Together, CAR and PXR are responsible for co-ordinating the metabolism and clearance of xenobiotics and some endogenous compounds, by regulating the expression of various metabolic and transport proteins [7,8]. A third member of the class II nuclear receptors, farnesoid X receptor (FXR), is responsive to bile acids and their derivatives and regulates the cellular machinery in the liver and intestines that is involved in both bile acid production and clearance [9].

Nuclear receptors have been poorly classified in pigs, although similarities in sequence homology and ligand complements

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between the human and pig orthologs of pig and human CAR [10], PXR, and FXR [11] have been reported. These similarities between human and pig receptors suggest that pigs may be a viable model for humans in toxicological and pharmacological studies. Of particular interest is the potential for porcine nuclear receptors to modulate specific pig phenotypes, including the accumulation of the malodorous compounds 5α-androst-16-en-3-one (androstenone, AND) and 3-methylindole (skatole, 3MI) in fat depots. These two compounds are the major components of boar taint, an unpleasant odor and taste generated upon heating of fat from uncastrated male pigs [12,13]. AND is produced in the testes from 5,16-androstadien-3β-ol, which is produced from pregnenolone via the andien-β-synthase reaction, which is carried out by cytochrome P450 17A1 (CYP17A1) in conjunction with cytochrome B5 [14]. Alternatively, pregnenolone can be converted by CYP17A1, via the 17α-hydroxylase reaction, to 17α-hydroxypregnenolone. This product is further processed, via the C17, 20-lyase reaction of CYP17A1, to dehydroepiandrosterone (DHEA). Boar taint can be reduced by castration of male pigs, which removes the potential for androstenone production; however, due to animal welfare issues and decreases in production efficiency caused by castration, elimination of boar taint through alternative methods would be of great benefit to the pork industry.

The transactivation of porcine CAR, PXR, and FXR by various ligands has been previously reported [10,11]. Both porcine CAR and porcine FXR were transactivated by a number of endogenous ligands, with CAR being inhibited by a few sex steroids and androstadienol, a precursor of AND. FXR was activated by the bile acid CDCA and progesterone. PXR was transactivated by a number of endogenous compounds, including progesterone, several estrogenic compounds, several sex steroids, as well as androstadienone and 3α-AND [11]. This transactivation by endogenous compounds may result in extensive up-regulation of genes favoring AND formation and increase boar taint. As well, it has been shown previously that 3MI is a potent inverse agonist of CAR, PXR, and FXR [11], and as such may modulate the production of AND by affecting nuclear receptor activity. As such, various physiological factors could play a role in regulating AND production in the testes by altering nuclear receptor activity, and as such determining the downstream affects of CAR, PXR, and FXR transactivation may help elucidate what the effects of these alterations could be.

To better understand the potential role of nuclear receptors in the metabolism of boar taint compounds, the downstream effects of nuclear receptor transactivation in pigs is required. Here we determined the effects of transactivation of CAR, PXR, and FXR on the transcription of key genes of interest and on the metabolism of pregnenolone in primary Leydig cells from mature boars, using known agonists for the three nuclear receptors of interest. (6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl)oxime (CITCO) is a commonly used synthetic CAR agonist, and has been shown to be affective at transactivating porcine CAR at a concentration of 1 μM [10]. Rifampicin (RIF) is an antibiotic agent that has been shown to be a potent PXR agonist, and is commonly used in that capacity at a concentration of 10 μM, including in studies looking at porcine PXR [11]. Chenodeoxycholic acid (CDCA) is a primary bile acid that has been shown to be a potent FXR agonist; studies focusing on porcine FXR determined that it was the most effective agonist, at a concentration of 100 μM, in transactivating porcine FXR [11]. 3MI was also used as a treatment to determine the direct effects it could have on the production of AND, and thus the generation of boar taint. The transcripts selected include porcine orthologs of genes involved in endobiotic and xenobiotic metabolism in humans, genes involved in steroidogenesis, as well as genes that were differentially regulated in boars displaying high or low levels of boar taint [15,16]. The downstream effects of these transcriptional changes on the metabolism of pregnenolone to sex steroids and 16-androstene steroids were also determined.

2. Materials and methods

2.1. Research animals

Animals for preparations of primary Leydig cells were a total of 9 Yorkshire boars weighing approximately 120 kg. They were acquired from the Arkell Swine Research Facility of the University of Guelph and used in accordance with the guidelines of the Canadian Council of Animal Care. Testes were removed immediately following slaughter and transported whole and on ice to the lab for further processing.

2.2. Isolation of primary Leydig cells

Isolation of Leydig cells was performed as previously described [17], with slight modifications. Briefly, testes were rinsed with cold water, decapsulated, and sliced to generate 1 cm^2 pieces approximately 1 mm thick. 180 g of tissue was incubated at 37 °C with 500 mL of TC 199 media (without phenol red) containing 0.1% L-glutamine, 1% bovine serum albumin (BSA), 1% glucose, 500 mg of Type IA collagenase, 25 mg of DNAse, and 25 mg of trypsin inhibitor. Digestion took place for 45–60 min with constant stirring and the mixture was then filtered through nylon mesh of 150 μm pore size followed by 75 μm pore size. Leydig cells were then purified using Percoll gradients as previously described [17] and cell viability was determined with a trypan blue exclusion test. Typical cell viability was 90%. Cells were then seeded on 10 cm tissue culture dishes at a density of 7 million cells/plate in complete TC 199 media containing 0.1% L-glutamine, 1% BSA, and 1% glucose supplemented with 10% FBS and 1% penicillin/streptomycin.

2.3. Treatment of primary cells, isolation of total RNA, and hormone quantitation

4 h after plating the cells, the media was replaced with complete TC 199 containing 0.05% (v/v) dimethyl sulfoxide (DMSO), 1 μM CITCO, 10 μM RIF, 100 μM CDCA, or 10 μM 3MI. Treatments were carried out in quadruplicate. 20 h after ligand treatment the media was replaced with 12 mL complete TC 199 containing [7-^{3}(H)N]-pregnenolone (20 μM, 16 μCi/μmol) on half of the plates per treatment group. The media was removed from the remaining plates and total RNA was extracted from the cells using Tri-Reagent (Sigma Chemical Co., St. Louis, MO, USA). RNA quality was assessed using the Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). RNA from was reverse transcribed to cDNA using SuperScript II reverse transcriptase with oligo dT primers (Invitrogen Corp., Burlington, ON, CAN) and used for real-time PCR as described below. 24 h after the application of pregnenolone, the steroid metabolites were extracted from each 2 mL media sample with 2× 4 mL ether and dried under a nitrogen stream. Extracted steroids were dissolved in 85% acetonitrile: 15% H_2O and subjected to HPLC to determine the amount of total sex steroids as defined previously [17]. This includes all steroids produced downstream of the cytochrome P450 17A1 (CYP17A1) reaction. Sex steroids (SS), including androgens and estrogens, eluted between 3 min and 7.5 min, and 16-androstenes (16A) in each treatment group, eluted starting at 12 min, with 3β-androstenol (3β-AND), 3α-androstenol (3α-AND), and AND eluting at 16.7, 17.5 and 18.5 min, respectively. Pregnenolone eluted at 7.6 min. A media sample containing [7-^{3}(H)N]-pregnenolone that had not been incubated with cells was used as a control and the percentages of pregnenolone remaining, SS produced, and 16A produced were calculated.