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Dried chicory root modifies the activity and expression of porcine hepatic CYP3A but not 2C – Effect of *in vitro* and *in vivo* exposure

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ABSTRACT

Hepatic cytochrome P450 expression and activity are dependent on many factors, including dietary ingredients. In the present study, we investigated the *in vivo* and *in vitro* effect of chicory root on hepatic CYP3A and 2C in male pigs. Chicory feeding increased the expression of CYP3A29 mRNA but not CYP2C33. Correspondingly, CYP3A activity was increased by chicory feeding, while CYP2C activity was not affected. Additionally, the *in vitro* effect of chicory extract on the CYP3A activity was investigated. It was shown that CYP3A activity in the microsomes from male pigs was inhibited, but this effect was eliminated by pre-incubation. In both male and female pigs the CYP3A activity was increased in the presence of chicory after pre-incubation. Furthermore, gender-related differences in mRNA expression and activity were observed. CYP3A mRNA expression was greater in female pigs; this was not reflected on activity. For CYP2C, no difference in mRNA expression was observed, while CYP2C activity was greater in female pigs. Surprisingly, the expression of the constitutive androstane receptor, pregnane X receptor and aryl hydrocarbon receptor did not differ with feed or gender. In conclusion, chicory root modifies the expression and activity of CYP3A *in vivo* and *in vitro*, while CYP2C is not affected.

1. Introduction

The hepatic cytochrome P450s (CYP450) is a family of enzymes, important for the Phase I metabolism of xenobiotics and endogenous compounds. Many factors are involved in the regulation of CYP450 activity such as age, genetic background, health status and environmental factors. Additionally, it has been shown that expression and activity of some hepatic CYP450s can be up- or down-regulated by bioactive compounds from plants (Chang, 2009; Delgoda and Westlake, 2004; Nowack, 2008; Zhou et al., 2003). An *in vitro* experiment using human hepatocytes showed that CYP2C19 activity was up-regulated upon treatment with extracts of St. John's Wort, Common Valerian, *Ginkgo biloba* and Common Sage (Hellum et al., 2009). Likewise, it has been shown using human hepatocytes that St. John's Wort induces CYP3A4 expression through activation of the pregnane X receptor (PXR) (Moore et al., 2000). Taken together, this shows that bioactive ingredients in the diet can act as ligands for the receptors regulating CYP450 expression, and thereby modify CYP450 expression and activity. However, regulation of CYP450 genes via its receptors is not straight forward, due to sharing of ligands and receptor cross-talk (Lewis, 2004; Pascussi et al., 2008). Moreover, interpretation from one species to another is complicated by intra-species differences in CYP450 gene regulation (Martignoni et al., 2006). Apart from the *in vivo* effect of diet, previous studies using *in vitro* models have shown that bioactive plant compounds also modify the activity of CYP450s on the kinetic level, e.g., by competitive inhibition (Doehmer et al., 2011; Obermeier et al., 1995; Teel and Huynh, 1998). Thus, introduction of bioactive compounds via feed can alter CYP450 activity, which in turn can affect the biological activity of drugs, endogenous hormones and other pathways of physiological importance.

The CYP3A and CYP2C isoforms are well studied in humans due to their involvement in the metabolism of a variety of drugs and endogenous compounds, including arachidonic acid, eicosanoids, progesterone, oestradiol, testosterone and cortisol (Lewis, 2004; Nebert and Russell, 2002; Waxman, 1996). Together with other factors, diet is a critical player in the regulation of these isoforms in humans. However, knowledge about dietary dependent regulation of porcine CYP3A and CYP2C is missing.

The addition of chicory in the diet to pigs has been suggested as an alternative to castration to prevent the occurrence of boar taint, an unpleasant odour in meat from male pigs. For the successful implementation of chicory enriched diet in entire male pig production the side effects on liver metabolism have to be investigated in order to evaluate the safety of dietary chicory. Therefore, it is essential to investigate the hepatic expression and activity of CYP450 isoforms in the presence of chicory. Moreover, to evaluate

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Table 1
Primers and TaqMan probes for PCR.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	TaqMan probe (5′–3′)	Reference
CYP3A29	GGACACCATAAATCCTTACACTTACCT	GCAAACCTCATGCCAATGC	CCTTTGGGACTGGACCCCGCAA	Nannelli et al. (2010)
CYP2C33	TGGGAATCTGATGCAACTTAACC	AACAGGGCCGTACTGTTTGG	AAGGACATCCCTGCGTCTCTTTCCAAGTT	Tomankova et al. (2012)
AhR	AGCTGCACTGGGCGTTAAA	GCCACTCGCTTCATCAATTCT	CCTTCACAGTGTCCAGACTCTGGAC	Messina et al. (2009)
CAR	TTCATCCATCACCAGCACTTG	TGATGTCCGCGAAATGCA	CCCTGGTGCCTGAACTGTCTCTGCTC	Nannelli et al. (2010)
PXR	GCTGAACTGTGCTAGGCTTCTG	CCTCCCACGAGCCATGTT	ATGCACCGGGACACAAGTGAGGG	Nannelli et al. (2010)
GAPDH	GTCGGAGTGA ACGGATTTGG	CAATGTCCACTTTGCCAGAGTTAA	CGCCTGGTCACCAGGGCTGCT	Young et al. (2008)

the changes in CYP450 expression, the differences in constitutive expression of CYP3A and 2C between male and female pigs were investigated.

We have recently showed that feeding chicory root to pigs caused an increase in the expression and activities of CYP1A2 and 2A (Rasmussen et al., 2011e), as well as other hepatic enzymes (Rasmussen et al., 2012). In the present study, we investigated the *in vivo* and *in vitro* effect of chicory root on hepatic CYP3A and 2C. After 16 days of oral administration of chicory root to pigs we analysed the hepatic CYP450 mRNA expression and activity. Moreover the mRNA expression of the receptors regulating CYP450 expression was investigated. The *in vitro* effect of chicory on CYP450 activity was investigated in liver microsomes using specific probe substrates and a crude extract of chicory root.

2. Materials and methods

2.1. Chemicals

All substrates and chemicals used were purchased for Sigma–Aldrich and of highest purity available. Primers and probes were custom made by DNA Technology (Aarhus, Denmark).

2.2. In vivo treatment and sample handling

The description of experimental animals, feed composition and slaughter procedure is given in Rasmussen et al. (2011e). In brief: a group of male pigs were randomly allocated to either control ($n = 6$) group or experimental feed pigs ($n = 14$) 16 days before slaughter. Experimental feed pigs were fed energy matched diet containing 10% dried chicory root for the 16 days before slaughter. Likewise a group ($n = 4$) of female pigs receiving control feed was included in the experiment. At an age of 164 days all pigs were slaughtered and liver samples were taken and stored at −80 °C until further analysis. Microsomes for activity measurements were prepared according to (Rasmussen et al., 2011a) using sucrose-buffer and ultracentrifugation.

2.3. mRNA expression

Total RNA isolation and reverse transcription were done according to Rasmussen et al. (2011c). Briefly, total RNA was isolated from frozen liver tissue using a Spin column according to the manufacturer's instructions (Rnasey Mini Kit, WVR, Herlev, Denmark). Equal amounts of RNA was converted to cDNA using SuperScript II Rnase H Reverse Transcriptase and Oligo(dT)12–18 Primer (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instruction. Real-time polymerase chain reaction (PCR) and designing of primers and probes were done according to Rasmussen et al. (2011b,c) with the use of porcine specific sequences of genomic DNA (http://www.ensembl.org/Sus_scrofa/Info/Index). Primers and probes are given in Table 1. All samples were analysed in duplicates in a 348 well plates using a ABI 7900 HT sequence detection system (Applied Biosystems, Carlsbad, CA, USA). The PCR was conducted under the following conditions: 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

Relative mRNA expression was calculated by relating threshold cycles to a standard curve obtained by running a serial dilution of a cDNA sample. Relative mRNA expression was normalised against the mRNA expression of GAPDH. The average of control samples was arbitrary set to 1. The expression of GAPDH was not significantly affected by the treatment.

2.4. Activities of CYP3A and 2C

The activities of 7-benzyloxyresorufin O-dealkylase (BROD; CYP3A), 7-benzyloxyquinoline O-debenzylase (BQOD; CYP3A) and tolbutamide hydroxylase (TBOH; CYP2C) were measured. Validation of specificity and analytical procedures for porcine BROD and BQOD were described by Zlabek and Zamaratskaia (2011) and for tolbutamide by Skaanild and Friis (2008), Zamaratskaia et al. (2012). Briefly, to measure BROD and BQOD activity, the incubation mixtures (0.5 ml) contained 0.2 mg of microsomal protein in 0.5 M potassium phosphate buffer (pH 7.4), appropriate substrate (100 μM of BQ or 4 μM of BR) and 0.5 mM of NADPH. The microsomes and substrates were pre-incubated for 2 min prior to start of the reaction by addition of NADPH. The mixtures were incubated in a water bath at 37 °C for 10 min and the reactions were terminated with ice-cold 100% methanol (500 μL). To measure TBOH activity, the incubation mixtures (0.25 ml) contained 0.5 mg of microsomal protein in 50 mM Tris–HCl, 10 mM $MgCl_2$, 0.1 mM EDTA buffer (pH 7.4), 200 μM of tolbutamide and 1 mM of NADPH. The mixtures were incubated in a water bath at 37 °C for 40 min without pre-incubation and the reactions were terminated with the addition of 0.25 ml of 100% cold acetonitrile.

2.5. In vitro inhibition study

BROD and BQOD inhibition by selective inhibitors has previously been reported (Zlabek and Zamaratskaia, 2012). Direct effect of chicory root extract (CRE) on the activities of BROD and BQOD was investigated using microsomal preparations from male and female pigs. The pigs used for preparation of the used microsomes have not previously been exposed to chicory. CRE was prepared by mixing chopped dried chicory root with 96% ethanol in 1:1 for 24 h, followed by filtration. The resulting liquid phase was used as a crude extract of chicory. Three dilutions of CRE were used (0, 10 and 100 times dilutions). In each experimental set 3 pigs of each gender were included. Activities were determined with or without a 5 min pre-incubation step. For measurements of inhibition without pre-incubation, microsomes were mixed with CRE, NADPH and substrate (7-benzyloxyresorufin or 7-benzyloxyquinoline), while for measurements with pre-incubation the microsomes were incubated for 5 min with CRE and NADPH before addition of the substrate. The pre-incubation step was introduced to test for the effect of metabolites produced from the CRE. To ensure the same temperature of all incubations, the microsomes were kept at 37 °C for 5 min before addition of NADPH. Results are presented as percentage of activity in absence of CRE, but in the presence of <0.1% ethanol (control samples). All control incubations (no CRE) were determined at the same occasion as the incubation with CRE. Preliminary analysis confirmed that the activity was not affected by the presence of <0.1% ethanol (data not shown). All incubations were performed in duplicate.

2.6. Statistics

In the *in vivo* experiment groups was compared using Student's unpaired t-test. Data from the *in vitro* study were statistically evaluated using two-way ANOVA (SAS version 9.2; SAS Institute, Cary, NC, USA). The model included CRE dilution and gender as fixed factors, and individual microsomes pools incubation as a random factor. Likewise, interaction between CRE dilution and gender was also included in the model. Analyses were performed separately with or without the pre-incubation step. The differences were considered significant at $p < 0.05$.

3. Results

3.1. In vivo effect of chicory on CYP expression and activity

In human CYP3A4 and 2C9 isoforms of the CYP3A and 2C families has been shown to the most important once for the metabolism of drugs (Guengerich, 2007). The porcine versions of these isoforms, based on amino acid sequence match are CYP3A29/3A39 and CYP2C33, respectively (Achour et al., 2011). For CYP3A29 vs 3A39, 3A29 has been shown to most resemble the properties of the human CYP3A4 (Puccinelli et al., 2011).

In pigs fed dried chicory root, the mRNA expression of CYP3A29 was 2.0-fold greater ($p < 0.05$) than in control feed male pigs, while no difference was observed in CYP2C33 mRNA expression (Fig. 1). The CYP3A dependent activity was determined using two different reactions, BROD and BQOD (Table 2). In chicory fed pigs the BROD