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Relationship between CYP3A29 and pregnane X receptor in *landrace pigs*: *Pig* CYP3A29 has a similar mechanism of regulation to human CYP3A4

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ABSTRACT

The objective of this study was to provide evidence of the validity of utilizing *pigs* as a model to study the regulation of human CYP3A4, with special emphasis on drug-drug interactions. We determined the mRNA expression and distribution of CYP3A and metabolic nuclear receptors in different tissues isolated from *landrace pigs*. Our results showed that CYP3A and metabolic nuclear receptor mRNAs were most highly expressed in liver tissues. The expression of the metabolic nuclear receptor pregnane X receptor (PXR) had a significant correlation with expression of CYP3A29, an analog of human CYP3A4. The correlation between their transcriptional levels was further demonstrated using LPS and TNF-α. The mRNA and protein expression of CYP3A29 and PXR in HepLi cells was significantly reduced by LPS and TNF-α treatment. CYP3A29 promoter activity was dramatically elevated by PXR over expression, whereas LPS and TNF-α treatment inhibited the enhanced CYP3A29 promoter activity that was induced by PXR; presumably through inhibition of PXR promoter activity. Furthermore, the inhibition of CYP3A29 promoter activity by LPS and TNF-α treatment was blocked by knockdown of PXR or retinoid X receptor (RXR). These data suggest high similarity in the regulation mechanism of *pig* CYP3A29 and human CYP3A4. Our research provided a significant evaluation to determine whether *pigs* are suitable as an experimental animal model.

1. Introduction

Various endogenous and exogenous substances including procarcinogens, xeno-estrogens or hormones may be bio-transformed by phase I and/or phase II metabolizing enzymes. Phase I metabolism leads to modification of the metabolite through hydroxylation, epoxidation, hydrogenation, demethylation, removal of the sugar moiety (hydrolysis) and, isomerization. During phase II metabolism, metabolites are conjugated with moieties such as glucuronic acid, sulfate, acetyl, glutathione or, methyl groups (Barc et al., 2013). After further modifications, metabolites are excreted from cells. The hepatic cytochrome P450 proteins (CYPs) are a family of enzymes important for Phase I metabolism of xenobiotics and endogenous compounds. Members of the CYP super family have been extensively studied in human and rodents (Mudra and Parkinson, 2001). *Pigs* are considered to be an important animal model for humans due to the physiological and anatomical similarities to humans (Shang et al., 2013). It has been demonstrated that CYP1A, CYP2A, CYP3A, CYP2C, CYP2D and CYP2E1 are expressed in the liver microsomes of miniature *pigs* (Achour et al., 2011; Anzenbacher et al., 1998).

Cross-species differences in the induction of CYP3A expression in response to xenobiotics have complicated the development of assays to identify compounds that modulate CYP3A transcription (Jones et al., 2000). At present, few data concerning gene expression and regulation of *pig* CYP3A have been published. CYP3A4 is an important CYP isoform responsible for metabolizing > 50% of clinical drugs in humans (Rendic and Di Carlo, 1997; Rodriguez-Antona et al., 2010; Shimada et al., 1994). Previous studies revealed that CYP3A, CYP2A and CYP2C enzymes in *pigs* have similar properties to human analogs (Nielsen et al., 2017; Soucek et al., 2001). In particular, CYP3A expressed in liver microsomes was comparable to human CYP3A4, determined by detecting liver microsome enzymatic activities (Anzenbacher et al., 1998). However, there was a lack of data on specific regulation of CYP3A expression by nuclear receptors.

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PXR can accept a wide variety of ligands exhibiting significant structural differences. This makes PXR a useful tool for sensing changes in the chemical environment. PXR regulates the transcription of numerous enzymes required for phase I and phase II drug metabolism and drug transporters, such as CYP3A, CYP2B and CYP2C (Chen et al., 2012; Wang et al., 2013; Zhou et al., 2009). The human and mouse forms of PXR share 76% amino acid identity in their ligand-binding domains (LBDs) but display markedly different activation profiles in response to xenobiotics (Jones et al., 2000). PXR, or other nuclear receptors, function as ligand-activated transcription factors. Together with RXR, PXR forms a heterodimer, which binds to specific DNA elements regulating transcription of numerous genes such as CYP3A in humans (Zhou et al., 2009). Thus, the question remains whether *pig* PXR and RXR show higher homology with human forms of the proteins and if *pig* PXR and RXR can regulate the expression of CYP3A29.

Therefore, this study aimed to investigate expression of CYP3A29 and metabolic nuclear receptors: PXR, RXR, Constitutive androstane receptor (CAR), Estrogen receptor (ER), Farnesoid X receptor (FXR), Peroxisome proliferator-activated receptor (PPAR), in different tissues isolated from *landrace pigs*, and explore the relationship between CYP3A29 and the nuclear receptor PXR.

2. Material and methods

2.1. Reagents

TNF-α was purchased from PROSPEC (ProSpec-Tany Techno Gene Ltd., Ness-Ziona, Israel). LPS was purchased from Sigma-Aldrich (Sigma-Aldrich Company Ltd., St. Louis, MO, U.S.A). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin were purchased from Gibco (Life Technologies, Grand Island, NY, U.S.A). 3-(4, 5-Dimethylthyazolyl-2)-2, 5-diphenyl-tetrazolium bromide (MTT) was purchased from MP Biomedicals (MP Biomedicals, Santa Ana, CA, U.S.A). TRIzol® reagent was purchased from Invitrogen (Life Technologies, Grand Island, NY, U.S.A). PXR antibody was purchased from GeneTex (GeneTex Inc., Irvine, CA, U.S.A). CYP3A and β-actin antibodies were purchased from Santa Cruz (Santa Cruz Biotechnology, California, CA, U.S.A). The Dual-luciferase reporter assay system was obtained from Promega (Promega, Madison, WI, U.S.A). Lipofectamine 2000 Transfection Reagent was purchased from Invitrogen (Life Technologies, Carlsbad, CA, U.S.A).

2.2. Animals

Four *landrace pigs* (50 ± 3 kg body weight, 3 months, castrated) were purchased from the Breeding Swine Testing Center (Wuhan, China) and acclimatized for one week with free access to basic diet and water under standardized temperature (26 °C) and light environment (12 h light/12 h dark). The diet was bought from Shanghai Xinnong (Shanghai Xinnong feed Co. Ltd., Shanghai, China). All *pigs* were esthetized and sacrificed by bleeding of the carotid artery prior to collecting tissue samples including the heart, liver, kidney, spleen, muscle, small intestine, lung and skin. We repeated our experiment 3 times, and used 4 *pigs* each time. The experiment interval was 1–2 months. Our experimental protocols were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals and approved by the Animal Care Ethics Committee of our institution and followed such guide lines. All of the experiments were conducted with an effort to minimize the number of animals used, and the suffering caused by the procedures employed in the present study. All tissues were freshly frozen in liquid nitrogen and transferred to −80 °C.

2.3. Cell culture and treatment

Immortalized porcine hepatocyte line, Hep-Li, was obtained from Zhejiang University (Hangzhou, China) and maintained in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin solution in an atmosphere containing 5% CO_2 at 37 °C.

2.4. Real-time polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using TRIzol (Life Technologies, Grand Island, NY, U.S.A) and DNA was reverse transcribed (RT) with the Superscript reverse transcriptase (Takara, Otsu, Japan). Supplemental Table 1 lists the primers used. The qRT-PCRs were performed using iQ™ SYBR Green PCR Super mix (Takara) in the Bio-Rad CXF real-time PCR detection system (Bio-Rad, Hercules, CA, U.S.A). Primer sequences are shown in Supplementary File 1. RT-PCR was carried out at 95 °C for 1 min, followed by 30 cycles of 95 °C for 5 s, 58 °C for 10 s, and 72 °C for 35 s.

2.5. Western analysis

Total proteins from cultured cells were extracted by RIPA buffer (Thermo Scientific, Waltham, MA, U.S.A). The protein concentration was measured by BCA protein assay (Thermo Scientific, Waltham, MA, U.S.A). 40 μl protein was separated on 12% gel by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% BSA in 0.1% Tween20 for 60 min, then incubated with rabbit polyclonal antibodies against anti-*pig* CYP3A29 (1: 500 dilution) and anti-*pig* PXR (1: 500 dilution) overnight. After washing in TBST buffer, the membrane was incubated with goat anti-rabbit IgG secondary antibody (1: 5000 dilution) at room temperature for 1 h. Signals were detected by Clarity Western ECL Substrate (Bio-Rad, Hercules, CA, U.S.A) and quantified by a DNR Bio Imaging System (DNR Industries, Anaheim, CA, U.S.A).

2.6. Transient transfection

HepLi cells were seeded in 24-well plates and cultured until 80% confluence. Transfections were performed with Lipofectamine™ 2000 Transfection Reagent (Life Technologies, Carlsbad, CA, U.S.A). Transfection mixtures contained 500 ng of promoter reporter vector with or without over expression vectors (pcDNA3.1 was used as control for over expression vectors)/siRNA and 25 ng of pRL-TK plasmid. Cells were transfected for 6 h, and the medium was replaced with fresh medium containing drugs or DMSO. Transfected cells were incubated for another 12 h and reporter enzyme activity was determined with a dual-luciferase reporter assay system (Promega, Madison, WI, U.S.A), according to manufacturer's instructions. Results were calculated from three or more independent transfection experiments. SiRNA sequences are shown in Supplementary Table 2.

2.7. Statistical analysis

The mRNA expression of CYP3A29, PXR and RXR-α was analyzed by qRT-PCR, the luciferase enzyme activity was determined with a dual-luciferase reporter assay system. Data are presented as mean ± standard deviation (SD) from at least three independent experiments. Statistical analysis was performed using SAS software version 9.1 (SAS Institute, Cary, NC, U.S.A). One-way analysis of variance (ANOVA) followed by Duncan's multiple comparison tests or Student's *t*-test was used to determine significant differences between each group. P-values < 0.05 was considered statistically significant.

3. Results

3.1. Expression of CYP3A and metabolic nuclear receptors in different tissues from landrace pigs

The expression profiles or other characteristics of CYPs vary across