Chapter 4

An in vivo and in vitro comparison of CYP gene induction in mice using liver slices and quantitative RT-PCR

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Adapted from Toxicology in Vitro 20 (2006): 125-131
Abstract

The scope of this study was to compare in vitro and in vivo cytochrome P450 (CYP) gene induction in mice, using liver slices as an in vitro model. We have chosen to study mice to be able to better interpret CYP induction during long-term safety studies in this species. Mouse liver slices were incubated with β-naphthoflavone (βNF), phenobarbital (PB) or dexamethasone (DEX) for 24 hours. In addition in an in vivo study, mice were treated with the same compounds for three days. The mRNA expression of cyp1a1, cyp1a2, cyp2b10 and cyp3a11, which are important for drug metabolism and inducible by xenobiotics, were investigated in vivo and in vitro by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR). Both in mouse liver slices and in vivo, βNF was found to be a potent inducer of cyp1a1 and to a lesser extent of cyp1a2. All three compounds induced cyp2b10 mRNA levels, while the cyp3a11 mRNA level was induced only by DEX. Overall, these data demonstrated a good predictive in vitro-in vivo correlation of CYP induction.

Introduction

Cytochrome P450 enzymes (CYP) play a critical role in the oxidative metabolism of a variety of endogenous and exogenous compounds, including most drugs. The expression of CYP genes is regulated by physiological, genetic, and environmental factors. In addition, CYP genes can be affected by drugs. Induction of CYPs by a drug is undesirable, as it can lead to enhanced metabolism of a co-administered drug that is a substrate of the induced CYP isoform. Further, CYP induction by non-genotoxic compounds may be related to liver tumour growth in rodents [1]. Although it is obvious that CYP induction in rodents is of less direct interest in human drug development, it is quite common that regulatory agencies require long and/or high dose treatment (six to twelve months) of rodents for safety assessment of pharmaceuticals [2]. Therefore, the availability of a mouse specific assay is of interest in situations where CYP induction in vivo rodents is suspected and the question raised whether or not this induction is rodent specific. In recent years much effort has been devoted to the development of in vitro systems to study CYP induction by xenobiotics. Various in vitro models for assessing CYP enzyme induction have been described and include precision-cut liver slices [3,4], primary hepatocytes [5], and reporter gene constructs [6]. In particular, the human and mouse PXR reporter gene assay allows a rapid evaluation of potential human/rodent specific CYP3A inducers during the early phase of drug development [7]. However, this assay based on PXR activation is mainly useful to investigate induction of the CYP3A isoform. Instead, in vitro induction studies using slices in combination with real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) allows the investigation of mRNA expression of every desired gene, including the CYP genes. The same methodology can be used to investigate mRNA CYP expression in vivo. Although mouse liver express many cyp450 forms [8], we have examined the induction of some selected CYP forms (cyp1a1, cyp1a2, cyp2b10 and cyp3a11), which are important for drug metabolism and inducible by xenobiotics [9]. In many instances, enzyme induction of the CYP supergene family (for example induction
of human CYP1A1) is accompanied by increased sensitivity to toxicity in target tissue and has been rationalized by induction of specific CYP forms that generate biologically reactive intermediates that are responsible for the observed toxicity, including carcinogenesis [10,11]. Therefore, the scope of this study was to evaluate the effect of some well described CYP inducers on some selected CYP isoforms in cultured mouse liver slices and to compare this with in vivo induction.

In a previous report [12] a similar approach was used, but only for rats. However, while the rat is the most important preclinical rodent species used for toxicology and pharmacology studies, mice are extensively used in the field of oncology research, such as in tumour growth models. Therefore, here we describe an in vitro – in vivo evaluation of CYP expression in mice.

Materials and Methods

Chemicals. The following compounds were obtained from the sources indicated: sodium phenobarbital, dexamethasone, β-naphthoflavone, d-glucose, insulin, hydrocortisone-21 hemisuccinate, gentamicin sulphate, and DMSO were from Sigma-Aldrich (St. Louis, MO, USA). L-methionine, fungizone, 5 x first strand buffer, RnaseOUT, Superscript, dATP, dGTP, dCTP, dTTP, random primers, DTT, BSA and Williams’ Medium E were obtained from Gibco (Paisley, Scotland, UK). Fetal calf serum was from Biological Industries Co (Kibbutz Beit Haemek, Israel). RNA 6000 Nano Assay was from Agilent Technologies (Palo Alto, CA, USA). RiboGreen® RNA Quantitation kit was from Molecular Probes (Eugene, OR, USA). Qiagen Rneasy® mini kit were from Qiagen Ltd (Crawley, UK). RNAalater™ was from Ambion (Austin, TX, USA). TaqMan® Universal PCR Master Mix Reagents and TaqMan® probes were obtained from Applied Biosystems (Foster City, CA, USA). The oligonucleotide primers were synthetised by Nerviano Medical Sciences Labs (Nerviano, Italy), except for 18S rRNA that was obtained from Applied Biosystems (Foster City, CA, USA).

Animals. Male CD-1 mice from Charles River (Como, Italy) were maintained under a 12-h light/dark cycle in standard cages and bedding with free access to standard commercial food pellets (4RF21 pellets from Mucedola, Settimo Milanese, MI, Italy) and tap water. Groups of three male mice (30-35 g) were treated with β-NF (80 mg kg⁻¹ day⁻¹, i.p., corn oil), PB (80 mg kg⁻¹ day⁻¹, i.p., saline) and DEX (100 mg kg⁻¹ day⁻¹, i.p., corn oil) for three days. One group of three control animals received only corn oil (vehicle), while a second group of three control animals received only saline (vehicle). Animals were sacrificed 24 h after the last treatment. Samples (about 30 mg) of the livers from mouse were stored in RNAalater™ and kept at 4°C for 24 h until RNA extraction.

Liver slice preparation. After i.p. anaesthesia with ketamine (67 mg/kg), hyalazine (15 mg/kg) and acepromazine (1 mg/kg), the livers from mice were excised and stored in ice-cold Williams’ Medium E until use (max. 0.5 h). Mouse liver slices (diameter 8 mm) were prepared in ice-cold Williams’ Medium E that was oxygenated with 95% O₂ / 5% CO₂ and supplemented with extra glucose (25 mM), using a Krumdieck tissue slicer [13]. The slices obtained were subsequently stored in ice-cold Williams’ Medium E until use (within 0.5 h after the preparation).

Incubations. Liver slices were individually incubated in 6 well culture plates (Falcon, France) in 3.2 ml Williams’ Medium E under 95% O₂ / 5% CO₂ atmosphere (one slice per well) at 37°C while shaking horizontally at 90-100 times min⁻¹. For each experiment, the slices were incubated in triplicate. The culture medium consisted of Williams’ Medium E containing glucose (25 mM), gentamicin (50 µg/ml), insulin (1 µM), hydrocortisone (10 µM), fungizone (2.5 µg/ml), methionine (0.5 mM) and fetal calf serum (5% v/v). After 1 hour, treatment was started by replacing the culture medium with medium containing the required concentrations of the test compounds (βNF 50 µM, PB 1 mM and DEX 100 µM) for 24 hours. The test compounds were dissolved in DMSO and added to the culture medium so that the final DMSO concentration was 0.1% (v/v) in all wells including the control incubations. After culture, three slices from each treatment were transferred in RNAalater™ and stored at 4°C for 24 hours until RNA extraction.
ATP content. The ATP content was determined as described before [14] using the ATPLite-M kit from Perkin Elmer (Boston, USA) and a TopCount NXT Luminescence Instrument from Perkin Elmer (Boston, USA).

RNA preparation from mouse liver and tissue. Total RNA was extracted from cultured mouse liver slices and from mouse liver samples using Qiagen Rneasy® mini kit. Briefly, 600 µl of lysis buffer from Qiagen was added to either a small sample (~30 mg) of liver or to one liver slice (~15 mg) and immediately homogenized using a conventional rotor-stator homogenizer. Then, the samples were extracted according to the Qiagen procedures. In addition, digestion of DNA, during RNA purification, was performed by adding DNase I (Rnase-Free Dnase Set according to Qiagen protocol). The quality of the isolated RNA was assessed using the RNA 6000 Nano Assay and the Agilent 2100 bioanalyzer (Palo Alto, CA, USA). The bioanalyzer uses gel electrophoresis in the confines of a microfabricated chip and laser induced fluorescence detection using an intercalating dye, which is added to the polymer. RNA concentration was determined using a RiboGreen™ RNA Quantitation kit. The yield of the extraction was 7.6 ± 1.1 µg total RNA/15 mg liver slice and 9.0 ± 0.5 µg total RNA/15 mg liver tissue. The quality of the isolated RNA was assessed by the 260/280 nm absorbance ratio (range 1.8-2.0 indicates a highly pure sample) [3].

Reverse transcription. The reaction mixture (final volume 40 µl) was prepared with final concentrations as follows: 1 x first strand buffer, 64 units RnaseOUT, 200 units Superscript, 0.6 mM of dNTP (dATP, dGTP, dCTP and dTTP), 0.75 µg random primers, 10 mM DTT and 16 ng BSA. To this mixture 1 µg of total RNA extracted was added. The reverse transcription reaction was performed for 10 min at 25°C, 60 min at 42°C and 30 min at 37°C.

Design of primers and probes. The cDNA sequences of mouse cyp1a1, cyp1a2 and cyp3a11 were obtained from GenBank accession no’s. NM 009992, NM 009993 and NM 007818, respectively. Taqman primer and probe sets for cyp2b10 were from [9]. PCR primers and probe sequences were designed using PrimerExpress software (Applied Biosystems) and shown in Table 1. Nucleotide primers and probe sequences were checked against the NCBI BLAST database to ensure specificity for the selected gene.

Table 1. Taqman® primer and probe sequences of mouse CYP mRNA

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers/Probes</th>
<th>5’ → 3′ Sequence</th>
<th>Amplicon Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cyp1a1</td>
<td>Forward</td>
<td>ATAAAGGTCATCATCACGATTTGCTTTTGG</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGTACATGAGGCTCCACGAGAT</td>
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</tr>
<tr>
<td></td>
<td>Probe</td>
<td>CACAGTCACAAGTGC</td>
<td></td>
</tr>
<tr>
<td>cyp1a2</td>
<td>Forward</td>
<td>CGTGACAGAACTTGCAGAGAAG</td>
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<td></td>
<td>Reverse</td>
<td>CGATGTTCAGCATCTCTCAG</td>
<td></td>
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<tr>
<td></td>
<td>Probe</td>
<td>CCAAGACATGGCAGCCTCG</td>
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<tr>
<td>cyp2b10</td>
<td>Forward</td>
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<td>TGAAGTCATCTGATGCTATGTCATT</td>
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</tr>
<tr>
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<td>Probe</td>
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<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>ACAGAGAAGTAAATTGC</td>
<td></td>
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</table>
Real-time quantitative RT-PCR. Real-time quantitative RT-PCR was performed, employing a TaqMan 7900 sequence detector system (Applied Biosystems). The PCR reaction was performed in a 384 well plate. The reaction mixture (13.5 µl) was added in each well to give the following concentration: 1 x master mix reagents, 900 nM of each primer and 200 nM probe for each CYP mRNA assay. cDNA (1.5 µl) was added to each well and the final volume was 15 µl. The thermal cycle condition was 50°C for 2 min, 95°C for 10 min to activate AmpliTaq Gold DNA polymerase, denaturation at 95°C for 15 sec and anneal/extension at 59°C for 1 min (40 cycles). Quantitative PCR for 18S rRNA was also performed to normalize for RNA loading. Ct values (the PCR cycle number required for fluorescence intensity to exceed an arbitrary threshold in the exponential phase of the amplification) were then determined of a series of standards. A standard curve was generated by plotting Ct versus the log of the amount of total RNA added to the reaction (0.035-35 ng) and used to compare the relative amount of a particular cyp mRNA in the control and treated samples.

Statistical analysis. Differences among group mean values were assessed using two-tailed, two sample t test, assuming equal variance. A difference of $P < 0.05$ was considered statistically significant.

Results and discussion

This study is focused on mice which are extensively used in oncology for their wide range of genetic models of human diseases [15]. When mice are used in long-term safety studies, sometimes tumours are observed that are suspected to be related with CYP gene induction [16]. Moreover, in some cases toxicity in the target tissue may be explained by enzyme induction of specific CYP forms that metabolise pro-carcinogens to biologically reactive intermediates, responsible for the observed toxicity, including carcinogenesis [17]. To investigate if there is a relation between hepatic enzyme induction and liver tumorigenesis, measurement of CYP mRNA levels are very useful. However, a mouse tumor caused by induction of specific CYPs may not be of human significance because of the well known species differences between human and mouse CYP gene regulation, for example as a result of species specific PXR activation [7]. We have chosen liver slices as an in vitro investigative tool because of its applicability to several species, including man [13]. To test if cultured liver slices may be useful to predict CYP gene induction, we compared results obtained in vitro to in vivo gene induction values. The incubation length was selected at 24 hours as a compromise to be able to measure CYP induction (taking advantage of the sensitivity of the real time RT-PCR method), but to avoid a decrease of basal CYP expression. Unnatural low basal CYP levels, such as seen after prolonged culture of virtually all present in vitro liver models may result in a artificially high fold induction over control.

To determine the relative viability of the liver slices, both ATP content as well as RNA integrity was assessed. ATP contents of the slices was maintained during the incubation period as shown in Fig. 1. RNA extracts from freshly excised liver tissue, directly after slicing and after 24 hours of slice incubation were analyzed for integrity by gel electrophoresis.
CYP induction in mouse liver slices

Figure 1. ATP content of liver slices during 24 h of incubation. Data are expressed as mean ± SEM from two independent experiments, in which at each time point the mean of three slices were determined.

As shown in Figure 2, total RNA extracted from slices incubated for 24 hours showed a typical, intact RNA pattern, comparable to that extracted from liver tissue or slices that were not incubated.

Figure 2. Total RNA integrity assessment from fresh liver tissue (A) and from liver slices incubated for 0 (B) and 24h (C). The fluorescence peaks at 41 and 48 sec from the gel electrophoresis run time represent the 18S and 28S bands, respectively.
After in vivo treatment, βNF strongly induced gene expression of cyp1a1 (∼880-fold, Fig. 3). Also, induction was also observed towards cyp1a2 and cyp2b10 (∼22-and ∼17-fold, Fig. 3). Phenobarbital significantly increased mRNA levels of cyp2b10 (∼25-fold, Fig. 3) and showed a minor induction of cyp3a11 (∼2 fold, Fig. 3). Levels of cyp2b10 were largely increased (∼380-fold, Fig. 3) after treatment with DEX. There was no effect of DEX on cyp1a1 and cyp1a2, while cyp3a11 levels were induced (∼7-fold, Fig. 3).

![Graph showing gene expression levels](image)

**Figure 3.** Effect of classic inducers on expression of CYP mRNAs in mouse liver, assayed by real-time RT-PCR. After treatment with βNF, PB and DEX for three days, the levels of cyp1a1, cyp1a2, cyp2b10 and cyp3a11 were investigated. Total RNA (3.5 ng) was loaded for one-step real-time RT-PCR assay and the levels of CYP mRNAs were normalized for RNA loading by taking the ratio of 18S and expressed as fold induction over untreated animals. Results are the mean ± SEM for groups of three rats. Values significantly different from control: *p < 0.05.

After in vitro treatment of precision-cut mouse liver slices, βNF markedly induced cyp1a1 gene expression (∼150-fold, Fig. 4). Cyp2b10 and cyp1a2 mRNA levels were also increased by βNF, by ∼18 and ∼5 fold induction, respectively (Fig. 4). PB showed a significant induction of cyp2b10 (∼130-fold, Fig. 4) but was only slightly inducing cyp1a2 (∼ 2-fold, Fig. 4). Finally, cyp2b10 mRNA levels were more than 500-fold increased by DEX (Fig. 4), whereas a small induction was observed for cyp3a11 (∼5-fold, Fig. 4).
Figure 4. Effect of classic inducers on expression of CYP mRNAs in mouse liver slices, assayed by real-time RT-PCR. After treatment the liver slices with βNF (50 µM), PB (1 mM) and DEX (100 µM) for 24 hours, the levels of cyp1a1, cyp1a2, cyp2b10 and cyp3a11 were investigated. Total RNA (3.5 ng) was loaded for one-step real-time RT-PCR assay and the levels of CYP mRNAs were normalized for RNA loading by taking the ratio of 18S and expressed as fold induction over untreated slices. Results are the mean ± SEM (n=6) of two separate experiments. Values significantly different from control: * p < 0.05.

To illustrate better the relationship between CYP induction observed in vivo and in vitro, the data are presented in Table format (Table 2). All three compounds tested showed qualitatively similar induction in vivo and in vitro towards the CYP isoforms tested, except for PB towards cyp3a11 and cyp1a2. Also the extent of induction (quantitative) was remarkably similar between in vivo and in vitro. Only PB seems to be more potent in vitro when compared to in vivo towards cyp2b10. However, it should be mentioned that mRNA induction of cyp genes does not necessarily means equal extent of induction of enzyme activity although often a good concordance is found, for example using rat liver slices [18]. Information on cyp2b10 and cyp3a11 induction in mouse hepatocytes is in accordance with our results [19,20]. In this work, we reported strong induction of cyp1a1 by βNF in liver slices from outbred CD-1 mice, in accordance with a previous report about mouse liver slices from the C57B1/10ScN inbred strain, but different from the inbred B6C3F1/6 and C57B1/6 strains [4]. Mice strain differences with respect to cyp1a induction are well known to be caused by the considerable structural and functional variability of the Ah-receptor between inbred mouse strains [21,22].
**Table 2.** A schematic comparison of induction (qualitative and quantitative) observed *in vivo* and *in vitro* in mice after exposure to βNF, PB and DEX.

<table>
<thead>
<tr>
<th></th>
<th>cyp1a1</th>
<th></th>
<th>cyp1a2</th>
<th></th>
<th>cyp2b10</th>
<th></th>
<th>cyp3a11</th>
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<tbody>
<tr>
<td>βNF</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>PB</td>
<td>-</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
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<td>DEX</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

- no significant induction
+ < 25 fold induction
++ 25-100 induction
+++ > 100 fold induction

Also the *in vivo* induction of cyp1a1 and cyp1a2 by βNF and of cyp2b10 and cyp3a11 by PB and DEX observed here is in agreement with previous reports [23,24].

Thus, the current study underlines the potential of mouse liver slices to study CYP induction *in vitro*. In addition, in a previous report [12] we demonstrated that also rat liver slices are a useful tool to study induction in rodents. Therefore, we expect that slices from other species including human can be used to predict the *in vivo* situation. This is currently investigated in our laboratory.

In summary, the RT-PCR method described here is a rapid and suitable procedure to assess changes in CYP mRNA expression in whole liver and liver slices from mice. In addition, the induction profiles (qualitative and quantitative) observed *in vitro* are very similar compared to *in vivo*. 

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References


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[23] L. Corcos, Phenobarbital and dexamethasone induce expression of cytochrome P-450 genes from subfamilies IIb, IIC, and IIIa in mouse liver, Drug Metab. Dispos. 20 (1992) 797-801.