MEASUREMENT OF CYTOCHROME P450 GENE INDUCTION IN HUMAN HEPATOCYTES USING QUANTITATIVE REAL-TIME REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION

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ABSTRACT:
Drug-induced changes in expression of cytochrome (CYP) P450 genes are a key cause of drug-drug interactions. Consequently, preclinical prediction of these changes by novel compounds is an integral component of drug development. To date, in vitro models of CYP induction have used mRNA measurement, immunodetec-
tion, and substrate metabolism as reporters. Here, we describe the application of quantitative real-time reverse transcriptase poly-
merase chain reaction to study CYP1A1 and CYP3A4 gene induc-
tion in 5-day-old cultures of human hepatocytes by known CYP inducers. After 5 days in culture, CYP1A1 expression was significa-
tantly elevated (5.1- to 26-fold; \( P < .01 \)) in all four livers studied. In contrast, CYP3A4 mRNA levels consistently decreased during culture (80- to 300-fold; \( P < .001 \)). In three independent experiments, a 48-h exposure to 3-methylcholanthrene, omeprazole, and lansoprazole significantly induced CYP1A1 expression in comparison to untreated cultures (\( P < .05 \)). Rifampicin and solvent were without effect on CYP1A1 expression. Under identical experimental conditions, rifampicin and lansoprazole significantly elevated CYP3A4 mRNA expression (\( P < .05 \)), whereas 3-methylcholanthrene, omeprazole, and dimethyl sulfoxide were without significant effect. These data demonstrate the applicability of quantitative reverse transcriptase polymerase chain reaction to the determination of gene dynamics in human hepatocytes. This offers a highly specific alternative to quantification of drug effects on CYP expression using immunodetection and substrate metab-
olism.

Cytochrome P450s (CYPs)\(^1\) comprise a group of haem-containing proteins, some of which play a key role in the metabolism, and hence the elimination, of a variety of chemically diverse compounds, in-
cluding pharmaceutical agents. The regulation of gene expression is poorly understood for most CYPs, but it is well documented that certain drugs can selectively modulate the expression of a range of CYP genes (Denison and Whitlock, 1995). When the result of drug administration is the induction of a CYP gene, differential or alterna-
tive metabolism of other coadministered therapies may occur, result-
ing in change of efficacy and possibly toxicity. Estimation of CYP induction and prediction of drug-drug interactions is therefore an important consideration for the development of novel therapeutic agents (Park et al., 1996). Predicting the ability of a drug to modulate CYP expression at an early stage of its discovery and development should reduce the risk of failure in the clinic and, more importantly, permit the identification of alternative noninducing chemical struc-
tures.

Increasingly, preclinical evaluation of CYP induction is focused on

1 Abbreviations used are: CYP, cytochrome P450; QRT-PCR, quantitative real-time reverse transcriptase-polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction; Ct, threshold cycle; Cn, starting mRNA copy number; 3-MC, 3-methylcholanthrene; GAPDH, glyceraldehyde-3-phos-
phate dehydrogenase; DMSO, dimethyl sulfoxide.

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models using human tissue, most notably liver, because of docu-
mented differences in the effects of CYP inducers between humans and other species. To date, the most widely used model system has been cultured human primary hepatocytes, because they contain a full complement of CYPs, together with the appropriate receptors, drug response elements, and transcriptional factors (Strom et al., 1996). Cultures of human hepatocytes have been shown to retain many aspects of liver function, including CYP-mediated oxidation of drugs and CYP induction (for review see Li et al., 1997a).

Quantification of CYP induction has been achieved primarily by measuring changes in the metabolism of CYP-selective substrates or by direct quantification of CYP protein using specific antibodies and Western blotting. More recently, direct study of CYP mRNA expression has been used because up-regulation of CYP gene transcription and the subsequent increase in protein levels are thought to be pri-
marily responsible for the enhanced metabolic function of CYPs after exposure to inducers (Hankinson, 1995; Honkakoski et al., 1998; Lehmann et al., 1998). One possible exception is induction of CYP2E1 by ethanol and isoniazid, which is thought to result either from protein stabilization (Zand et al., 1993) or increased protein translation (Park et al., 1993). Although some quantitative data have been reported, precise quantification of mRNA expression by con-
ventional methods such as Northern blotting is unreliable, and use of reverse transcriptase-polymerase chain reaction (RT-PCR) requires elaborate methodology, which limits their use for routine prediction of CYP induction.

The human CYP protein superfamily is encoded by at least 48
different genes. In addition, 14 human CYP pseudogenes, which do not encode functional protein, have been described. Thus, to obtain a comprehensive assessment of the ability of a drug to modulate CYP function through modulation of transcription requires a method that can dissect the effect of a drug on single CYP isoforms. Although it is possible to quantify single CYP proteins using selective antibodies, immunodetection of CYP proteins is plagued by uncertainty regarding antibody specificity because CYP families possess a high degree of protein sequence homology. Their use is, therefore, restricted to the few CYPs for which selective antibodies exist. The task is more difficult using metabolic substrates, given the polymorphic nature of certain CYPs in the human population (notably CYP2C9, CYP2C19, and CYP2D6), donor variability of CYP expression, and lack of absolute substrate specificity.

Here we describe the application of quantitative real-time reverse transcriptase-polymerase chain reaction (QRT-PCR) to the measurement of drug induction of two CYP isoforms, CYP1A1 and CYP3A4, in primary cultures of human hepatocytes. CYP1A1 was chosen for study because of its well documented induction by aromatic hydrocarbons (Hankinson, 1995), and CYP3A4 because it is the most abundant form of the hepatic CYPs (Shimada et al., 1994), and is responsible for the oxidative metabolism of two-thirds of pharmaceutical drugs tested (Cholerton et al., 1992). The activity of 3-methylcholanthrene (3-MC), rifampicin, omeprazole, and lansoprazole was assessed because all are known CYP inducers in vivo and in human primary hepatocytes (Li et al., 1997a).

**Materials and Methods**

**Chemicals.** Phenol red-free Williams’ E medium, rat tail collagen, 3-MC, rifampicin, and lansoprazole were obtained from Sigma. Collagenase A was purchased from Boehringer. Omeprazole was extracted from Losec capsules using dimethyl sulfoxide (DMSO). Oligonucleotide primers and fluorogenic probes were purchased from PE Biosystems (Warrington, UK). TriZol and the remaining tissue culture materials were obtained from Life Technologies (Paisley, UK). All other reagents used in this study were of analytical grade and purchased from commercial sources.

**Human Liver.** The human livers used, together with donor-related information, are listed in Table 1. All livers were obtained through medically induced hepatectomy. Immediately after resection, exposed vessels were cannulated and the specimen was perfused with University of Wisconsin solution. A catheter was introduced into the most prominent hepatic blood vessel and secured with sutures. The remaining vessels on the cut surface were ligated to improve perfusion of the tissue. The liver was placed in University of Wisconsin solution and transported on ice to our laboratory. Hepatocytes were isolated by collagenase digestion essentially as described by Strom et al. (1982). Isolated hepatocytes were resuspended in Williams’ E medium supplemented with insulin (0.01 μg/ml) and hydrocortisone (0.005 μg/ml), and initial cell viability was measured by Trypan blue dye exclusion. The hepatocyte preparations used in this study had >80% initial cell viability (Table 1). The cells were plated into collagen-coated 6-well plates (2 × 10^6 cells/well in 2 ml of medium) and cultured for 3 days before induction experiments. Medium was changed daily.

**Extraction of Total RNA from Hepatocytes.** Total RNA was extracted from hepatocytes using TriZol, a commercially available mixture of phenol and guanidine isothiocyanate, according to the protocol described by the manufacturer (Life Technologies). The concentration and purity of the RNA were determined by measurement of the optical densities at 260 and 280 nm. A ratio of >1.7 for A260/A280 was required for these studies. The RNA solutions were diluted to a working concentration of 1 μg/μl in nuclease-free water with the addition of RNase inhibitor (N808-0119; PE Biosystems).

**Principles of TaqMan Technology.** A quantitative analysis of specific mRNA expression was performed by QRT-PCR using the ABI Prism 7700 Sequence Detection System (PE Biosystems). The system uses a fluorogenic probe to generate sequence-specific fluorescent signals during PCR. The probe is an oligonucleotide with fluorescent reporter and quencher dyes attached, designed from the mRNA sequence to hybridize to a region between the forward and reverse PCR primers. While intact, the intensity of reporter fluorescence is suppressed by the quencher. If the probe forms part of a replication complex, the fluorescent reporter is cleaved from the quencher by 5' → 3' exonuclease activity inherent in Taq polymerase.

The starting mRNA copy number (Cn) of a target sequence is established by determining the fractional PCR threshold cycle (Ct) number at which the fluorescent signal generated during the replication process passes above a threshold value. The initial amount of target mRNA in each sample is then estimated from the experimental Ct by interpolation from a standard curve generated using known amounts of genomic DNA (Fig. 1).

**Transcription Detection.** Forward and reverse primers and the fluorogenic TaqMan probe were designed using Primer Express software (PE Biosystems) to amplify a 141 base pair fragment from the human CYP1A1 mRNA sequence (GenBank accession number AF040258).
Forward: 5’ TGGTCCTCCTCTTCAGCTTGT 3’
Reverse: 5’ ATTTTCCCTACTATCAATAGGTTCT 3’
Probe: 5’ CAAAGCATGCAAGTCAATGCAGGCT 3’

A pair of primers and a TaqMan probe were also designed to amplify an 86 base pair fragment from the human CYP3A4 mRNA sequence (GenBank accession number D11131).

Forward: 5’ CTTCATCCAATGGAGCTGATAAAT 3’
Reverse: 5’ TCCCAAGTATAACCTCTACACAGACAA 3’
Probe: 5’ CCGGGGATCTTCGATCAGTATTG 3’

In addition, a pair of primers and a TaqMan probe were designed to amplify a 78 base pair portion of the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript that spans an exon/exon boundary. GAPDH is a ubiquitously expressed ‘housekeeping’ gene, and was used to provide an internal marker of mRNA integrity within the experiment.

Forward: 5’ GAAAGTGGAAGTGGAGTCAAC 3’
Reverse: 5’ CAGAGTTAAAGAGCAGGCTTGT 3’
Probe: 5’ TTTGGTGCGGTATTGGCCGCT 3’

The CYP probes were labeled with the fluor 6-carboxyfluorescein, the GAPDH probe with the fluor JOE. Both fluoros were quenched with carboxytetramethyl rhodamine.

Total RNA samples were treated with RNase-free DNase I (amplification grade; Life Technologies) according to the manufacturers’ instructions. For each RNA sample, 100 ng was then used as template for first strand cDNA synthesis. The RNA in a volume of 4 μl and in the presence of reverse primers for CYP1A1, CYP3A4, and GAPDH, 1 × PCR buffer II (PE Biosystems), and 5 mM MgCl₂ was heated to 72°C for 5 min and cooled slowly to 55°C. After addition of all other reagents, the 6-μl reaction was incubated at 37°C for 30 min followed by an enzyme inactivation step of 90°C for 5 min. The final reaction conditions for reverse transcription were as follows: 1 × PCR buffer II; 5 mM MgCl₂; 1 mM dATP, dGTP, and dCTP; 2 mM dTTP; 12.5 U MuLV reverse transcriptase (Life Technologies). The resulting cDNA was subjected to PCR amplification in the ABI 7700 Sequence Detection System to identify either CYP1A1 or CYP3A4 PCR product in conjunction with GAPDH transcripts in a single reaction. Final reaction conditions were 4% glycerol; 0.66 × TaqMan buffer A (PE Biosystems); 6 mM MgCl₂; 358 μM dATP, dUTP, dGTP, and dCTP; 2.5 U AmpliTaq Gold; and 0.16 U of AmpErase UNG (uracil N-glycosylase). An initial enzyme activation step of 94°C for 12 min was followed by 45 PCR cycles each of 95°C for 15 s, 60°C for 30 s.

Transcription Quantification. Standard curves have been generated for >100 different targets using 3, 6, 15, 30, and 60 ng of sheared genomic DNA, equivalent to 1,000, 2,000, 5,000, 10,000, and 20,000 copies of the human male genome, respectively. Reactions were carried out in duplicate under identical conditions to those described above. The criteria for successful primer/probe design are extremely rigorous, and it has been our experience that the efficiency of amplification with sets that conform is very high. Primer/probe sets were designed according to the parameters incorporated in the Primer Express software (PE Biosystems). The primer/probe sets were homology-searched to ensure that they were specific for the target mRNA transcript using an NCBI BLAST search. We have determined the frequency distribution of the data points from a large number of standard curves generated with different primer/probe sets and have found that they are very tightly grouped. Therefore, we have used these data to generate a global standard curve that is used to quantify all target mRNA copy numbers (Fig. 1). The relationship between log Ct and Ct is linear. Using interpolation, the Ct at which the accumulated fluorescent signal exceeded a predetermined threshold level above background was compared with the global standard curve to generate a starting copy number for the CYP1A1 and CYP3A4 transcripts.

Statistical Analysis. The effects of test compounds on CYP1A1 and CYP3A4 mRNA expression were determined in hepatocytes from three different donors. Each determination was performed in triplicate, and data are presented as means ± S.E. The effect of culture on CYP mRNA expression was determined in hepatocytes from three different donors. Each determination was performed in triplicate, and data are presented as means ± S.E. The effect of culture on CYP mRNA expression was determined in hepatocytes from three different donors. Each determination was performed in triplicate, and data are presented as means ± S.E. The effect of culture on CYP mRNA expression was determined in hepatocytes from three different donors. Each determination was performed in triplicate, and data are presented as means ± S.E.

Results

Quantification of CYP1A1 and CYP3A4 mRNA. Sufficient total RNA was obtained from 4 million human hepatocytes for parallel quantification of CYP1A1 and CYP3A4 mRNA. Both CYP1A1 and CYP3A4 genes were abundantly expressed, readily permitting quantification using real-time RT-PCR (Fig. 2). The maximum fluorescent signal obtained with the CYP1A1 TaqMan probe was approximately 10, whereas that for CYP3A4 was lower, at about 8. The difference is most probably due to differential 6-carboxyfluorescein labeling of the probes, because studies using genomic DNA indicate that they amplify with similar efficiencies. When interpreting amplification plots, it is important to realize that samples expressing higher levels of message achieve threshold fluorescence at low cycle numbers (i.e., low Ct values), whereas low expression is associated with high Ct values. The highest Ct obtained with any sample in this study was 26, much lower than the number of cycles followed in the experiment (45), clearly demonstrating that the sensitivity of the technology is adequate for reliable quantification of CYP mRNA expression. In addition, positive GAPDH amplification was measured for all samples (data not shown), confirming the integrity of RNA used in the assays.

Effect of Culture on CYP mRNA Expression. CYP1A1 and CYP3A4 mRNA expression was quantified both immediately after hepatocyte isolation and after 5 days in medium to determine the effect of culture. Immediately post-isolation, the lowest expression of
CYP1A1 was detected in donor 1 (6940 copies/100 ng of total RNA), whereas donor 4 expressed 9-fold higher levels (60,600 copies/100 ng of total RNA). After 5 days of culture, CYP1A1 expression increased by between 5.1- and 26-fold in the cells from the four livers studied; this increase was statistically significant \((P < .01); \text{Fig. 3A}\). In direct contrast, culture of hepatocytes was associated with a highly significant reduction of CYP3A4 expression \((P < .001); \text{Fig. 3B}\), corresponding to a decrease to about 1% of initial levels. In freshly isolated hepatocytes, expression of CYP3A4 mRNA was approximately two orders of magnitude higher than CYP1A1.

**Effect of Inducers on CYP mRNA Expression.** The levels of gene expression for CYP1A1 and CYP3A4 were studied in 5-day-old cultures of human hepatocytes after a 48-h exposure to known CYP inducers. Data for CYP expression in three different preparations of hepatocytes are shown in Figs. 4 and 5. None of the treatments produced any visible toxic effect (i.e., cell death, loss of adhesion) on hepatocytes. The methods of mRNA quantification used by these groups were relatively elaborate to overcome intrinsic features of RT-PCR that lead to semiquantitative data. The main problem is the inherent saturation that occurs in the PCR amplification process, which can complicate “endpoint” assays, and lead to incorrect interpretation of starting mRNA abundance (Murphy et al., 1990; Wiesner et al., 1992). This is exemplified in Fig. 2B, where the amount of fluorescence after 45 cycles of PCR demonstrates that CYP3A4 mRNA was present in all three samples, but the magnitude of the signal is similar in all cases, and does not reflect the marked rifampicin-induced CYP3A4 gene expression. Numerous methods have been developed to achieve quantification using RT-PCR; for example, use of competitive DNA fragments (Siebert and Larrick, 1993) or termination assays in which the PCR process is stopped after a predetermined number of cycles (Mannes and Li, 1997). None, however, offers the reliability or simple detection of PCR product in real time associated with QRT-PCR used in this study.

In this study, we applied QRT-PCR to the analysis of CYP1A1 and CYP3A4 gene induction in primary hepatocytes in a process that allows the parallel assessment of multiple gene targets in single extracts of hepatocyte RNA. Real-time measurement of the amount of PCR product at each cycle of PCR is achieved by measuring cleavage of a fluorescent TaqMan probe using the ABI Prism 7700 Sequence Detection System (PE Biosystems). PCR amplification is performed in 96-well plates, and accumulation of PCR product is quantified in real-time by fluorometric detection. This permits the parallel assess-
ment of mRNA expression of all the major CYP isoforms, together with other phase I enzymes, such as alcohol dehydrogenase, monoamine oxidase, and aromatases, and phase II enzymes, such as glucuronyltransferase, sulfotransferase, glutathione transferase, and N-acetyl transferase. The importance of phase II induction is currently being reappraised in the light of reports of UDP-glucuronyltransferase inducibility (Abid et al., 1997; Masubuchi et al., 1997). Rifampicin and ganciclovir have also been reported to increase zidovudine metabolism, possibly via induction of zidovudine glucuronidation (Burger et al., 1994).

CYP1A1 and CYP3A4 mRNA levels in freshly isolated human hepatocytes were about 10,000 and 1,000,000 copies per 100 ng of

FIG. 4. Quantification of CYP1A1 mRNA expression after drug exposure for 2 days.

Data for individual donors are presented (mean ± S.E. of triplicate observations). Messenger RNA abundance is expressed as the Log Cn in 100 ng of total RNA.
total RNA, respectively, suggesting that functionally, CYP3A4 may be the more abundant isoform (Fig. 2). However, because determinations of CYP mRNA expression made in this study were not directly compared with either protein abundance or functional activity, a direct correlation cannot be made. We have also found no literature data with which to compare our determination of basal CYP1A1 and CYP3A4 mRNA expression. Mattes and Li (1997) estimated CYP3A (primers recognized CYP3A3 and CYP3A4 cDNA transcripts) expression at 73,000 copies per 100 ng of total RNA in a single preparation of human hepatocytes, after 7 days of culture, somewhat

Fig. 5. Quantification of CYP3A4 mRNA expression after drug exposure for 2 days.
Data for individual donors are presented (mean ± S.E. of triplicate observations). Messenger RNA abundance is expressed as the Log Cn in 100 ng of total RNA.
lower than that obtained by QRT-PCR in this study. There are many differences between the two studies, most notably different culture times, quantification method, and RNA extraction methodology, making any quantitative comparison unreliable. In addition, Mattes and Li did not prevent or correct for possible contribution of genomic DNA contamination in their assay, so that their measurements using RT-PCR amplification may be an overestimate of CYP3A mRNA. All samples in this study were treated with DNase to remove any contaminating DNA before reverse transcription of mRNA to cDNA.

In models of hepatic CYP induction, primary hepatocytes are routinely cultured for 2 to 3 days before the start of treatment with inducer (Li et al., 1997a). One of the reasons for culture before exposure to inducer is the rapid decline in metabolic activity that is observed during the first few days post-isolation, enabling drug-induced changes in CYP activity to be measured against a lower baseline, using immunoblotting and substrate metabolism (Grant et al., 1987; Li et al., 1995). In our experiments, CYP3A4 mRNA expression declined considerably during culture (to about 1% of initial levels), which correlates well with previous studies using immunoblotting (Silva et al., 1998) and substrate metabolism (Li et al., 1995). In direct contrast, CYP1A1 mRNA levels increased significantly in all donors (5- to 26-fold increase). There is little literature evidence with which to compare this finding. Interestingly, CYP1A1 activity as measured by O-dealkylation of ethoxyresorufin has been shown to decline in a similar manner to CYP3A4 in human hepatocytes (Grant et al., 1987). It is, however, difficult to differentiate the metabolic effects of CYP1A1 from its isomorph CYP1A2, with which it shares a similar substrate profile. This reduction in ethoxyresorufin metabolism may therefore be attributable to loss of CYP1A2 activity, masking an up-regulation of CYP1A1.

To investigate the applicability of QRT-PCR to measurement of CYP induction, the effects of four known inducers on CYP1A1 and CYP3A4 expression were measured. 3-MC, omeprazole, and lansoprazole all significantly increased CYP1A1 expression after a 48-h exposure in three donors. These data are consistent with the known action of 3-MC (Morel et al., 1990; Donato et al., 1995; Masubuchi et al., 1998), omeprazole (Diaz et al., 1990; Curi-Pedrosa et al., 1994; Masubuchi et al., 1998), and lansoprazole (Curi-Pedrosa et al., 1994; Masubuchi et al., 1998) in human hepatocytes. The inactivity of rifampicin on CYP1A1 expression also correlates with literature findings (Curi-Pedrosa et al., 1994; Silva et al., 1998). Likewise, up-regulation of CYP3A4 expression by rifampicin (Morel et al., 1990; Gillum et al., 1993; Kocarek et al., 1995; Greuet et al., 1997; Li et al., 1997b) and lansoprazole (Curi-Pedrosa et al., 1994; Masubuchi et al., 1998) and the trend toward induction by omeprazole (Curi-Pedrosa et al., 1994) are consistent with previously published information. Furthermore, the inability of 3-MC to affect mRNA levels for CYP3A4 supports the use of this approach to define the profile of CYP induction activity for test compounds.

In conclusion, we have described the application of QRT-PCR to the investigation of CYP induction in cultures of human hepatocytes. The technology is able to quantify CYP mRNA with ample sensitivity, permitting the determination of changes in mRNA expression caused by treatment with known inducers of CYP1A1 and CYP3A4 enzymes. Being 96-well-based, several hundred QRT-PCR reactions can be performed daily, allowing screening of chemical series and not just selected lead molecules. In addition, parallel analysis of multiple mRNA species in the same hepatocyte total RNA extracts reduces intersample variation, lowers hepatocyte usage, and permits the profiling of families of genes. QRT-PCR therefore permits a comprehensive assessment of the ability of a drug to modulate hepatic gene expression, including those encoding CYP enzymes, thus offering a sensitive, specific, and rapid alternative to quantification of gene induction by immunodetection or substrate metabolism.


