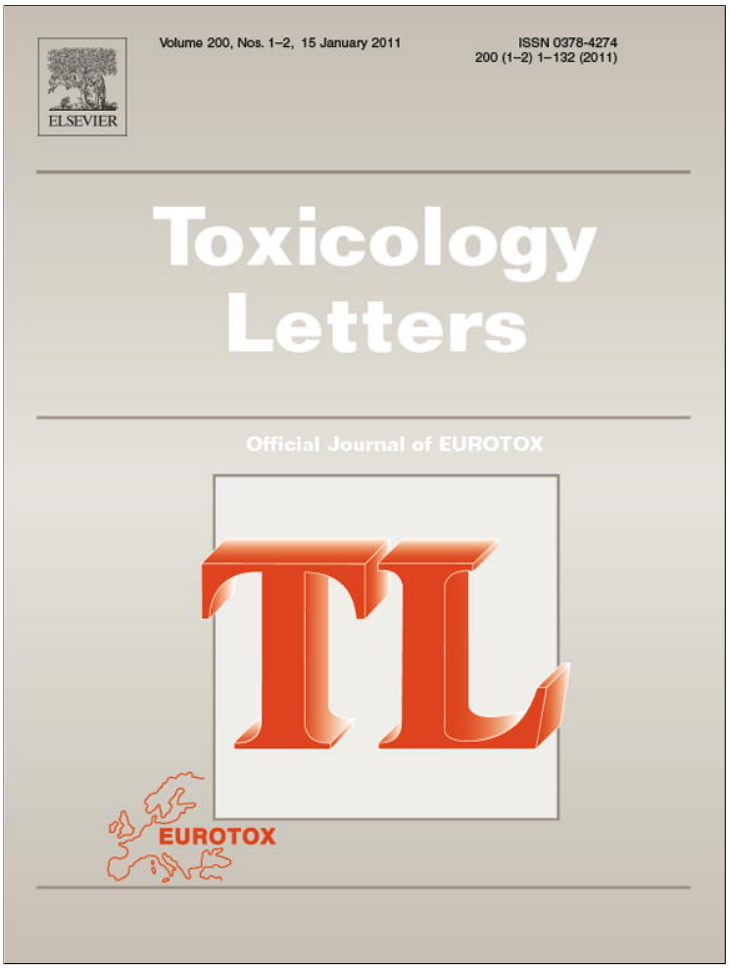

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In vivo effect of dried chicory root (*Cichorium intybus* L.) on xenobiotica metabolising cytochrome P450 enzymes in porcine liver

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ABSTRACT

Cytochrome P450 (CYP) enzymes are widely studied for their involvement in metabolism of drugs and endogenous compounds. In porcine liver, CYP1A2, 2A and 2E1 are important for the metabolism of skatole. Feeding chicory roots to pigs is known to decrease the skatole concentration in plasma and fat. In the present study we investigated the effect of chicory on CYP mRNA and protein expression, as well as their activity. Male pigs were feed dried chicory root for 16 days before liver samples were collected. By the use of RT-PCR and Western blotting we showed that the mRNA and protein expression of CYP1A2 and 2A were increased in chicory fed pigs. The mRNA expression of CYP2E1 was increased, while there was no effect on protein expression. Activity of CYP1A2 and 2A were increased in chicory feed pigs; this was not the case for CYP2E1 activity. In conclusion; oral administration of chicory root for 16 days to pigs increased the mRNA expression of CYP1A2, 2A and 2E1; and the protein expression of CYP1A2 and 2A. The activities of CYP1A2 and 2A were increased.

1. Introduction

The cytochrome P450 enzymes (CYP) are a superfamily of enzymes important for the metabolism of endogenous compounds and xenobiotics. High concentrations of CYPs are mostly found in the liver, but also in the small and large intestine, lung and brain. CYPs are extensively studied for their involvement in drug metabolism and activation of pro-carcinogens. Inter-individual variation in e.g. CYP dependent drug metabolism has been observed (Zanger et al., 2008) and is often explained by the genetic profile of the individual. However there is growing evidence for a regulatory effect obtained by dietary compounds. The expression and activity of hepatic CYP can be up- or down-regulated by bioactive compounds from plants (reviewed by Chang, 2009). One extensively studied case is the inhibitory effect of grape fruit juice on CYP3A4 activity and consequently lower clearance of drugs metabolised by that CYP isoform (Kiani and Imam, 2007). An *in vitro* experiment using human hepatocytes showed that CYP2C19 activity was up-regulated upon treatment with extracts of St. Johns Wort, Common Valerian, *Ginkgo biloba* and Common Sage, while there was no effect of Cone Flower or Horse Chestnut (Hellum et al., 2009). The same study reported that CYP2E1 activity was up-regulated by St. Johns Wort, while all the previously mentioned plants had no or down-regulating effect on CYP2E1 activity.

The perennial herb chicory (*Cichorium intybus* L.) has been shown to posses several health beneficial properties (reviewed by Bais and Ravishankar, 2001) including anti-carcinogenic (Pool-Zobel et al., 2002), anti-inflammatory (Cavin et al., 2005), antioxidant (Gazzani et al., 2000), as well as protection against immunotoxicity induced by ethanol (Kim et al., 2002) and a prebiotic effect depending on its content of inulin (reviewed by Kolida et al., 2002). Chicory is used as a coffee substitute and the roots are often used as a source for inulin. Moreover, one group of documented bioactive component in chicory is sesquiterpene lactones (Rodriguez et al., 1976; Beek et al., 1990).

The aim of the present study was to investigate whether oral administrations of chicory root increases the activity and expression of hepatic CYP enzymes in entire (uncastrated) male pigs. Differences in CYP1A2, 2A and E1 dependent activity, mRNA and protein expression were evaluated in liver samples from the pigs. The selected enzymes are of particular importance in pigs because of their role in the metabolism of skatole, one of the major compounds responsible for boar taint (Zamaratskaia and Squires, 2009). Moreover, pigs could be used as a suitable model for humans, because their physiological characteristics are similar to humans (Lunney, 2007).

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Table 1
Composition and nutrient content of the control (CON) and experimental diet (DCR).

	CON	DCR
Components (% of total weight)		
Dried chicory root[a]	–	10.0
Barley	60.0	50.0
Soybean cake	16.7	19.0
Wheat	12.1	11.3
Oat	5.0	3.0
Vegetable fat	1.9	1.2
Melasses	1.0	3.2
Calculated analysis (g/kg)		
FEsvin[b]	1.0	1.0
Dry matter	863.6	871.3
Crude protein	156.0	161.8
Crude fat	42.2	32.0
Total fibre	165.1	226.1
Inulin	–	47.1
Tryptophan	2.0	2.1

[a] Dried at 65 °C.
[b] Feeding unit pig.

2. Materials and methods

2.1. Chemicals

Coumarin, *p*-nitrophenol, ethoxyresorufin, and methoxyresorufin were purchased from Sigma–Aldrich (St. Louis, MO, USA). The CYP1A2, 2A and 2E1 antibodies were purchased from Acris Antibodies (Herford, Germany), Santa Cruz (Santa Cruz, CA, USA) and Abcam (Cambridge, UK), respectively. Primers and probes were custom made by DNA Technology (Aarhus, Denmark). All chemicals and solvents were of analytical grade and were obtained from common commercial sources.

2.2. Experimental animals, feeding strategies and sampling

All animals were entire (uncastrated) male pigs of a crossbreed between Landrace × Yorkshire sire and Duroc boars. 20 pigs were raised under the same conditions, fed *ad libitum* with a commercially available diet (CON; Table 1) and kept in the same pen until 16 days before slaughter. 6 pigs were randomly allocated to the control group and kept in a separate pen where they remain fed *ad libitum* with the control diet (CON). The remaining 14 pigs were fed *ad libitum* with an energy matched diet containing 10% dried chicory root (DCR; Table 1) for the last 16 days before slaughter. At an age of 164 days (weighing approximately 130 kg) all pigs were slaughtered in a commercial slaughter house. After stunning with CO_2, the pigs were burned, scalded at approximately 60 °C and eviscerated and samples of the liver tissue were collected. All samples were taken approximately 20 min after stunning. The liver samples were stored at −80 °C until further analysis. There were no differences between the groups with regard to use of medicine, signs of diseases, health problems, carcass weight and lean meat content.

2.3. Preparation of liver microsomes

Liver microsomes were prepared by ultracentrifugation according to Rasmussen et al. (2010b,c). Briefly, tissue was homogenized in a buffer containing 250 mM sucrose, 50 mM Tris–HCl, 150 mM KCl, 1 mM EDTA, 2 mM PMSF (pH 7.4) and centrifuged at 10000 × *g* (4 °C). The supernatant was then centrifuged at 100000 × *g* for 60 min at 4 °C and the resulting pellet was suspended in appropriate buffer (100 mM Tris-base, 1% SDS, pH 9.5 for Western blotting, or 50 mM Tris-base, 1 mM EDTA, 250 mM sucrose, 20% glycerol, pH 7.4 for activity measurements). Total protein concentration was determined using a BCA kit (Pierce) according to the manufacturer's instruction.

2.4. Measurement of specific CYP activity

The activity of CYP1A was measured as the rates of *O*-dealkylation of ethoxyresorufin (EROD) or methoxyresorufin (MROD) to resorufin with a modified method according to Zamaratskaia and Zlabek (2009). 0.2 mg total protein was incubated in 0.5 ml 50 mM phosphate buffer (pH 7.4) at 37 °C with either 2 μM ethoxyresorufin or methoxyresorufin dissolved in dimethylsulfoxide (DMSO), giving a final concentration of DMSO at 0.4%. The reaction was started by adding 1 mM NADPH and stopped with 100% ice-cold methanol after 5 min for EROD or 10 min for MROD. The formation of resorufin was analysed by HPLC (Zamaratskaia and Zlabek, 2009).

The activity of CYP2A was measured as the rate of hydroxylation of coumarin (COH) to 7-hydroxycoumarin, while the activity of CYP2E1 was measured as the rate of hydroxylation of *p*-nitrophenol (PNPH) to *p*-nitrochatecol. Both assays were done according to Rasmussen et al. (2010b).

All CYP activity measurements were done in duplicates.

Table 2
Cytochrome P450 dependent activity in liver microsomes from entire male pigs feed control (CON) or experimental (DCR) diet. Activities are given as pmol/min/mg protein. EROD, ethoxyresorufin *O*-deethylase; MROD, methoxyresorufin *O*-demethylase; COH, coumarin hydroxylation; PNPH, *p*-nitrophenol hydroxylase. Level of significance: $^{**}p < 0.01$ and $^{*}p < 0.05$.

	CON	DCR
CYP-dependent activity		
EROD	38.1 ± 4.6	39.1 ± 3.8
MROD	8.2 ± 0.6	12.2 ± 0.8**
COH	3.0 ± 1.1	13.0 ± 1.7*
PNPH	20.6 ± 9.1	29.5 ± 3.9

The activity of the CON group has been published before (Rasmussen et al., 2010b).

2.5. Western blotting

Equal amounts of protein were separated on a 12% Bis-Tris gel (BioRad, Hercules, CA, USA) and electroblotted onto a PVDP membrane. After 5 min of blocking in TBS-buffer (50 mM Tris-base, 0.5 M NaCl, pH 7.4) with 2% Tween20, the membrane was treated with primary antibody diluted in TBS-buffer containing 0.1% Tween20 overnight at 4 °C. The membrane was then washed 3× 10 min in TBS-buffer containing 0.1% Tween20 and incubated for 90 min at room temperature with a secondary antibody attached with Alexafluor488. Before scanning on a Molecular Imager® FX (Bio-Rad, Hercules, CA, USA) scanner, the membrane was washed 6× 5 min in TBS-buffer with 0.1% Tween20. The primary antibodies used are raised against a human epitope and have previously been shown to be specific in identifying porcine CYPs (Rasmussen et al., 2010b).

All samples to be compared were quantified relative to a standard sample. The average of the control group was arbitrary set to 100 and the experimentally fed group expressed relative to that.

2.6. Reverse transcription

RNA was isolated with Rnasey Mini Kit (WVR, Herlev, Denmark) according to the manufactures instruction. The isolated RNA was quantified by measuring the absorbance at 260 nm and purity assessed by the 260 nm/280 nm ratio, which was always above 1.9. Reverse transcription using SuperScript II Rnase H Reverse Transcriptase and Oligo(dT)$_{12–18}$ Primer (Invitrogen, Carlsbad, CA, USA) were used to convert RNA to cDNA. cDNA were diluted in RNAase free water and stored at −20 °C until further use.

2.7. Quantitative PCR

The amount of cDNA was quantified by polymerase chain reaction (PCR) using TaqMan probes. cDNA, primers and probes were mixed with TaqMan®2X Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). The sequence of primers and probes were the same as used by Rasmussen et al. (2010b). The PCR consisted of the following temperature profile: 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

The relative mRNA expression was calculated from the obtained values for threshold cycles related to a standard curve obtained by running a serial dilution of one cDNA sample. The relative mRNA amount was normalised against the mRNA expression of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and expressed as arbitrary units. The mRNA expression of GAPDH was not affected by the feed. Average of the control group was arbitrary set to 1 and the experimentally fed group expressed relative to that.

For all PCR analysis, samples with no DNA and genomic DNA were analysed to confirm that no unspecific amplification occurred. All PCRs were done in duplicates.

2.8. Statistical analysis

Student's unpaired *t*-test was used to test for differences between groups.

3. Results

3.1. Cytochrome activity in microsomes

In pigs fed with chicory root the CYP2A dependent COH activity was increased approximately 4 times ($p < 0.01$), while the CYP2E1 dependent PNPH activity did not differ ($p > 0.05$) between the two groups (Table 2). Two different substrates were used to evaluate the activity of CYP1A. Ethoxyresorufin has been shown to be metabolised by CYP1A1, while methoxyresorufin is more specific to CYP1A2 metabolism (Messina et al., 2008; Chirulli et al., 2007). There was no difference ($p > 0.05$) in EROD activity in the micro-

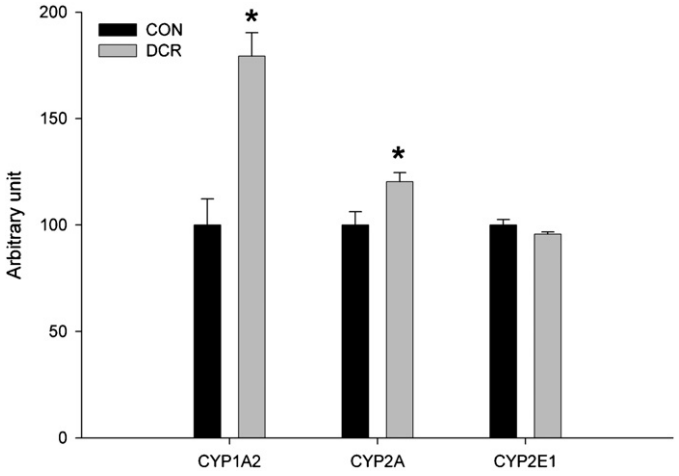

Fig. 1. Protein expression of cytochrome P450 isoform 1A2, 2A and 2E1 in microsomes from entire male pigs feed control diet (CON) or experimental diet (DCR). Levels of significance: $^{*}p < 0.05$.

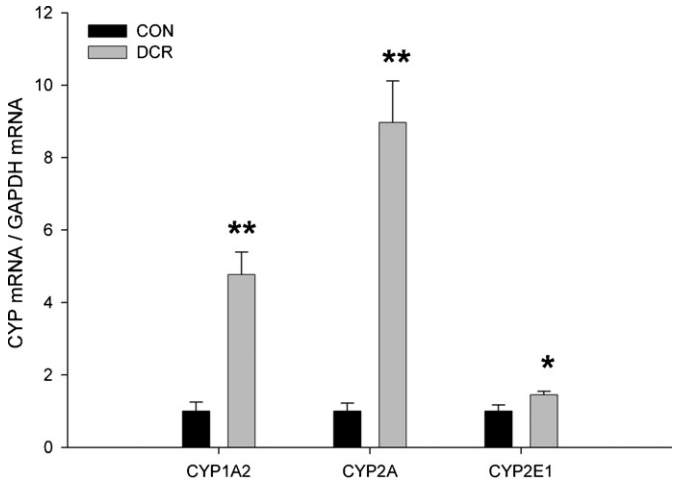

Fig. 2. mRNA expression of cytochrome P450 isoform 1A2, 2A and 2E1 in liver tissue from entire male pigs feed control diet (CON) or experimental diet (DCR). Levels of significance: $^{**}p < 0.01$; $^{*}p < 0.05$.

somes from entire male pigs fed either chicory root or control diet (Table 2). The MROD activity was increased approximately 1.5 times ($p < 0.01$) in the microsomes from entire male pig fed chicory root compared to control fed pigs (Table 2).

3.2. Protein and mRNA expression of CYP1A2, 2A and 2E1

The expression of CYP1A2 and CYP2A in entire male pigs fed chicory root was 79 and 20% greater, respectively ($p < 0.05$) (Fig. 1) compared to control pigs. There were no differences between the expression of CYP2E1 in the two groups (Fig. 1).

Semi quantitative PCR showed that the group of pigs fed chicory root had greater mRNA expression of CYP1A2 (4.7 ± 0.6 fold; $p < 0.01$), 2A (9.0 ± 1.2 fold; $p < 0.01$) and 2E1 (1.5 ± 0.1 fold; $p < 0.05$) (Fig. 2).

4. Discussion

The regulation of CYP activity involves multiple mechanisms at different levels and is mainly studied in humans, rats and mice. However, the information about regulation of CYP by bioactive compounds in pigs is still limited. This is to the best of our knowledge the first study to investigate the effect of dietary compounds on the expression and activity of hepatic CYP in pigs. We showed that oral administration of dried chicory root for 16 days increased the mRNA levels of CYP1A2, CYP2A and CYP2E1 and protein expression of CYP1A2 and CYP2A. Additionally, the MROD and COH were greater in the pigs fed chicory root.

Porcine CYP2A and 2E1 have been extensively studied for their involvement in the skatole metabolism (reviewed by Zamaratskaia and Squires, 2009). Moreover CYP1A2 has also been shown to metabolise skatole in both humans and pigs (Matal et al., 2009). *In vitro* experiments have showed that in microsomes prepared from male pigs the activity of CYP2E1 was inhibited in the presence of testicular steroids (Rasmussen et al., 2010a,b; Zamaratskaia et al., 2007). Decreased skatole metabolism in mature male pigs leads to accumulation of high skatole concentrations in the adipose tissue, negatively affecting the sensory quality of the meat. Feeding chicory root to sexually mature male pigs has previously been shown to decrease the concentration of skatole in both plasma and adipose tissue (Hansen et al., 2002; Hansen et al., 2006; Byrne et al., 2008; Lanthier et al., 2006). This effect has partly been ascribed to a prebiotic effect due to high inulin content. However, our study for the first time suggests that chicory root may increase the hepatic skatole-metabolism through the induction of CYP expression and activity. Feeding entire male pigs with chicory root for 16 days increased the CYP1A2 and CYP2A dependent activity. Moreover, we showed that the protein and mRNA expression of CYP1A2 and CYP2A were also increased. In the present study we found no effect of chicory root on CYP2E1 dependent activity and protein expression. However, the expression of CYP2E1 mRNA was increased 1.5 fold. Taken together these results show, that transcription and translation of CYP2A and CYP1A2 are increased by chicory root, while CYP2E1 is not. Increased expression of CYP proteins are in most cases achieved by de novo synthesis regulated by the involvement of cytosolic receptors like pregnane X receptor (PXR), constitutive androstane receptor (CAR) and aryl hydrocarbon receptor (AhR) (reviewed by Honkakoski and Negishi, 2000).

It is worth noting that the specificities of the substrates used to asses CYP activity in this study for the different pig CYP450s are debated. Thus, it has been suggested that *p*-nitrophenol can be metabolized by both CYP2E1 and CYP2A in porcine liver microsomes (Skaanild and Friis, 2007). Furthermore the specificity of EROD and MROD in pigs is not fully elucidated (Zamaratskaia and Zlabek, 2009). Nevertheless, the used substrates are commonly used to asses specific CYP activity and we believe that the results provide solid evidence on the importance of chicory root in the modulation of porcine CYP450s.

The sesquiterpene lactone artemisinin, origin from the genus *Artemisia*, has showed to be an agonist for CAR and thereby regulating CYP expression (Simonsson et al., 2006). The experimental set-up used in the present study could not be used to elucidate the mechanisms underlying the observed CYP induction. We suggest that the induction could be due to the presence of one or more agonists for the PXR, CAR or AhR, given that chicory contains sesquiterpene lactones. However, several other possible mechanisms of CYP induction exists including indirect effect of altered hormonal or endogenous substrate concentrations through a prebiotic effects on the intestinal microbial flora, or altered mRNA turnover rates. However further studies are needed to elucidate the underlying mechanisms.

5. Conclusion

The current study provided the first evidence that feeding dried chicory root to pigs induced the mRNA expression of CYP1A2, CYP2A and CYP2E1. Protein concentrations of CYP1A2 and CYP2A as well as MROD and COH rates were also significantly increased.

Conflict of interest

The authors declare that there are no conflicts of interest.

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