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.)

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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

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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