Food Chemistry 146 (2014) 255–263

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Regulation of cytochrome P450 mRNA expression in primary porcine hepatocytes by selected secondary plant metabolites from chicory (*Cichorium intybus* L.)

Martin Krøyer Rasmussen *, Christina Lindgaard Klausen, Bo Ekstrand

Department of Food Science, Aarhus University, Denmark

ARTICLE INFO

Article history:
Received 15 January 2013
Received in revised form 4 July 2013
Accepted 11 September 2013
Available online 19 September 2013

Keywords:
Bioactive compounds
Primary hepatocytes
Cryopreservation
Boar taint
Gene regulation
Detoxification
Liver metabolism

ABSTRACT

Chicory (*Cichorium intybus*) has been shown to induce enzymes of pharmacokinetic relevance (cytochrome P450; CYP). The aim of this study was to investigate the effects of selected secondary plant metabolites with a global extract of chicory root, on the expression of hepatic CYP mRNA (1A2, 2A19, 2C33, 2D25, 2E1 and 3A29), using primary porcine hepatocytes. Of the tested secondary plant metabolites, artemisinin, scoparone, lactucin and esculetin all induced increased expression of specific CYPs, while esculin showed no effect. In contrast, a global extract of chicory root decreased the expression of CYP1A2, 2C33, 2D25 and 3A29 at high concentrations. The results suggest that purified secondary metabolites from chicory affect CYP expression and thereby might affect detoxification in general, and that global extracts of plants can have effects different from individual components.

1. Introduction

Chicory (*Cichorium intybus* L.) is an important source of the polysaccharide inulin and has been shown to possess a wide range of pharmacological properties, including hepato-protective, anti-inflammatory, anti-malarial and anti-diabetic effects (Ahmed, Al-Howiriny, & Siddiqui, 2003; Bais & Ravishankar, 2001; Cavin et al., 2005; Tousch et al., 2008). Additionally, research has shown that chicory feeding increased the expression and activity of hepatic xenobiotica (drugs, antibiotics) metabolizing enzymes in pigs (Rasmussen, Zamaratskaia, & Ekstrand, 2011a). The aerial parts of the plant are often consumed as part of salads, while the roots are processed and used as coffee substitutes or food ingredients.

Metabolism of the diverse classes of xenobiotics is usually divided into two phases: phase I, oxidative modification, and phase II, derivatisation. Phase I is often catalysed by enzymes belonging to the cytochrome P450 (CYP) superfamily. Hence, CYPs are extensively studied for their involvement in drug metabolism and detoxification (Guengerich, 2007). Proteomic analysis of the porcine liver has determined that the most abundant CYP families are CYP1A, 2A, 2C, 2D, 2E and 3A (Achour, Barber, & Rostami-Hodjegan, 2011). Apart from their impact on drug metabolism, porcine CYPs are also studied for their ability to metabolize skatole. The compound skatole is a metabolite of tryptophan produced in the gut; after absorption to the blood stream it is metabolized in the liver by CYPs. If the hepatic clearance of skatole is insufficient, it will accumulate in the adipose tissue of the pig. High concentrations of skatole in fat are associated with poor meat quality of sexually mature male pigs, a phenomenon known as boar taint. Several different CYPs have been shown to catabolise skatole (Matal, Matuskova, Tunkova, Anzenbacherova, & Anzenbacher, 2009; Terner, Gilmore, Lou, & Squires, 2006; Wiercinska, Lou, & Squires, 2012).

Differences in CYP expression and activity between individuals have been observed and are often explained by the genetic profile of the individual, but other factors are also important, e.g., gender and age. Moreover, there is growing evidence that bioactive compounds, e.g., secondary plant metabolites, affect liver function and CYP activity. It has been shown that St. John's wort, *Ginkgo biloba*, wormwood (*Artemisia*), common sage (*Salvia*) and chicory (*Cichorium*) influence the expression and/or activity of hepatic CYPs (Chang, 2009; Chang & Waxman, 2006; Hellum, Hu, & Nilsen, 2009; Rasmussen, Zamaratskaia, Andersen, & Ekstrand, 2012; Rasmussen et al., 2011a). Moreover, the secondary plant metabolites, artemisinin and scoparone (from wormwood), have been shown to possess pharmacological properties (Burk et al., 2005; Huang, Zhang, & Moore, 2004). Chicory root has been shown to contain numerous secondary metabolites, e.g., the sesquiterpene

* Corresponding author. Address: Department of Food Science, Aarhus University, Blichers allé 20, P.O. box 50, DK-8830 Tjele, Denmark. Tel./fax: +45 89 99 19 00.
E-mail address: Martink.rasmussen@agrsci.dk (M.K. Rasmussen).

0308-8146/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodchem.2013.09.068

lactone lactucin, and the coumarins esculin and esculetin (Bais & Ravishankar, 2001). Thus, knowledge about the impact of plants (used for consumption) on hepatic CYP expression/activity is very important due to potential herb–drug interactions. The aim of this study was to investigate the effects of selected secondary plant metabolites from chicory on porcine CYP mRNA expression *in vitro* and compare that to the effects of secondary plant metabolites from wormwood (*Artemisia*).

The expression of CYPs is generally believed to be regulated by xenobiotic receptors (XR), such as the aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR) and pregnane X receptor (PXR), regulating CYP1A, 2A and 3A expression, respectively. However, extensive cross-talk between receptors does take place (reviewed by Pascussi et al. (2008)). By treating hepatocytes with standard activators of these receptors and selected plant secondary metabolites, the potential for CYP mRNA induction was investigated. The effect of a global chicory root extract was compared to that of the standard CYP inducers and the secondary plant metabolites.

2. Materials and methods

2.1. Ethical and animal welfare aspects

The pigs used for hepatocyte isolation were treated in accordance with the guidelines from the Danish Inspectorate of Animal Experimentation.

2.2. Isolation of porcine hepatocytes

Five female cross-bred (Landrace × Yorkshire sire and Duroc boar) piglets (11.1 ± 0.5 kg) were used in this study. The pigs were killed with a bolt pistol, followed by immediate exsanguination, before the liver (weight 200–250 g) was removed and transported on ice to the laboratory. Time from killing to the arrival of the liver at the laboratory was not more than 10 min. Hepatocytes were isolated by a protocol adopted from Monshouwer, Witkamp, Nijmeijer, Van Amsterdam, and Van Miert (1996) and Seglen (1972). After arrival at the laboratory, the liver was transferred to a laminar flow-bench and cannulated with a tube in the large central vein. The liver was then perfused with 1 l of basis-buffer (Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (DPBS; Invitrogen) supplemented with 10 mM HEPES, 1% glucose; saturated with O_2; pH 7.4) treated with 1 mM EGTA, followed by perfusion with 1 l of basis-buffer. Afterwards 0.75 l of basis-buffer containing 4.75 mM $CaCl_2$ and 0.05% collagenase (Type II; Worthington, USA) was perfused under recirculation for 15 min. The liver was then cut into small pieces with a scalpel and transferred to a bottle containing basis-buffer with 4.75 mM $CaCl_2$ and 0.025% collagenase and incubated under gentle stirring for 15 min. To the resulting cell suspension. ice-cold basis-buffer with 2% BSA (1:1) was added, and it was then filtered through nylon mesh with a mesh size of 200 μm^2, followed by filtering through nylon mesh with a mesh size of 50 μm^2. Cells were then isolated by a 5 min centrifugation at 100*g* (4 °C) and washed in William E medium (WME; Invitrogen) containing 10% (volume/volume) foetal calf serum (FCS; Sigma). After isolation of the cells, they were seeded directly or cryopreserved according to the procedure described below.

2.3. Cell number, viability, cryopreservation and thawing of hepatocytes

Cell number was evaluated by counting the cells in a haemocytometer, combined with light microscopy. Cell viability was estimated by their ability to exclude trypan blue, regarding cells not able to exclude trypan blue as dead.

The isolated cells were diluted in WME with 10% FCS, 2 mM L-glutamine (Sigma) and 1 μM insulin (Sigma), obtaining a final density of 10^7 cells/ml. DMSO was slowly added to the cell suspension reaching a final concentration of 10% (v/v). Afterwards 1.0 ml of cell suspension was put in cryotubes (NUNC) and placed in propanol-containing boxes and frozen at −80 °C freezer overnight. The next day, tubes were transferred to a tank containing liquid nitrogen.

Thawing was done by heating the tubes in a 37 °C water bath and, when the content was thawed, it was transferred to 50 ml tubes with WME containing 10% FCS (37 °C). The cells were then sedimented by centrifugation at 100*g* for 5 min (37 °C).

2.4. Culturing of hepatocytes and effect of time in culture

For evaluation of the effect of cryopreservation and time in culture, either freshly isolated or cryopreserved hepatocytes were suspended in WME with 10% FCS, 2 mM L-glutamine, 100 units/ml of penicillin (Sigma), 100 μg/ml of streptomycin (Sigma), 20 μg/ml gentamicin (Sigma), 2.5 μg/ml of ampotericin B (Sigma) and 1 μM insulin. 100,000 cells/cm^2 were seeded into collagen (Type I, BD Biosciences)-coated wells (6 well plates; NUNC) and subjected to 37 °C and 5% CO_2 in an incubator. 24 h after seeding, dead and unattached cells were removed by washing the wells with DPBS (Ca^{2+}, Mg^{2+}; 37 °C) and fresh medium was added. This time point was regarded as day 1. Medium was renewed every 24 h and samples collected equivalent to days 2, 3, 6 and 7. Due to low attachment of the cryopreserved hepatocytes isolated from one pig (Table 1; Pig B), a Percoll purification (Kreamer et al., 1986) was performed before seeding.

2.5. Induction

To test the functionality of the isolated hepatocytes we determined their response to standard CYP inducers. We used 100 μM β-naphthoflavone (Sigma), 100 μM CITCO (6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl) oxime; Sigma), 50 μM dexamethasone (Sigma) and 5 nM TCPOBOP (1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene; Sigma) dissolved in DMSO, giving a final concentration in the media of 0.1% DMSO. Likewise, 1–100 μM artemisinin (Mediplantex, Hanoi, Vietnam), lactucin (Extrasynthese, France), esculin (6,7-dehydroxycoumarin; Sigma), esculetin (Sigma) and 10–100 μM scoparone (6,7-dimethoxycoumarin; Sigma) were dissolved in DMSO, giving a final concentration of 0.1% DMSO in the media.

A crude extract of dried chicory root was made by mixing (1:3 w/v) dried chicory root with 96% methanol. After 24 h of extraction, the extract was filtered and the methanol removed under vacuum, resulting in a 50 times reduction in volume. The final

Table 1
Viability of freshly isolated and cryopreserved porcine hepatocytes.

	Viability (%)	
	Fresh	Cryopreserved
Pig A	95	79
Pig B	92	72
Pig C	84	69
Pig D	85	74
Pig E	86	71
Average	88.4	73.0
SD	4.8	3.8

extract was given an arbitrary concentration of 100,000 units. For all added concentrations of extract, methanol was added to the media, giving a final concentration of methanol at 0.1%.

Cryopreserved hepatocytes were seeded at a density of 100,000 cells/cm^2 into 12 well collagen-coated plastic plates. The cells were seeded in WME with 5% FCS, 2 mM L-glutamine, 100 unit/ml of penicillin, 100 μg/ml of streptomycin, 20 μg/ml of gentamicin, 2.5 μg/ml ampotericin B and 1 μM insulin. After 24 h of attachment, the wells were washed with DPBS and fresh medium, with or without inducers/extract, was added for another 24 h before the cells were lysed.

The control samples were treated with either 0.1% DMSO or methanol. In preliminary experiments, it was shown that DMSO and methanol, at these concentrations, did not affect the CYP mRNA expression (data not shown). All treatments were analysed in duplicate for each cell isolate.

2.6. Quantitative PCR

RNA isolation and reverse transcription were done using commercially available kits. Briefly, total RNA was isolated by lysing the cell in the wells with RLT-buffer (WVR, Herlev, Denmark) and running the lysate on a Spin column according to the manufacturer's instructions (Rnasey Mini Kit, WVR, Herlev, Denmark). Equal volumes of RNA were 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. Primers and probes for CYP1A2, 2A19, 2E1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are given in Rasmussen, Zamaratskaia, and Ekstrand (2011b), while CYP3A29 is given in Nannelli et al. (2010). For determination of CYP2C33, we used forward primer, reverse primer and TaqMan probes with the following sequences (5′–3′): TGGGAATCTGATGCAACTTAACC, AACAGGGCCGTACTGTTTGG and AAGGACATCCCTGCGTCTCTTTCCAAGTT, respectively. For determination of CYP2D25, the corresponding sequences were: TTGGAAGGACTGAAGGAGGAAGT, CCCGGGATATGCCTGAGAA and CCTCATGCGCCAGGTGCTGGA. Primers and probes were designed with Primer Express version 2 (Applied Biosystems, Carlsbad, CA, USA), using porcine specific genomic DNA sequences (www.ensembl.org/Sus_scrofa/Info/Index). The samples were analysed in duplicate in 348 well plates, using an ABI 7900 HT sequence detection system (Applied Biosystems, Carlsbad, CA, USA) 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 arbitrarily set to 1. The obtained Ct-values for GAPDH were not significantly affected by any of the treatments.

2.7. Statistical analysis

To evaluate the effect of cryopreservation and time in culture on CYP mRNA expression, two-way ANOVA was used, followed by Tukey's *post hoc* test to identify differences in CYP mRNA expression. The effect of inducers and secondary plant metabolites was evaluated by one-way ANOVA, followed by Tukey's *post hoc* test. To evaluate the effect of the chicory root extract compared to the control samples, the Student's *t*-test with Bonferroni's correction was used (a corrected *p*-level of $p < 0.0083$ was regarded as significant).

All statistical tests were done using R (version 2.11.0).

3. Results

3.1. Isolation of hepatocytes

The total yield of hepatocytes was between 2×10^8 and 5×10^9 cells. When comparing the morphology of freshly isolated and cryopreserved hepatocytes, no differences were seen, whereas the viability before seeding was lowered by cryopreservation (on average from 88% to 73%, $p < 0.01$, see Table 1).

3.2. CYP mRNA expression in hepatocytes; effect of time on culture

For CYP1A2, 2A19, 2D25 and 2E1, there was a significant decrease in mRNA expression on day 2 in culture compared to day 1 (Fig. 1). This reduction was maintained for the rest of the period. For CYP2C33 and 3A29, there was no significant difference in mRNA expression between the days in culture. However, for these CYPs, large variations between the cell isolates were observed. For all CYPs investigated, no differences in mRNA expression between freshly isolated and cryopreserved hepatocytes, in relation to their time in culture, were observed.

3.3. Induction of CYP mRNA

To test the potential of the model to modify mRNA expression of the investigated CYPs, the hepatocytes were treated with standard activators of the receptors regulating gene expression. The used inducers were 100 μM β-naphthoflavone (Fig. 2A), 100 μM CITCO (Fig. 2B), 50 μM dexamethasone (Fig. 2C) or 5 nM TCPOBOP (Fig. 2D). Treating hepatocytes with β-naphthoflavone significantly increased the mRNA expression of CYP1A2 5.1-fold compared to control. Likewise, the mRNA expression of CYP1A2 was significantly larger than the expression of all other investigated CYP isoforms. Treatment of hepatocytes with CITCO significantly increased the mRNA expression of CYP1A2 4.0-fold compared with the control. Furthermore, the mRNA expression of CYP1A2 was significantly larger than for all other investigated CYP isoforms. In the dexamethasone-treated hepatocytes, mRNA expression of CYP1A2 and 3A was significantly increased, 4.9- and 5.3-fold, respectively, compared with the control, while the 4.3-fold increase in CYP2C33 expression almost reached significance ($p = 0.054$). There was no effect on CYP mRNA expression of treating hepatocytes with TCPOBOP (ANOVA, $p > 0.05$).

3.4. Secondary plant metabolites from chicory and wormwood, and chicory root extract

Treatment of hepatocytes with artemisinin significantly increased the mRNA expression of CYP1A2, 2C33, 2D25 and 3A29, up to 3.5-, 2.0-, 1.5-, and 3.4-fold, respectively, compared with the control (Fig. 3A). Scoparone significantly increased the mRNA expression of CYP1A2 and 2D25, up to 4.3- and 1.8-fold, respectively, compared with the control (Fig. 3B). Apart from that, no other effects on CYP mRNA expression were observed.

For the chicory secondary metabolite, lactucin increased the mRNA expression of CYP2A19, 2D25 and 2E1, up to 2.6-, 2.0- and 3.1-fold, respectively, compared with the control (Fig. 3C). Esculetin increased the CYP1A2 mRNA expression up to 2.2-fold (Fig. 3D) while the expression of the other investigated CYP isoforms remained unchanged. Esculin showed no effect on CYP mRNA expression (Fig. 3E).

The extract of chicory decreased the mRNA expression of CYP1A2 when administered at the three highest concentrations (Fig. 4A). Moreover, chicory extract also decreased the mRNA expression of CYP2C33, 2D25 and 3A29 when administered at

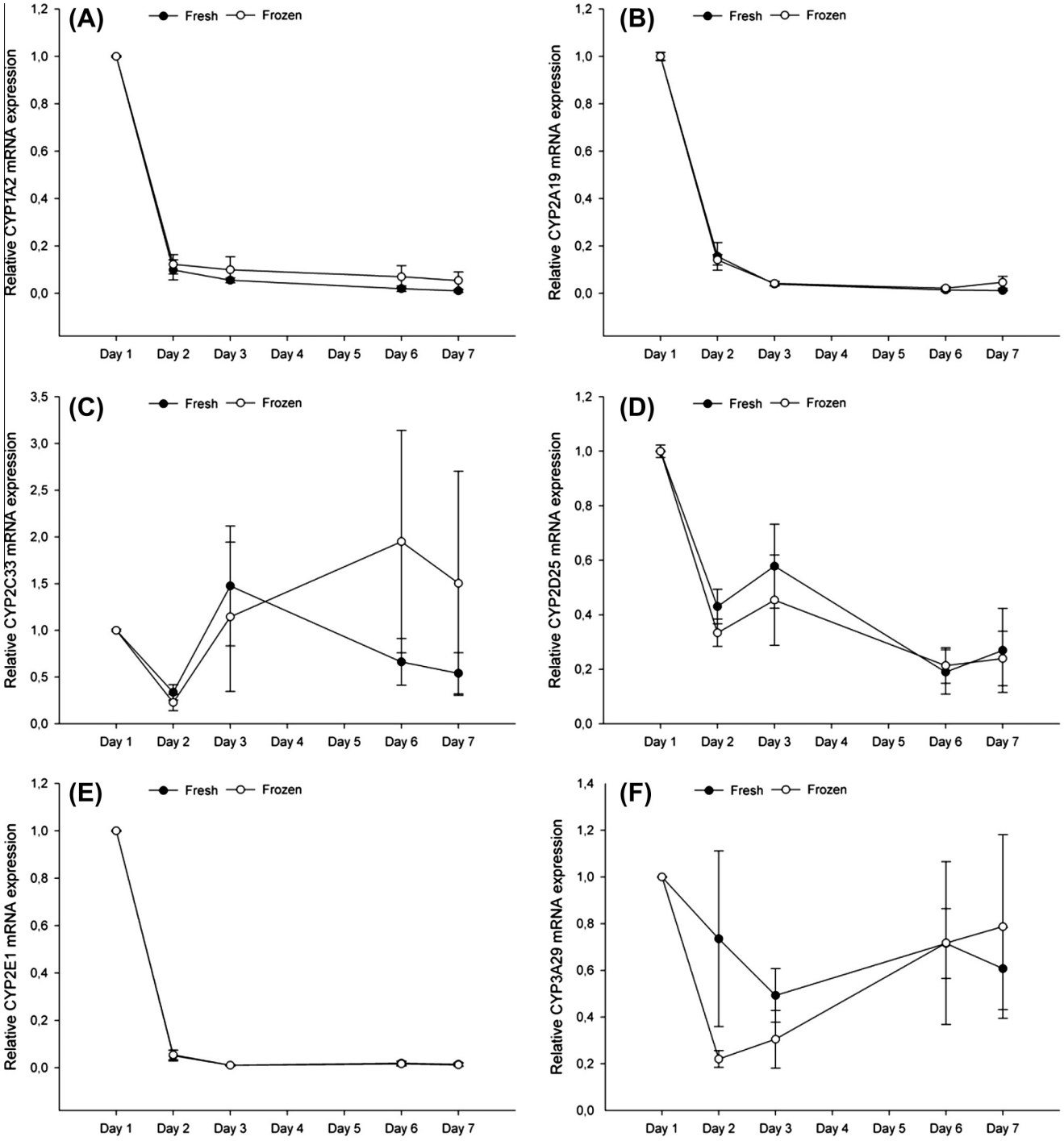

Fig. 1. Cytochrome P450 mRNA expression in freshly isolated (closed circles) and cryopreserved (open circles) primary porcine hepatocytes ($n = 4$). mRNA expression of (A) CYP1A2, (B) 2A19, (C) 2C33, (D) 2D25, (E) 2E1 and (F) 3A29 was determined on days 1 (24 h after seeding), 2, 3, 6 and 7 in culture. Data are means ± SEM.

the highest concentration (Fig. 4C, D and F). No effect of the chicory root extract was found on CYP2A19 and 2E1 expression.

4. Discussion

The aim of the present study was to use primary hepatocytes isolated from pigs to investigate regulation of CYP mRNA expression by treatment with selected secondary plant metabolites. In order to validate the hepatocyte model, the effects of time in culture and standard CYP inducers on CYP expression were investigated. The major findings were that 1) mRNA expression of the investigated CYPs with time in culture was not affected by cryopreservation; 2) treatment with standard CYP inducers and selected secondary plant metabolites increased CYP expression whereas 3) a global chicory root extract lowered the expression of some of the investigated CYPs, but not all.

Several studies have investigated the effect of time in culture on overall and specific CYP protein expression and activity in porcine hepatocytes (Gillberg, Skaanild, & Friis, 2006; Keatch, Nelson, Mason, Hayes, & Plevris, 2002; Koebe, Mueller-Hoecker, Koebe, & Schildberg, 1995; Loven, Olsen, Friis, & Andersen, 2005; Monshouwer, van't Klooster, Nijmeijer, Witkamp, & van Miert, 1998;

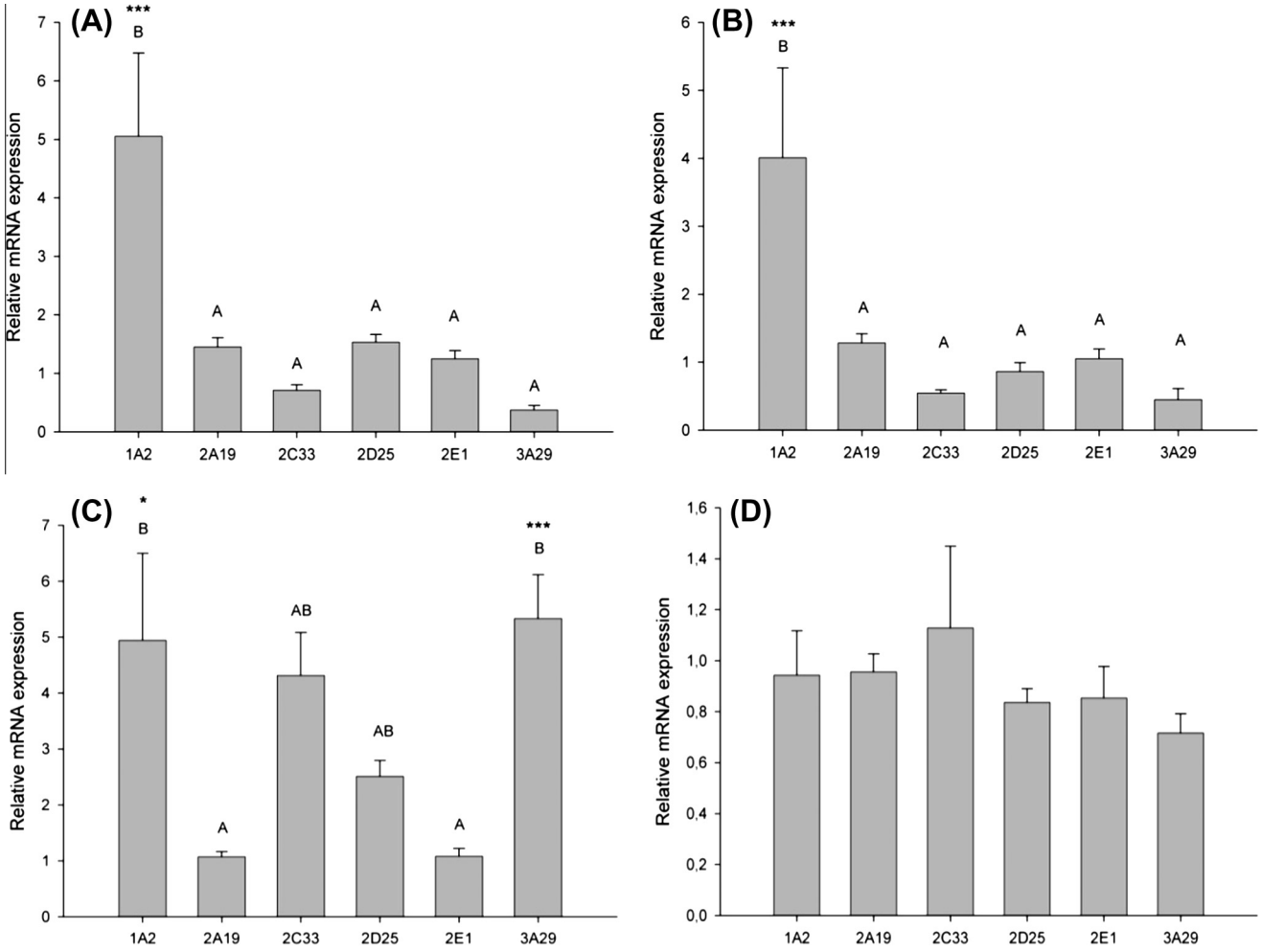

Fig. 2. Induction of cytochrome P450 mRNA in primary porcine hepatocytes by standard inducers. Hepatocytes ($n = 4$) were treated with (A) 100 μM β-naphthoflavone, (B) 100 μM CITCO, (C) 50 μM dexamethasone or (D) 5 nM TCOPBOP for 24 h. Data are means ± SEM. $^{*}p < 0.05$ and $^{***}p < 0.001$ significantly different from control (no inducers). Bars not sharing a superscript are different from each other ($p < 0.05$). Significance was evaluated using one-way ANOVA, followed by Tukey's *post hoc* test.

Skaanild & Friis, 2000; Zhou, Tokiwa, Kano, & Kodama, 1998). However, to our knowledge, this is the first study to investigate the effect of time in culture on porcine CYP mRNA expression, while the studies mentioned above have all investigated the protein expression or activity. In the present study, we showed that compared to day 1 after seeding, the mRNA expression of CYP1A2, 2A19, 2D25, 2E1 was reduced by approximately 90% after day 2 in culture. Following the next 5 days in culture, the mRNA expression remained stable at a low level compared to day 1. The mRNA expression of CYP2C33 and 3A29 was not changed by time in culture. These findings are in accordance with previous results on CYP activity (Gillberg et al., 2006; Loven et al., 2005; Skaanild & Friis, 2008), showing that, in porcine hepatocytes, the CYP2A-dependent activity is reduced by up to 80% during the first day in culture. Additionally, the study by Skaanild and Friis (2000) showed a concurrent reduction in CYP2A-dependent activity and protein expression. Moreover, it has been shown that CYP1A-dependent activity decreases with time in culture (Koebe et al., 1995; Zhou et al., 1998), while CYP2E1 protein expression is not changed within 24 h after seeding (Doran, Whittington, Wood, & McGivan, 2002). As the present study showed that mRNA expression of CYP1A2 and 2A19 is reduced, it could be suggested that the reduction in CYP-dependent activity is caused by a reduction of the transcriptional level. The unchanged expression of CYP3A29 mRNA found in the present study is in accordance with Gillberg et al. (2006) and Skaanild and Friis (2000), that showed constant protein expression and CYP3A-dependent activity for up to 75 h of culturing. However, other studies have shown that both increased (Loven et al., 2005) and decreased (Monshouwer et al., 1998) CYP3A-dependent activity. Taken together, the present findings suggest that the reduction of CYP protein expression and activity, found with time in culture, is due to reduced mRNA expression. In the present study we showed that, for none of the investigated CYPs, were there differences between time-dependent CYP mRNA expression in freshly isolated and cryopreserved hepatocytes. This suggests that cryopreserved hepatocytes can be used to study CYP mRNA expression.

To test the ability and the magnitude by which the hepatocytes can regulate CYP mRNA, we treated them with standard inducers. β-Naphthoflavone and dexamethasone are commonly used to activate the AhR and PXR, respectively, while CITCO and TCPOBOP are used to activate CAR. However, as supported by the current results, the single inducer can activate other XRs, either directly or indirectly, via cross-talk or via their metabolites. In the present study, β-naphthoflavone proved to be very specific in inducing an increase only in CYP1A2 mRNA expression, probably by activating the AhR. Porcine CYP1A induction by β-naphthoflavone has been shown previously both *in vivo* (Messina, Nannelli, Fiorio, Longo, & Gervasi, 2009; Nannelli et al., 2009) and *in vitro* (Monshouwer et al., 1998). TCPOBOP has previously, as in the current study, been

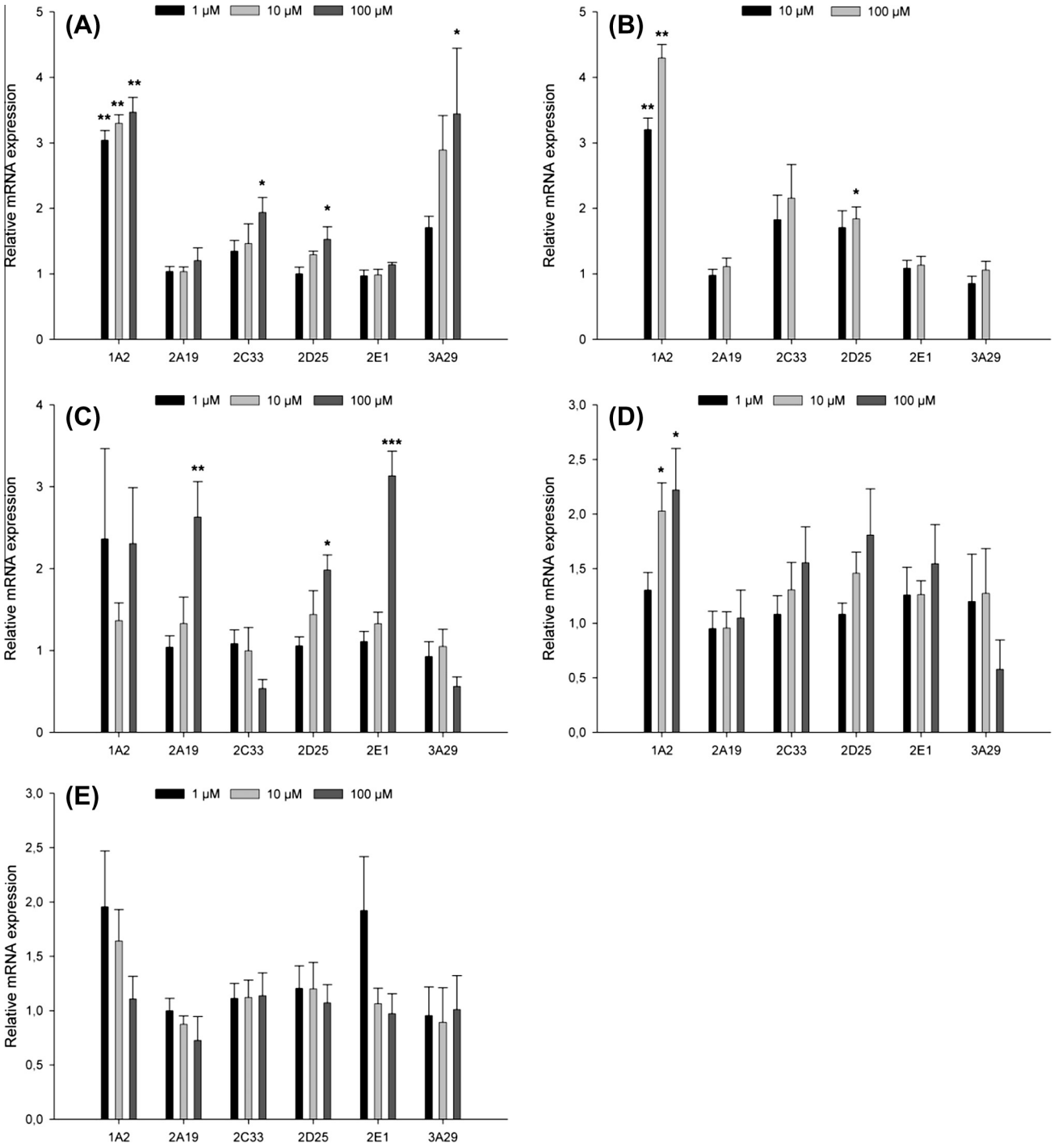

Fig. 3. Induction of cytochrome P450 mRNA in primary porcine hepatocytes by 1–100 μM artemisinin (A), lactucin (C), esculetin (D) and esculin (E) and 10–100 μM scoparone (B). Hepatocytes ($n = 4$) were treated with the secondary plant metabolites for 24 h at concentrations indicated in the Figure. Data are means ± SEM, $^{*}p < 0.05$ $^{**}p < 0.01$ significantly different from control (no secondary plant metabolites). Significance was evaluated using one-way ANOVA, followed by Tukey's *post hoc* test.

shown to have no effect on CYP2A expression (Gillberg et al., 2006) in porcine hepatocytes, in contrast to murine hepatocytes. Moreover, the study by Gillberg et al. (2006) showed that CITCO induced CYP2A-dependent activity. Surprisingly, our study showed no changes in CYP2A19 mRNA in hepatocytes treated with CITCO, while an increase in CYP1A2 mRNA expression was observed. This discrepancy between the two studies can be explained by the different investigated response, i.e., mRNA expression vs. enzyme activity. Dexamethasone has previously been shown to increase CYP3A in porcine hepatocytes (Monshouwer et al., 1998). In the current study, dexamethasone induced CYP3A29 and 1A2 mRNA expression, while the observed increase in 2C33 almost reached significant levels ($p = 0.054$). This result is supported by studies using human hepatocytes, showing that dexamethasone treatment increased CYP3A4, CYP2C8 and CYP1A1 (Lai, Wong, & Wong, 2004; Pascussi, Gerbal-Chaloin, Fabre, Maurel, & Vilarem, 2000). This suggests that dexamethasone has a complex mode of action in relation to XR activation and gene regulation. Taken together, the results

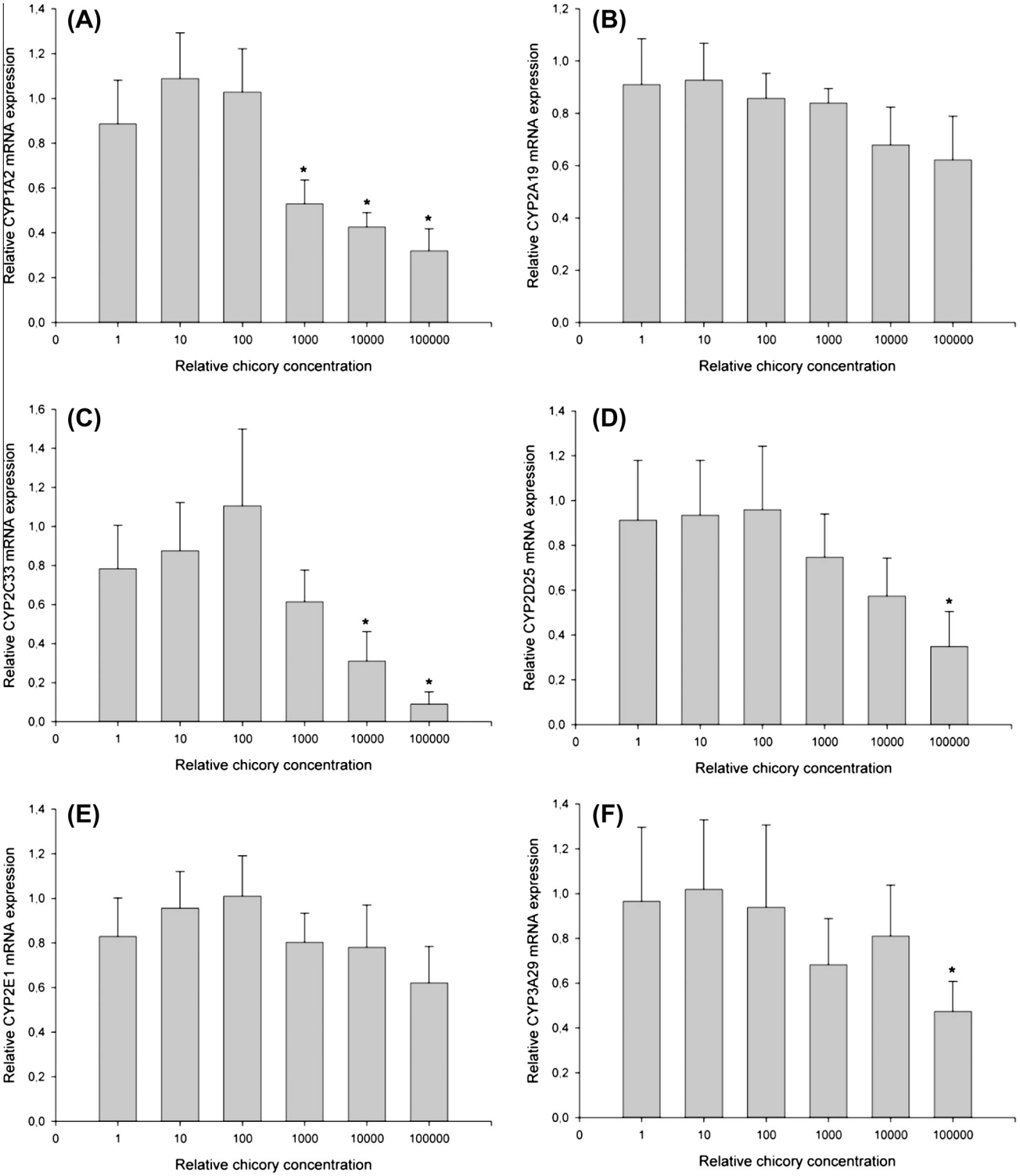

Fig. 4. Cytochrome P450 mRNA expression in primary porcine hepatocytes treated with a methanolic extract of chicory root. Hepatocytes ($n = 4$) were treated with different dilutions of the original extract for 24 h as indicated in the Figure. Data are means ± SEM. *Significantly different from control samples (no chicory extract). Significance was evaluated using Student's *t*-test with Bonferroni's correction.

show that, even though CYP expression is suppressed with time in culture, the hepatocytes are still able to increase CYP mRNA, by at least 5-fold, when treated with XR activators.

To investigate the potential of inducing CYPs with secondary plant metabolites, we treated hepatocytes with a sesquiterpene lactone, artemisinin, and a coumarin derivative, scoparone. Artemisinin increased CYP1A2 and 3A29 mRNA expression by up to 3.5 times, while CYP2C33 and 2D25 mRNA expression was increased up to 2 times. This suggests that artemisinin simultaneously activates AhR, CAR and PXR. This is further supported by a study using a reporter gene assay, which showed that artemisinin activates human CAR and PXR, as well as induces CYP3A4 and 2C19 mRNA expression in human hepatocytes (Burk et al., 2005). Simonsson et al. (2006) also found that artemisinin activated the human CAR and induced a weak but reproducible increase in CYP1A2 mRNA in mice treated with artemisinin for 4 days. Our

results show that scoparone treatment increased the mRNA expression of CYP1A2 and 2D25, suggesting an activation of both AhR and CAR. Previous studies, using human and mice hepatocytes, have shown that scoparone can induce CYP2B6 and CYP2B10 mRNA expression by activating CAR (Huang et al., 2004; Yang, Yang, Shi, Deng, & Yan, 2011).

It is still not known if the secondary plant metabolites found in chicory (lactucin, esculetin and esculin) are able to modify the expression of hepatic CYP mRNA. In the present study we showed that the sesquiterpene lactone, lactucin, and the coumarin esculetin both induce CYP expression. Lactucin increased the expression of CYP2A19, 2D25 and 2E1. Expression of these CYPs is expected to be controlled by CAR. This suggests that lactucin can activate CAR. Esculetin might activate AhR, due to the observed increase of CYP1A2 mRNA. The esculetin metabolite, esculin, did not affect the mRNA expression of any of the investigated CYP isoforms. Thus, previously shown effects of feeding chicory root can be explained by the presence of chicory secondary metabolites activating XRs and inducing CYP expression (Rasmussen et al., 2011a). In the group of pigs which had been given 10% dried chicory root in their feed for the last 16 days before slaughter, there was up to a 9-fold increase in CYP1A2 and 2A19 mRNA and a small increase in CYP2E1 mRNA. Correspondingly, there was a subsequent increase in CYP1A2 and 2A19 protein expression and activity. Surprisingly, the effects of the global chicory root extract on hepatic CYP mRNA expression found in the present study are not consistent either with our previous *in vivo* results (Rasmussen et al., 2011a) or some of our present results for individual secondary metabolites from chicory. However, using global plant extracts, in *in vitro* models introduces some biases. Due to the reported presence of high amounts of sesquiterpene lactones in a methanolic extract of chicory root (Cavin et al., 2005), methanol was chosen as solvent. This, however, excludes compounds not easily soluble in methanol, which might possess CYP-inducing properties. Another explanation is *in vivo* modification (e.g., in the gastrointestinal tract) of the chicory compounds during absorption, potentially making them ligands for the CYP-regulating receptors. Likewise, more complex events, e.g., changes in hormone concentration caused by chicory, can also affect CYP expression. This is supported by previous studies showing decrease in androstenone levels with chicory feeding (Byrne, Thamsborg, & Hansen, 2008; Rasmussen, Brunius, Zamaratskaia, & Ekstrand, 2012).

It is noteworthy that the same CYP isoforms that were up-regulated in the dexamethasone- and artemisinin-treated hepatocytes, were down-regulated by high concentrations of the global chicory extract. This may suggest that these CYPs have a common mechanism for regulation and that the chicory extract acts as an inverse agonist for the transcription factors for these CYPs.

In conclusion, the results show that purified secondary plant metabolites, from e.g. chicory, can increase the expression of CYP mRNA in porcine hepatocytes. In contrast, a global extract of chicory root decreased the expression of several CYP isoforms.

Acknowledgements

The authors greatly appreciate the excellent technical assistance by Bente Andersen, Department of Food Science, Aarhus University. This study was partially funded by the Future Food Initiative, and the GUDP NO-CAST Project, Grant No. 3405-10-OP-00134.

References

Achour, B., Barber, J., & Rostami-Hodjegan, A. (2011). Cytochrome P450 pig liver pie: Determination of individual cytochrome P450 isoform contents in microsomes from two pig livers using liquid chromatography in conjunction with mass spectrometry. *Drug Metabolism and Disposition, 39*(11), 2130–2134.

Ahmed, B., Al-Howiriny, T. A., & Siddiqui, A. B. (2003). Antihepatotoxic activity of seeds of *Cichorium intybus*. *Journal of Ethnopharmacology, 87*(2–3), 237–240.

Bais, H. P., & Ravishankar, G. A. (2001). *Cichorium intybus* L. – Cultivation, processing, utility, value addition and biotechnology, with an emphasis on current status and future prospects. *Journal of the Science of Food and Agriculture, 81*(5), 467–484.

Burk, O., Arnold, K. A., Nussler, A. K., Schaeffeler, E., Efimova, E., Avery, B. A., et al. (2005). Antimalarial artemisinin drugs induce cytochrome P450 and MDR1 expression by activation of xenosensors pregnane X receptor and constitutive androstane receptor. *Molecular Pharmacology, 67*(6), 1954–1965.

Byrne, D. V., Thamsborg, S. M., & Hansen, L. L. (2008). A sensory description of boar taint and the effects of crude and dried chicory roots (*Cichorium intybus* L.) and inulin feeding in male and female pork. *Meat Science, 79*(2), 252–269.

Cavin, C., Delannoy, M., Malnoe, A., Debefve, E., Touché, A., Courtois, D., et al. (2005). Inhibition of the expression and activity of cyclooxygenase-2 by chicory extract. *Biochemical and Biophysical Research Communications, 327*(3), 742–749.

Chang, T. (2009). Activation of pregnane X receptor (PXR) and constitutive androstane receptor (CAR) by herbal medicines. *The AAPS Journal, 11*(3), 590–601.

Chang, T. K. H., & Waxman, D. J. (2006). Synthetic drugs and natural products as modulators of constitutive androstane receptor (CAR) and pregnane X receptor (PXR). *Drug Metabolism Reviews, 38*(1–2), 51–73.

Doran, E., Whittington, F. W., Wood, J. D., & McGivan, J. D. (2002). Cytochrome P450IIE1 (CYP2E1) is induced by skatole and this induction is blocked by androstenone in isolated pig hepatocytes. *Chemico-Biological Interactions, 140*(1), 81–92.

Gillberg, M., Skaanild, M. T., & Friis, C. (2006). Regulation of gender-dependent CYP2A expression in pigs: Involvement of androgens and CAR. *Basic & Clinical Pharmacology & Toxicology, 98*(5), 480–487.

Guengerich, F. P. (2007). Cytochrome P450 and chemical toxicology. *Chemical Research in Toxicology, 21*(1), 70–83.

Hellum, B. H., Hu, Z., & Nilsen, O. G. (2009). Trade herbal products and induction of CYP2C19 and CYP2E1 in cultured human hepatocytes. *Basic & Clinical Pharmacology & Toxicology, 105*(1), 58–63.

Huang, W., Zhang, J., & Moore, D. D. (2004). A traditional herbal medicine enhances bilirubin clearance by activating the nuclear receptor CAR. *Journal of Clinical Investigation, 113*(1), 137–143.

Keatch, S. A., Nelson, L. J., Mason, J. I., Hayes, P. C., & Plevris, J. N. (2002). Examination of cytochrome P450 content of primary porcine hepatocyte cultures with various media formulations for use in bioartificial liver devices. *Journal of Artificial Organs, 5*(1), 37–43.

Koebe, H. G., Mueller-Hoecker, J., Koebe, S., & Schildberg, F. W. (1995). Viable porcine hepatocytes from slaughterhouse organs. *Artificial Cells Blood Substitutes and Immobilization Biotechnology, 23*(5), 613–626.

Kreamer, B., Staecker, J., Sawada, N., Sattler, G., Hsia, M., & Pitot, H. (1986). Use of a low-speed, iso-density percoll centrifugation method to increase the viability of isolated rat hepatocyte preparations. *In Vitro Cellular & Developmental Biology – Plant, 22*(4), 201–211.

Lai, K. P., Wong, M. H., & Wong, C. K. C. (2004). Modulation of AhR-mediated CYP1A1 mRNA and EROD activities by 17β-estradiol and dexamethasone in TCDD-induced H411E cells. *Toxicological Sciences, 78*(1), 41–49.

Loven, A. D. B., Olsen, A. K., Friis, C., & Andersen, B. (2005). Phase I and II metabolism and carbohydrate metabolism in cultured cryopreserved porcine hepatocytes. *Chemico-Biological Interactions, 155*(1–2), 21–30.

Matal, J., Matuskova, Z., Tunkova, A., Anzenbacherova, E., & Anzenbacher, P. (2009). Porcine CYP2A19, CYP2E1 and CYP1A2 forms are responsible for skatole biotransformation in the reconstituted system. *Neuroendocrinology Letters, 30*, 36–40.

Messina, A., Nannelli, A., Fiorio, R., Longo, V., & Gervasi, P. G. (2009). Expression and inducibility of CYP1A1, 1A2, 1B1 by β-naphthoflavone and CYP2B22, 3A22, 3A29, 3A46 by rifampicin in the respiratory and olfactory mucosa of pig. *Toxicology, 260*(1–3), 47–52.

Monshouwer, M., van't Klooster, G. A. E., Nijmeijer, S. M., Witkamp, R. F., & van Miert, A. S. J. P. (1998). Characterization of cytochrome P450 isoenzymes in primary cultures of pig hepatocytes. *Toxicology in Vitro, 12*(6), 715–723.

Monshouwer, M., Witkamp, R. F., Nijmeijer, S. M., Van Amsterdam, J. G., & Van Miert, A. S. J. P. (1996). Suppression of cytochrome P450- and UDP glucuronosyl transferase-dependent enzyme activities by proinflammatory cytokines and possible role of nitric oxide in primary cultures of pig hepatocytes. *Toxicology and Applied Pharmacology, 137*(2), 237–244.

Nannelli, A., Rossignolo, F., Tolando, R., Rossato, P., Longo, V., & Gervasi, P. G. (2009). Effect of β-naphthoflavone on AhR-regulated genes (CYP1A1, 1A2, 1B1, 2S1, Nrf2, and GST) and antioxidant enzymes in various brain regions of pig. *Toxicology, 265*(3), 69–79.

Nannelli, A., Rossignolo, F., Tolando, R., Rossato, P., Pellegatti, M., Longo, V., et al. (2010). Expression and distribution of CYP3A genes, CYP2B22, and MDR1, MRP1, MRP2, LRP efflux transporters in brain of control and rifampicin-treated pigs. *Molecular and Cellular Biochemistry, 337*(1), 133–143.

Pascussi, J. M., Gerbal-Chaloin, S., Duret, C., Daujat-Chavanieu, M., Vilarem, M. J., & Maurel, P. (2008). The tangle of nuclear receptors that controls xenobiotic metabolism and transport: Crosstalk and consequences. *Annual Review of Pharmacology and Toxicology, 48*(1), 1–32.

Pascussi, J. M., Gerbal-Chaloin, S., Fabre, J. M., Maurel, P., & Vilarem, M. J. (2000). Dexamethasone enhances constitutive androstane receptor expression in

human hepatocytes: Consequences on cytochrome P450 gene regulation. *Molecular Pharmacology, 58*(6), 1441–1450.

Rasmussen, M. K., Brunius, C., Zamaratskaia, G., & Ekstrand, B. (2012). Feeding dried chicory root to pigs decrease androstenone accumulation in fat by increasing hepatic 3β hydroxysteroid dehydrogenase expression. *The Journal of Steroid Biochemistry and Molecular Biology, 130*, 90–95.

Rasmussen, M. K., Zamaratskaia, G., Andersen, B., & Ekstrand, B. (2012a). Dried chicory root modifies the activity and expression of porcine hepatic CYP3A but not 2C – Effect of in vitro and in vivo exposure. *Food and Chemical Toxicology, 50*(11), 4175–4179.

Rasmussen, M. K., Zamaratskaia, G., & Ekstrand, B. (2011a). In vivo effect of dried chicory root (*Cichorium intybus* L.) on xenobiotica metabolising cytochrome P450 enzymes in porcine liver. *Toxicology Letters, 200*(1–2), 88–91.

Rasmussen, M. K., Zamaratskaia, G., & Ekstrand, B. (2011b). Gender-related differences in cytochrome P450 in porcine liver – Implication for activity, expression and inhibition by testicular steroids. *Reproduction in Domestic Animals, 46*(4), 616–623.

Seglen, P. O. (1972). Preparation of rat liver cells: I. Effect of Ca2+ on enzymatic dispersion of isolated, perfused liver. *Experimental Cell Research, 74*(2), 450–454.

Simonsson, U. S. H., Lindell, M., Raffalli-Mathieu, F., Lannerbro, A., Honkakoski, P., & Lang, M. A. (2006). In vivo and mechanistic evidence of nuclear receptor CAR induction by artemisinin. *European Journal of Clinical Investigation, 36*(9), 647–653.

Skaanild, M. T., & Friis, C. (2000). Expression changes of CYP2A and CYP3A in microsomes from pig liver and cultured hepatocytes. *Pharmacology & Toxicology, 87*(4), 174–178.

Skaanild, M. T., & Friis, C. (2008). Analyses of CYP2C in porcine microsomes. *Basic & Clinical Pharmacology & Toxicology, 103*(5), 487–492.

Terner, M. A., Gilmore, W. J., Lou, Y. P., & Squires, E. J. (2006). The role of CYP2A and CYP2E1 in the metabolism of 3-methylindole in primary cultured porcine hepatocytes. *Drug Metabolism and Disposition, 34*(5), 848–854.

Tousch, D., Lajoix, A. D., Hosy, E., Azay-Milhau, J., Ferrare, K., Jahannault, C., et al. (2008). Chicoric acid, a new compound able to enhance insulin release and glucose uptake. *Biochemical and Biophysical Research Communications, 377*(1), 131–135.

Wiercinska, P., Lou, Y., & Squires, E. J. (2012). The roles of different porcine cytochrome P450 enzymes and cytochrome b5A in skatole metabolism. *Animal, 6*(Special Issue 05), 834–845.

Yang, D., Yang, J., Shi, D., Deng, R., & Yan, B. (2011). Scoparone potentiates transactivation of the bile salt export pump gene and this effect is enhanced by cytochrome P450 metabolism but abolished by a PKC inhibitor. *British Journal of Pharmacology, 164*(5), 1547–1557.

Zhou, X. D., Tokiwa, T., Kano, J., & Kodama, M. (1998). Isolation and primary culture of adult pig hepatocytes. *Methods in Cell Science, 19*(4), 277–284.