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Effects of nuclear receptor transactivation on boar taint metabolism and gene expression in porcine hepatocytes

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ABSTRACT

The accumulation of the testicular steroid androstenone (AND) and tryptophan degradation product skatole (3MI) in fat results in boar taint, an off odor and flavor in boar meat. Increasing boar taint metabolism in the liver may help limit the deposition of AND and 3MI in fat, thereby improving meat quality. The effects of transactivation of the nuclear receptors constitutive androstane receptor (CAR), pregnane X receptor (PXR), and farnesoid X receptor (FXR) on the expression levels of several transcripts of interest and the metabolism of AND and 3MI in primary porcine hepatocytes were tested. 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 and transcripts previously shown to be differentially expressed in boars with high boar taint levels. Transactivation of CAR, PXR, or FXR resulted in altered expression of several transcripts, including increased expression of *cytochrome P450 (CYP) 2B22* by CAR, of *CYP2A19*, *CYP2B22*, *CYP2C33*, and *CYP2C49* by PXR, of *CYP2C33* and *CYP2E1* by FXR, and of *CYP19A2* by all three receptors. Only transactivation of PXR had a significant effect on AND metabolism, resulting in $7.5 \pm 1.5\%$ of the initial level of AND remaining compared to $21.4 \pm 3.1\%$ remaining with control dimethyl sulfoxide (DMSO) treatment. FXR had the greatest effect on 3MI metabolism, increasing the expression of *CYP2E1* by 1.29-fold and increasing the production of the key metabolite 6-hydroxy-3-methylindole (6-OH-3MI), while decreasing 5-hydroxy-3-methylindole (5-OH-3MI) production. 3-Hydroxy-3-methyloxindole (HMOI) production was increased by CAR transactivation, while indol-3-carbinol (I3C) production was increased by PXR and FXR transactivation, and by treatment with 5β-dihydrotestosterone (5β-DHT). From this, it can be concluded that selective transactivation of PXR and FXR may be a viable means of decreasing boar taint by increasing the hepatic metabolism of AND and 3MI.

1. Introduction

The accumulation of 5α-androst-16-en-3-one (androstenone, AND) and 3-methylindole (skatole, 3MI), in the fatty tissue of boars results in boar taint, which is an unpleasant odor and taste in the meat [1,2]. AND is a naturally occurring steroid synthesized in the testes from pregnenolone by the activities of CYP17A1; from there AND is transported to the submaxillary salivary gland, where it is released into the saliva to act as a pheromone that influences sow reproduction [3–5]. In the salivary gland and hepatocytes, AND is converted to two other 16-androstene steroids, 3β-androstenol (3β-AND) and 3α-androstenol (3α-AND)[6,7], with 3β-AND as the major product produced by the 3-hydroxysteroid dehydrogenase enzymes present in the liver [8]. Both of the androstenols are further metabolized to phase II metabolites through conjugation with polar groups.

3MI is a tryptophan degradation product generated by gut microflora and is absorbed into the bloodstream from the gut and deposited in fatty tissues [9]. 3MI is extensively metabolized in the liver, with seven different metabolites identified. These metabolites include the primary metabolites 3-hydroxy-3-methylindolenine (3-OH-MI) and 3-hydroxy-3-methyloxindole (HMOI), indole-3-carbinol (I3C), 2-aminoacetophenone (2AAP), 3-methyloxyindole (3MOI), 5-hydroxy-3-methylindole (5-OH-3MI) and 6-hydroxy-3-methylindole (6-OH-3MI) [10–12]. The sulfoconjugate of 6-OH-3MI was shown to be the major circulating 3MI metabolite in pigs that exhibit low 3MI levels in fat, whereas HMOI was major metabolite in pigs that exhibited high levels [13]. This suggests that metabolism of 3MI through 6-OH-3MI is the major pathway for 3MI clearance. Several porcine cytochrome P450s (CYP) have been implicated in the metabolism of 3MI, including CYP1A1, CYP2A19, CYP2C33v4, CYP2C49, CYP2E1 and CYP3A, with CYP2A19, CYP2E1, and CYP2C49 potentially being the most physiologically important

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due to their ability to generate 6-OH-3MI [14]. Of the *in vitro* tested porcine P450s, CYP2A19 was shown to be the most extensive metabolizer of 3MI; however, *in vivo* expression levels of individual P450s may result in CYP2E1 in having a more extensive role than CYP2A19, due to higher hepatic expression levels [14].

Boar taint is usually prevented by castrating male piglets at a very young age. This directly reduces the production of AND, which in turn is thought to increase hepatic metabolism of 3MI; 3MI metabolism is negatively affected by the presence of AND through both decreased *CYP2E1* expression [15,16] and direct inhibition of CYP2E1 enzyme activity [17]. However, castrates have decreased growth rate, feed efficiency, and lean meat production compared to intact boars due to the loss of testicular steroids, so castrates are economically less favorable [18]. Physical castration is also an animal welfare concern, for it is commonly done without anaesthetics, which is stressful and painful to the piglet, and can lead to infections at the incision site. As such, alternate methods to physical castration are required; one option for reducing boar taint would be maximizing the metabolic clearance of the two primary boar taint compounds.

The nuclear receptor family of transcription factors, which are modulators of ligand-induced transcription of key genes, have been implicated in controlling a variety of physiological processes, including lipid metabolism and energy homeostasis, bile acid and xenobiotic metabolism, steroidogenesis, reproduction and development, and CNS, circadian, and basal metabolic functions [19]. Two receptors that are primarily transactivated by exogenous compounds, and are therefore important targets in toxicological studies, are the constitutive androstane receptor (CAR) and pregnane x receptor (PXR). Both are highly expressed in the liver and are responsible for co-ordinating the metabolism and clearance of both exogenous and endogenous compounds through regulation of the expression of various metabolic and transport proteins [20,21]. Both phase I metabolic enzymes, including the cytochrome P450 class of monooxygenases, and phase II metabolic enzymes are differentially regulated through the transactivation of CAR and PXR, and as such may aid in the metabolic clearance of AND and 3MI from boars.

A third member of the nuclear receptor family, the farnesoid X receptor (FXR), is highly expressed in the liver and intestinal tract, and is responsive to bile acids and their derivatives. FXR regulates both bile acid production and clearance in the liver and intestines, and is therefore one of the key components in bile acid homeostasis [22]. FXR has also been shown to play a role in hepatic metabolism, by up-regulating hydroxysteroid sulfotransferase (SULT2A1) [3,23]. FXR also binds to a motif in the promoter region of the *Pxr* gene [24], and therefore may play a role in hepatic metabolic processes through up-regulation of PXR.

Nuclear receptors have been poorly classified in pigs, though recent research has determined partial ligand complements for pig CAR [25], PXR, and FXR [26]. As such, their potential as pharmacological targets for the control of boar taint is largely unexplored. To better understand the potential of nuclear receptors as modulators of boar taint, a better understanding of the downstream effects of nuclear receptor transactivation in pigs is required. This study was designed to determine the effects of transactivation of CAR, PXR, and FXR on the regulation of specific transcripts of interest in primary porcine hepatocytes, 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 effective at transactivating porcine CAR at a concentration of 1 μM [25]. Rifampicin (RIF) is an antibiotic agent that has been shown to be a potent PXR agonist, and is commonly used at a concentration of 10 μM, including studies with porcine PXR [26]. 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 [26]. 3MI was also used as a treatment to determine the direct effects it could have on the metabolism of AND, and thus the extent of boar taint. The transcripts selected include porcine orthologs of genes involved in endobiotic and xenobiotic metabolism in humans, and genes that are differentially regulated in livers of boars with high or low levels of boar taint [27–29]. The effects of receptor transactivation on the metabolism of AND and 3MI by primary porcine hepatocytes were also studied, to determine if the changes in gene expression induced by receptor transactivation affect the metabolism of boar taint compounds. The effects of 5α-dihydrotestosterone (5α-DHT), 5β-DHT, and estradiol (E2) on the metabolism of 3MI were also tested, since they were previously shown to alter porcine nuclear receptor activity, with 5α-DHT and 5β-DHT significantly decreasing CAR activity, and all three steroids significantly increasing PXR activity [25,26].

2. Materials and methods

2.1. Research animals

Animals used for preparations of primary hepatocytes 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. Animals were a total of 10 uncastrated Yorkshire boars weighing approximately 120 kg. Livers were removed immediately following slaughter for further processing.

2.2. Isolation of primary hepatocytes

Primary hepatocytes were isolated as described previously [8] with slight modifications. Briefly, a liver lobe was cannulated and blanched with Hanks' balanced salt solution (without Ca^{+}, Mg^{2+}, HCO_3^{-}, and phenol red) containing 1 mM EGTA and 10 mM HEPES pH 7.4. Blanching occurred for 10 min at 37 °C with a flow rate of 25 ml/min, and the liver was then rinsed of the blanch solution with Hanks' balanced salt solution containing 10 mM HEPES. The lobe was then digested for 30 min at 37 °C using Williams' Media E, pH 7.4, containing 10 mM HEPES and 0.5 mg/ml Type I collagenase.

After digestion, the lobe was gently dissected in a sterile hood to liberate the hepatocytes from the collagen matrix. Cells were collected in Williams' media E, pH 7.4, containing 10 mM HEPES, 0.02 units/ml insulin, 1% penicillin/streptomycin, and 10% fetal bovine serum (FBS), filtered through sterile nylon mesh, and then centrifuged at 15 × *g* for 3 min. The cells were rinsed with the collection media, and then re-centrifuged at 15 × *g* for 3 min. The cells were counted using a hemocytometer, and cell viability determined using exclusion of 0.04% trypan blue. Viability was generally about 90%. Cells were then plated in collection media in 10 cm tissue culture dishes at a seeding density of 12 million cells/plate.

2.3. Treatment of primary hepatocytes and isolation of total RNA

The primary hepatocytes were treated 4 h after plating. The media was replaced with Williams' media E, pH 7.4, containing 10 mM HEPES, 0.02 units/ml insulin, 1% penicillin/streptomycin, 10 mM pyruvate, and 0.35 mM L-proline and either 0.05% (v/v) dimethyl sulfoxide (DMSO) as control, 1 μM 6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime (CITCO), 10 μM rifampicin (RIF), or 100 μM chenodeoxycholic acid (CDCA). Treatments were carried out in duplicate. 20 h after treatment, the cells were lysed and total RNA extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. RNA quality was