Assessment of Temporal Biochemical and Gene Transcription Changes in Rat Liver Cytochrome P450: Utility of Real-Time Quantitative RT-PCR

Federico M. Goodsaid,[1,3] Jairam R. Palamanda,[2] Diana Montgomery,[2] George Mandakas,[1] Chunyan Gu,[2] Zhiling Li,[2] Xiaoli You,[2] Laura Norton,[2] Roger Smith,[1] Inhou Chu,[2] Tony Soares,[2] Kevin Alton,[2] Narendra S. Kishnani,[2] and I. Y. Rosenblum[1]

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Purpose. A conventional approach to assess cytochrome P450 (CYP) induction in preclinical animal models involves daily dosing for a least a week followed by Western blot and/or enzyme activity analysis. To evaluate the potential benefit of a third more specific and sensitive assay, real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR), with the objective of reducing the duration of the conventional 1-week study, we simultaneously assessed gene expression by qRT-PCR along with Western blots and enzyme activity assays as a time course in an *in vivo* model.

Methods. Rats were dosed daily for 8 days with model inducers of CYP1A, CYP2B, CYP3A, or CYP4A. Liver P450 levels were measured after 0.5, 1, 2, 4, and 8 days of dosing by qRT-PCR, Western blot, and enzyme activity.

Results: CYP1A, CYP3A, and CYP4A genes were maximally induced very rapidly (0.5–1 day), whereas the CYP2B gene was maximally induced after a lag time of 4 days. In all cases, fold changes in induction detected by qRT-PCR were greater than fold changes in protein levels and enzyme activities.

Conclusions. Maximal persistent and larger fold changes observed by qRT-PCR either preceded or occurred simultaneously with maximal sustained fold changes in protein levels as measured by Western blots and enzyme activity assays. Our data show that qRT-PCR provides increased sensitivity and specificity over conventional assays and may be key information for reliable assessment of drug-related changes in CYP induction during the transition from discovery to toxicology studies.

KEY WORDS: CYP induction; qRT-PCR; quantitative gene expression; Western blot; enzyme activity; proteomics; genomics.

INTRODUCTION

With the escalating cost of drug development, there is a continuing compelling need to screen candidate compounds not only rapidly but also thoroughly for various aspects that are important to the successful development of a candidate drug. Conventional screens typically used for this purpose include models to assess physicochemical characteristics, rapid pharmacokinetics, microsomal stability, and protein binding. A major consideration in selecting a candidate for development is its effect on induction of drug-metabolizing enzymes, in particular cytochrome P450s. Screening out candidates that are strong inducers of CYPs such as CYP 1A1, CYP 2B1, CYP 3A4, and CYP 4A1 is based on the potential for tumorigenesis (CYP 1A1, CYP 2B1, and CYP 4A1) in certain rodent models used in toxicologic evaluations and the potential for drug–drug interactions clinically (CYP 3A4) (1,2). A conventional approach to assessing cytochrome P450 (CYP) induction in preclinical animal models involves daily dosing for a least a week followed by Western blot and/or enzyme activity analysis. The importance and toxicologic significance of monitoring changes in expression of key drug-metabolizing enzymes is highlighted in a recent report on the toxicity of acetaminophen in a murine model where drug-induced activation of a receptor (constitutive androstane receptor, CAR) resulted in induction of acetaminophen-metabolizing enzymes and increased toxicity (3).

Induction, especially autoinduction, can also impact preclinical safety studies by reducing exposure to the drug candidate, thus limiting the achievement of acceptable exposure multiples expected by the regulatory agencies for the approval of a drug. Clinically, for example, rifampin and ritonavir (inducers of CYP 3A4) have been shown to substantially reduce (up to 95% reduction in AUC with rifampin) the plasma levels of coadministered drugs (4,5). Drugs most affected by such interactions are substrates for CYP 3A4 and P-glycoprotein, an efflux protein. Many such drugs are essential components of therapy in highly susceptible populations (geriatrics and patients with HIV). Because it is well recognized that in an era of unprecedented polypharmacy the clinical use of a drug may be severely limited by its interaction potential, it becomes critical to screen candidates very early using techniques that are not only sensitive but specific as well. Techniques with a high sensitivity for detecting induction are particularly valuable during discovery screening because typical dosing concentrations are lower (the amount of compound available is limited) than in toxicologic studies. The method of real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) is both highly sensitive and displays a large dynamic range. The use of qRT-PCR in discovery screening may ultimately enhance the early identification of P450-inducing compounds.

Quantitative gene expression has been applied previously to the measurement of CYP enzyme induction (6–8). Earlier studies have measured CYP gene expression changes using cultured cells (6,7). Bowen *et al.* (6) showed that quantitative gene expression for CYP 1A1 and 3A1 induction could be determined for human hepatocytes in culture, but no correlation with functional testing was included (6). Burczynski *et al.* (7) found a good correlation between quantitative gene expression for the measurement of CYP induction in rat hepatocytes with LC/MS/MS-based CYP enzyme assays (7). Results published previously (8) using CD1 mice have shown that quantitative gene expression measurements for CYP enzyme induction in liver tissues are possible for CYP 3A11,

[1] Department of Genetic and Molecular Toxicology, Schering-Plough Research Institute, P.O. Box 32, Lafayette, New Jersey 07848.
[2] Department of Drug Metabolism and Pharmacokinetics, Schering-Plough Research Institute, Kenilworth, New Jersey 07033.
[3] To whom correspondence should be addressed. (e-mail: federico.goodsaid@spcorp.com

ABBREVIATIONS: CYP, cytochrome P450 gene(s); qRT-PCR, quantitative reverse transcription polymerase chain reaction; AUC, area under the curve; BNF, β-naphthoflavone; SCH, Schering-Plough compound; DEX, dexamethasone; CLO, clofibrate; C_T, threshold cycle; EROD; 7-ethoxyresorufin; PROD, 7-pentoxyresorufin.

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CYP 2B10, CYP 2D9, CYP 2E1, and CYP 1A2. This study, however, did not comprehensively characterize biochemical changes in these P450 enzymes.

For this technology to gain acceptance in mainstream drug development, it is essential to establish that changes in gene transcription (mRNA) correlate with the more conventionally observed changes in protein levels and enzyme activity levels. This type of correlative analysis is critical because there are instances in which genomic and proteomic data could not be reconciled (9,10). A review of the existing literature suggests that a simultaneous correlative analysis of three key components of the effect of a compound, i.e., changes in mRNA, protein, and enzyme activity, as a time course in an *in vivo* model has not been reported. The goal of this paper is twofold: to report the time course and magnitude of changes in expression (mRNA and protein) and corresponding enzyme activity and to test whether increased sensitivity and selectivity can be achieved by monitoring specific gene expression changes using qRT-PCR, thus allowing possible reduction in the duration of the studies. To allow for comparison with literature data generated by different investigators and within our organization, known inducers were used as model compounds in this study at doses that would be expected to elicit maximal responses (11).

MATERIALS AND METHODS

Animal Model

This study was conducted in an AAALAC-accredited facility in compliance with the NRC "Guide for the Care and Use of Laboratory Animals" and the Animal Welfare Act. Male Sprague-Dawley rats (Crl:CD^R[SD]IGS BR VAF/Plus^R) from Charles River Laboratories approximately 6 weeks old were randomly assigned to groups according to body weight. These animals were housed individually in suspended stainless-steel cages and allowed access to tap water and Certified Rodent LabDiet^R 5002 (block) (PMI Nutrition International, Inc.) *ad libitum.*

Study Design

Table I summarizes the design of this study. Test articles were prepared as suspensions in 0.4% (w/v) aqueous methylcellulose or corn oil. Doses for β-naphthoflavone (BNF; CYP 1A inducer), dexamethasone (DEX; CYP 3A inducer), and clofibrate (CLO; CYP 4A inducer) were selected from a previously published study (11) at levels reported to yield strong induction responses. Doses for an experimental Schering-Plough compound (designated SCH; a CYP 2B inducer) were based on previous Schering-Plough studies that demonstrated strong 2B1/2B2 induction (unpublished data). SCH and CLO were administered orally (gavage). DEX and BNF were administered by intraperitoneal injection. All animals, excluding untreated controls, were dosed once daily for up to 8 days. Each daily dosing (day 0 was the first day of dosing) was performed at approximately the same time each day (±30 min).

Dose volumes administered to each animal were calculated based on the most recent individual body weight data. All rats were dosed up to and including the day of sacrifice. Rats were sacrificed (approximately 1 hour post-dose) after 12 h, and 1, 2, 4 and 8 days of exposure to the respective test compounds. These sampling time points were selected non-empirically and spread out over the 8-day dosing period in the absence of any *a priori* knowledge of expected changes.

Table I. Dosing of Model Inducers of Cytochrome P450 Enzymes

Test/control article[a]	Dose route	Number[d]	Dose (mg/kg)	Dose conc. (mg/ml)[e]
Control (untreated)	–	3	–	–
Control (0.4% aqueous methylcellulose)[b]	PO	15	0	0
Control (corn oil)[c]	IP	15	0	0
β-Naphthoflavone	IP	15	40	8
Dexamethasone	IP	15	50	10
Schering-Plough compound	PO	15	300	60
Clofibrate	PO	15	250	50

[a] Time points 0.5, 1, 2, 4, and 8 days with vehicle controls for each time point.
[b] Vehicle for oral (PO) dosing.
[c] Vehicle for intraperitoneal (IP) dosing.
[d] n = 3 rats per time point for qRT-PCR, Western blots, and enzyme assays.
[e] Dose volume was 5 ml for all treated rats.

Postmortem Observations

Rats were sacrificed by exsanguination during isoflurane-induced anesthesia. Tissue samples were collected from the untreated control group at the conclusion of the final necropsy. At each sacrifice time point, up to three rats per group per time point were sacrificed 1 h postdose. Liver tissue was divided so that gene expression, Western blots, and enzyme activity assays could be compared. Liver tissues to be tested by enzyme activity assays were fresh frozen in liquid nitrogen. Tissues for gene expression assays were placed in approximately 5 volumes of RNA Later™ (Ambion) an RNA-stabilizing solution, and then stored in a freezer set to maintain −80°C.

RNA Isolation and Conversion to cDNA

Total RNA was isolated with the Absolutely RNA™ (Stratagene) miniprep kit, which included DNAse treatment to remove genomic DNA. The concentration of the isolated total RNA was determined by measuring the optical density at A_{260} nm. Total RNA (2 μg/100 μl) was converted to cDNA with the RT-PCR Miniprep Kit (Applied Biosystems).

Real-Time qRT-PCR Analysis of Gene Expression

Primers and probes (Table II) were designed with the Primer Express software (Applied Biosystems) for the detection of rat CYP sequences for 1A1, 1A2, 2B1/2B2 (primers and probe are located in a region of sequence identity between the two genes), 3A1, 4A1, and for rat 18S ribosomal RNA. The location and specific sequences of the CYP primers and probes were chosen to exclude the detection of genomic DNA by placing one of the primers over a junction between two exons. Although the 18S primers and probe can

Table II. Probe and Primer Sequences Used for Real-Time Quantitative RT-PCR

Gene name location	Primer	Sequence (5′ to 3′)[a]	Specificity
18S RNA (GenBank® V01270)	Forward primer	AGTCCCTGCCCTTTGTACACA	
	Reverse primer	GCCTCACTAAACCATCCAATCG	
	Probe	VIC-CGCCCGTCGCTAC-MGBNFQ	
CYP 1A1 (GenBank® X00469)	Forward primer	AATCAAAGAGCACTACAGGACATTTG	13 mismatches from CYP 1A2 (K02422)
	Reverse primer	CAATGCTCAATGAGGCTGTCTG	9 mismatches from CYP 1A2 (K02422)
Exon 1/2 boundary	Probe	6FAM-AAGGGCCACATCCG-MGBNFQ	6 mismatches from CYP 1A2 (K02422)
CYP 1A2 (GenBank® K02422)	Forward primer	ATAACTTTGTGCTGTCTCTGCAGAAA	14 mismatches from CYP 1A1 (X00469)
	Reverse primer	GATGTCCTGGATACTGTTCTTGTTGA	9 mismatches from CYP 1A1 (X00469)
Exon 1/2 boundary	Probe	6FAM-AGTCCAGGAACACTATC-MGBNFQ	6 mismatches from CYP 1A1 (X00469)
CYP 2B1/2B2 (GenBank® J00719 and M34452)	Forward primer	GAGCTGTTCTACCGGACCTTTTC	Identical to CYP 2B2 (M34452)
	Reverse primer	AGTATTTCAGGAACCCAGAGAAGAACT	Identical to CYP 2B2 (M34452)
Exon 4/5 boundary	Probe	6FAM-TCCTAAGTTCATTCTCC-MGBNFQ	Identical to CYP 2B2 (M34452)
CYP 3A1 (GenBank® M10161)	Forward primer	GATTCTGTGCAGAAGCATCGAGT	4 mismatches from CYP 3A2 (U09742)
	Reverse primer	ATAGGGCTGTATGAGATTCTTTGTCTT	5 mismatches from CYP 3A2 (U09742)
Exon 8/9 boundary	Probe	6FAM-TCAGCTGATGATGAATG-MGBNFQ	3 mismatches from CYP 3A2 (U09742)
CYP 4A1 (GenBank® M57718)	Forward primer	GTTCAGGTGGATGGAAATTACAAGA	6 mismatches from CYP 4A2 (M57719)
	Reverse primer	TTGGAAGAAAAATTATAGATGGTATCATTC	11 mismatches from CYP 4A2 (M57719)
Exon 5/6 boundary	Probe	6FAM-ATTGGGAACTTGAATGAC-MGBNFQ	8 mismatches from CYP 4A2 (M57719)

[a] MGBNFQ, minor groove binder nonfluorescent quencher.

detect genomic DNA, these levels are more than five orders of magnitude less than the detected levels of 18S RNA (data not shown). Table II also lists the number of mismatches that each probe or primer has with an alternate target.

Quantitative RT-PCR reactions were performed using the reagents and protocol contained in the TaqMan® Universal PCR Master Mix (Applied Biosystems). Primers were used at a concentration of 900 nM. Probes were used at a concentration of 250 nM. Reactions were run in a volume of 25 μl, containing 30 ng of cDNA (based on the RNA concentration added to the reverse transcription reaction). Real-time PCR data were collected and analyzed on an Applied Biosystems 7700 Sequence Detection System instrument.

PCR efficiency for each of these amplicons was determined from a plot of cycle threshold (C_T) as a function of template concentration over a four-log range. The slope of each plot was used to calculate PCR efficiency (E) according to the equation: $N = N_0 \times E^n$. N is the concentration of PCR product molecules formed during amplification, N_0 is the initial concentration of molecules, n is the number of PCR cycles, and E is the efficiency of amplification. Ideally PCR efficiency is 2: two copies of template are being generated for each round of PCR. This equation can be transformed into: $n = (-1/\log E)(\log N_0) + (\log N/\log E)$. The slope of the ($C_T$) as a function of template concentration plot therefore is (−1/logE). The PCR efficiencies for the amplicons used in this study ranged from 1.81 to 2.00, compared to an ideal value of 2. In sum, the amplicons used in this study displayed PCR efficiencies of >90% (expressed as a percentage of 2).

Real-time PCR data were analyzed using the comparative C_T method as described in the instructions of the ABI PRISM 7700 User Bulletin #2 (P/N 4303859) from Applied Biosystems (12). The amount of each gene target in treated and control groups was normalized to an endogenous control (18S ribosomal RNA), the expression of which is proportional to the total RNA in the sample. Fold changes for treatment groups compared to control were calculated using the following equation:

$$\text{fold change} = 2^{-\Delta\Delta C_T}$$

$$\text{where, } \Delta\Delta C_T = \Delta C_{T(\text{treated})} - \Delta C_{T(\text{control})}$$

$$\Delta C_{T(\text{treated})} = C_{T(\text{CYP})} - C_{T(18S)} \text{ (rats dosed with model inducers).}$$

$$\Delta C_{T(\text{control})} = C_{T(\text{CYP})} - C_{T(18S)} \text{ (rats dosed with vehicle)}$$

p values were calculated for a two-tailed, two-sample equal-variance *t* test on $\Delta C_{T(\text{treated})}$ and $\Delta C_{T(\text{control})}$ for animals in each group and are reported on the graphs for $p < 0.05$.

Isolation of Rat Hepatic Microsomes

Liver specimens were homogenized in 1 mM EDTA, 250 mM sucrose, and 50 mM Tris-acetate buffer, pH 7.4. The liver homogenate was centrifuged at 4°C for 20 min at 10,000 × *g*. The microsomes were obtained by ultracentrifugation of the 10,000 × *g* supernatant at 101,000 × *g* for 60 min at 4°C. The isolated microsomes were resuspended in fresh 1 mM EDTA, 250 mM sucrose, and 50 mM Tris-acetate buffer, pH 7.4, followed by ultracentrifugation under the same conditions. The washed microsomal pellets were resuspended in 20% glycerol, 1 mM EDTA, and 50 mM Tris-acetate buffer pH 7.4. The resuspended microsomes were aliquoted and stored at −70°C until analysis. Hepatic microsomal protein was determined by Peterson's modification (13) of the Lowry method (14).

Enzyme Activity Assays

Alkoxyresorufin O-dealkylation reactions (EROD and PROD) were used to quantify the activity of rodent CYP 1A and CYP 2B isoforms (15,16). EROD (7-ethoxyresorufin) and PROD (7-pentoxyresorufin) activities were determined in 200-μl incubation mixtures contained in a 96-well plate containing 100 mM KPO_4 buffer, pH 7.4, 0.05 to 0.2 mg microsomal protein, and 1.5 μM of either 7-ethoxyresorufin or 7-pentoxyresorufin and 1 mM NADPH final concentrations. The mixtures were incubated at 37°C for 10 to 20 min and scanned in a microtiter plate fluorescent reader. The amount of resorufin formed from either ethoxy- or pentoxyresorufin

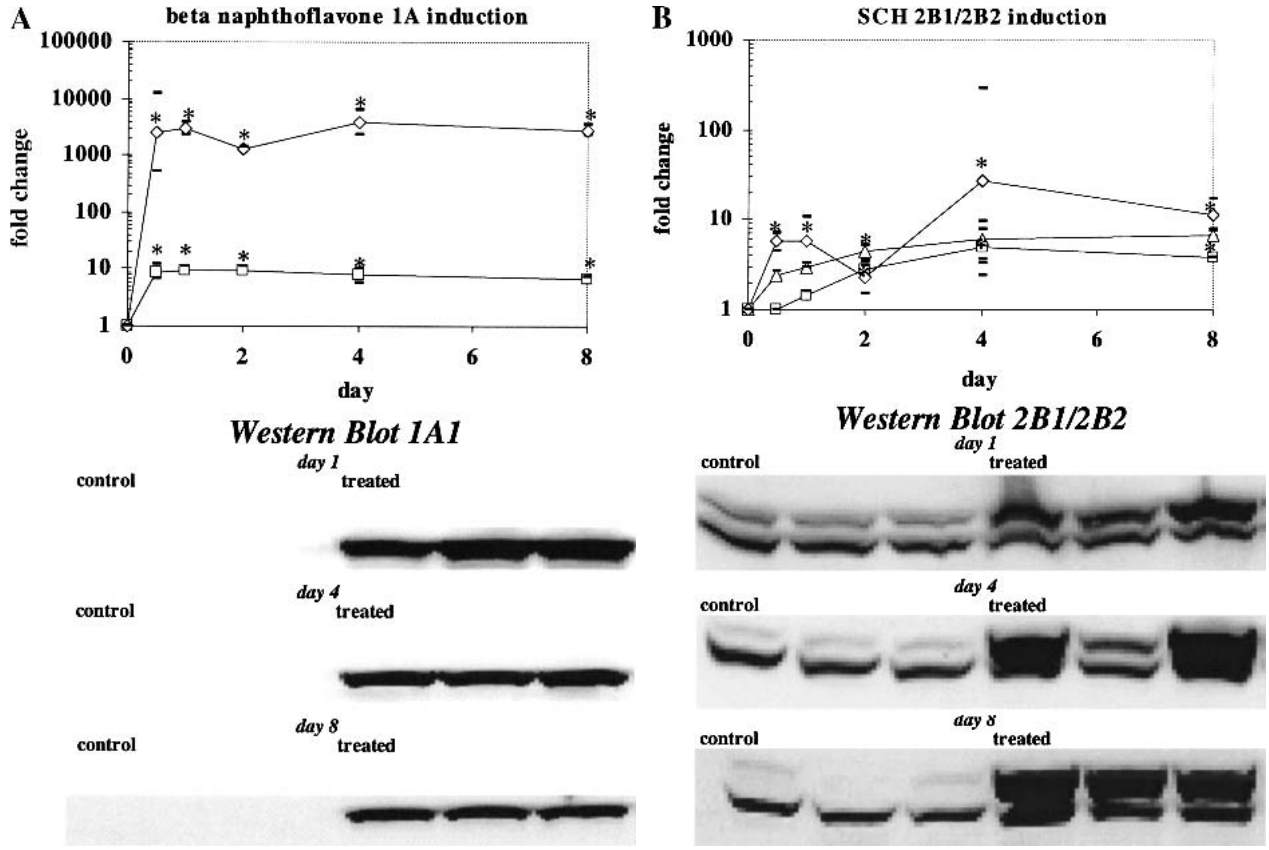

Fig. 1. Time course for fold changes in gene expression, Western blotting, and enzyme activity for the model inducers tested. This figure shows the average and standard error for the individual results corresponding to liver tissue isolated from three rats in each group. Photographs of the original Western blot strips for each individual animal are shown for days 1, 4, and 8. A, β-Naphthoflavone CYP 1A induction. Western blot quantification is not defined for this induction because baseline levels for CYP 1A1 are not detectable by Western blotting. The qRT-PCR data are from the CYP 1A1 specific probe and primer set. B, SCH, CYP 2B1/2B2 induction. For the activity assay (□), treated samples from days 2, 4, and 8 are significantly induced ($p < 0.05$) compared to the vehicle controls. For the qRT-PCR (◇), treated samples for days 0.5 through 8 are significantly induced ($p < 0.05$) compared to the vehicle controls. C, dexamethasone CYP 3A induction. D, clofibrate CYP 4A induction. Symbols: diamond, qRT-PCR; triangle, Western blot; square, activity assay. *$p < 0.05$.

was quantified using a microtiter plate fluorescence reader set at excitation λ 530 nm and emission λ 590 nm. Resorufin formation in samples was calculated by constructing a standard curve with known amounts of resorufin vs. intensity of fluorescence. The limit of quantitation of resorufin was 10 nM.

Testosterone 6β-hydroxylation by CYP 3A was measured by incubating testosterone at 100 μM with 0.25 mg/ml microsomal protein and 1 mM NADPH. The reaction was stopped by the addition of 100 μl of ice-cold methanol containing internal standard corticosterone (1 μg/ml). The reaction was stopped by the addition of 100 μl of ice-cold methanol and precipitation of the denatured proteins by centrifugation. The supernatant was analyzed for 6β-OH testosterone by LC-MS/MS using a Supelco Supelcosil LC-18 column (5 μm, 150 × 4.6 mm, Bellefonte, PA) with isocratic elution (34% MeOH + 57% H_2O + 9% THF). The m/z transition from 305.3 to 269 was monitored to quantify 6β-OH testosterone. The m/z transition from 347 to 329 was monitored to quantify corticosterone (Internal Standard).

Lauric acid ω-hydroxylation by CYP 4A was measured by incubating 0.1 to 0.2 mg of rat liver microsomes for 10 min at 37°C with 50 μM lauric acid in 200 μl of a 100 mM potassium phosphate buffer, pH 7.4, in the presence of 1 mM NADPH. The reaction was stopped by the addition of 100 μl of methanol containing DL-2-OH myristic acid (20 μg/mL) as internal standard. Following centrifugation to precipitate the protein, the supernatant was quantified for ω-hydroxylated lauric acid metabolite by LC-MS/MS using a MACMOD hydrobond AQ column (5 μm, 4.6 × 50, Chadds Ford, PA) with gradient elution. Two solvent mixtures were used for the gradient elution of 12-OH lauric acid. Solvent mixture A (SMA) consisted of 80% of 0.05% NH_4OH in H_2O and 20% ACN, and solvent mixture B (SMB) consisted of 80% ACN and 20% of 0.05% acetic acid in H_2O. The initial conditions were 100% SMA, which was held for 0.5 min after sample injection and then changed to 70% SMA and held for 0.5 min. Afterward, the solvent mixture was changed to 100% SMB and held for another 1.5 min and then switched back to the initial conditions for column equilibration. The m/z transition from 215.1 to 169.2 was monitored to quantify 12-OH lauric acid. The m/z transition from 243.5 to 197.2 was monitored to quantify DL-2-OH-myristic acid (Internal Standard).

The CYP 3A and CYP 4A activity assay products (6β-OH testosterone and ω-hydroxylated lauric acid) were monitored using a PE SCIEX 3000 triple quadrupole mass spectrometer (Ontario, Canada) with a Shimadzu LC-10ADvp pump (Columbia, MD) and a Leap Technology multiplate

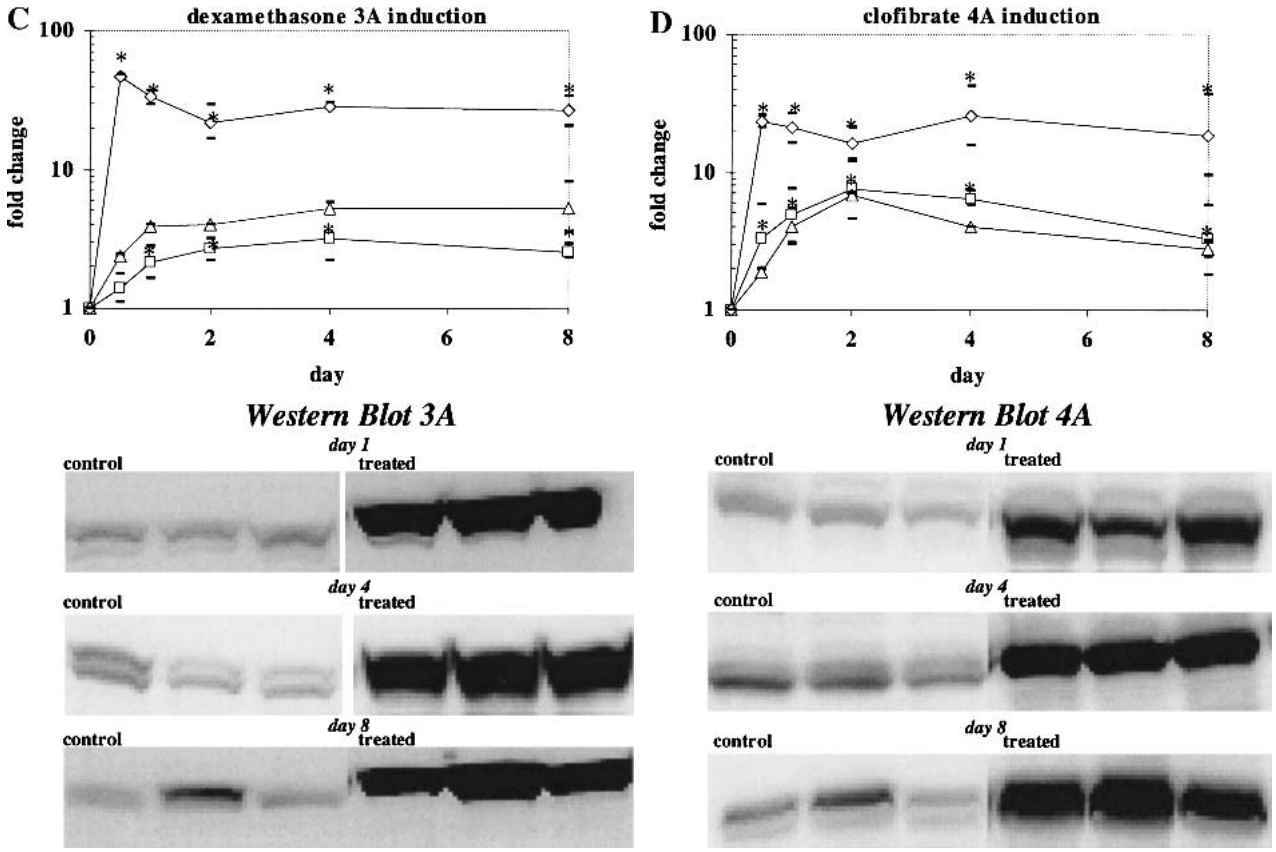

Fig. 1. Continued.

injector autosampler (Carrboro, NC). The mass spectrometer was operated under the multiple reaction monitoring (MRM) mode. The column elute was transferred via PEEK tubing (0.005″ ID, Upchurch Scientific, Oak Harbor, WA) through a Valco diverter valve to the ionization chamber of the mass spectrometer. The Valco diverter valve was used to minimize the contamination of the ion source by the buffer present in the matrix. A heated nebulizer was used for both assays because it has a higher tolerance for buffer contents and less tendency for signal suppression than electrospray. The flow rates for both assays were 1 ml/min, and the injection-to-injection cycle time was 5 min. For each assay, a calibration curve was constructed using linear regression. The concentration of the analyte was calculated by the SCIEX MacQuan program according to the peak area ratio between each analyte and an internal standard. 6β-OH Testosterone was detected at a concentration as low as 200 nM, and 12-OH lauric acid was detected at a concentration as low as 47 nM (in each case this was the lowest standard used for the assay).

All enzyme activity assays were conducted in triplicate, and the %CV values were less than 20%. p values were calculated for a two-tailed, two-sample equal-variance t test on enzyme activity results for treated and control animals in each group and are reported on the graphs for $p < 0.05$. Activities were determined as picomoles of metabolite formed per minute per milligram microsomal protein. The activities of hepatic microsomes isolated from drug-treated rats were compared to vehicle-treated microsomal activities (controls), and results expressed as a percentage of control.

CYP 1A, 2B, 3A, and 4A assays were validated by measuring activities using β-naphthoflavone-, Phenobarbital-, dexamethasone-, and clofibrate-induced microsomes purchased from Xenotech LLC (Kansas City, KS). These served as positive control markers for the various assays. Phenobarbital-induced microsomes produced between 15- and 30-fold induction of CYP 2B, and β-naphthoflavone, dexamethasone-, and clofibrate-induced microsomes produced 7- to 10-fold induction of CYP 1A, CYP 3A, and CYP 4A activities, respectively.

Western Blots

Anti-rat CYP (CYP 1A1, CYP 1A2, CYP 2B1/2, CYP 3A, and CYP 4A) antibodies were purchased from Xenotech LLC. Microsomal protein was resolved on a 10% polyacrylamide minigel in the presence of sodium dodecylsulfate (SDS) (17) obtained from BioRad Laboratories. The resolved proteins were electrophoretically transferred onto a nitrocellulose membrane (18). The nitrocellulose membrane was incubated sequentially with the primary antibodies, followed by incubation with a biotinylated antirabbit secondary antibody (for CYP 1A, CYP 2B, CYP 3A) or antigoat secondary antibody (for CYP 4A), and finally with streptavidin–horseradish peroxidase complex. CYP bands were detected by luminol chemiluminescence detection (Amersham).

RESULTS

Animal Dosing

Rats were dosed daily for eight consecutive days with either β-naphthoflavone (BNF; CYP 1A inducer), an experi-

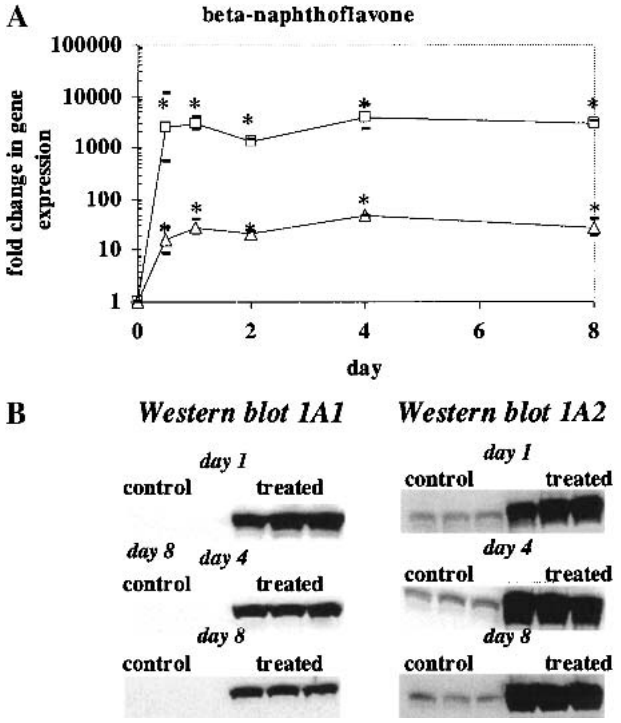

Fig. 2. Time course of gene expression and Western blots for CYP 1A1 and CYP 1A2 subtypes following treatment with β-naphthoflavone. This figure shows the average and standard error for the individual results corresponding to liver tissue isolated from three rats in each group. A, qRT-PCR of CYP1A1 (□), CYP1A2 (△). *$p < 0.05$. B, Western blots of CYP 1A1 and CYP 1A2. Photographs of the original Western blot strips for each individual animal are shown for days 1, 4, and 8.

mental Schering-Plough compound (designated SCH; a CYP 2B inducer), dexamethasone (DEX; CYP 3A inducer), clofibrate (CLO; CYP 4A inducer), and corresponding vehicle controls. Liver samples were collected after 0.5, 1, 2, 4, and 8 days of dosing.

Basal CYP levels by qRT-PCR and Western blots

CYP gene expression levels in livers from control rats were measured by qRT-PCR. A broad range of expression was observed in uninduced animals (Table III). For example, the basal expression of CYP 1A1 was lower than those of the other CYP genes listed in Table III. In general, typical C_T values range up to 40, with a higher C_T value corresponding to a more dilute target. Relative copy numbers for CYP genes were estimated based on an internal benchmark C_T of 38 corresponding to one copy of the target. In our studies, a C_T of 8 was consistently experimentally observed for 18S RNA. The difference of 30 in C_T values was used to estimate 10^9 copies of 18S RNA; each C_T ideally represents a twofold increase in transcript ($2^{30} = 10^9$ copies). The estimates for CYP copy numbers in Table III were based on the approximate CYP C_T values measured in this study for the untreated samples, and the benchmark C_T of 38 corresponding to one copy. The PCR efficiency for each of the CYP targets and 18S RNA was determined as described in the Materials and Methods section and found to be close to the theoretical value of 2 (data not shown).

Table III. Gene Expression Levels in Control Rats by Quantitative RT-PCR

Rat CYP gene	Baseline C_T (approx.)	Estimated relative number of copies of mRNA per 10^9 copies of 18S RNA[a]
1A1	31	100
1A2	21	100,000
2B1/2B2	24	10,000
3A1	21	100,000
4A1	25	5,000

[a] These estimates are based on approximate values for the C_T measured for the untreated samples and a determination of >90% PCR efficiency for all targets (data not shown). See Materials and Methods and Results for details.

Basal levels of CYP 2B1/2B2, CYP 3A, CYP 4A (Fig. 1B–D), and CYP 1A2 (Fig. 2B) proteins were detected by Western blot in livers of control animals; however, CYP 1A1 was not detected. It is noteworthy that qRT-PCR, in contrast, was able to detect the basal expression level of CYP 1A1 (C_T of 31, Table III). These data provide evidence that the qRT-PCR technique is more sensitive than the CYP1A1 Western blot.

Induction Changes following Treatment with Model Inducers

Quantitative RT-PCR, Western blot, and enzyme activity measurements, as a time course of CYP induction in liver by the model inducers, are presented in Fig. 1. To measure the statistical significance of qRT-PCR and enzyme activity signals from treated rats relative to vehicle control rats, p values were calculated for a two-tailed, two-sample equal-variance t test and are reported on the graphs for $p < 0.05$. For CYP 1A1, fold changes by Western blots could not be calculated because the Western blot was not able to detect CYP 1A1 in uninduced control livers. In general, as shown in Fig. 1, the fold change (dynamic range) of CYP induction as measured by qRT-PCR was consistently larger than such changes measured by Western blots or by enzyme activity assays (Fig. 1A,C,D). Induction of CYP 1A, CYP 3A, and CYP 4A genes measured by qRT-PCR ranged from 20-fold to as high as ~3000-fold. Induction changes in CYP 2B, in contrast, were smaller, with most changes being ≤10-fold.

Of the three techniques, qRT-PCR was the only one that consistently detected induction at the earliest time point (0.5 days), although in some instances either Western blots or enzyme activities also showed induction (Figs. 1 and 2). Induction of CYP 1A1/2, CYP 3A, and CYP 4A as detected by qRT-PCR reached maximal persistent levels early (0.5 days) and remained elevated throughout the 8-day time course. In contrast, induction changes in these genes as detected by Western blots and activity assays increased at a slower rate and reached maximal levels by day 2 to day 4 (Fig. 1A,C,D; Fig. 2B). For CYP 2B1/2B2, the profile of induction as measured by qRT-PCR was different from those obtained for the other CYP genes. Maximal changes occurred at 4 days. The profiles for the Western blot and enzyme assay (Fig. 1B), however, were similar to those for the other CYP genes (Fig. 1A,C,D; Fig. 2B).

All model inducers demonstrated induction changes of target P450s. Expression levels for all P450 gene targets were measured for each inducer. In addition to induction by BNF, CYP 1A1 was also induced by dexamethasone (not considered to be a model inducer of CYP 1A) as measured by qRT-PCR. Maximal gene induction changes occurred by day 1 for CYP 1A1 (Fig. 3A). No induction was observed for CYP 1A2 (Fig. 3). The magnitude of CYP 1A1 induction measured by qRT-PCR with dexamethasone (~10 to 100-fold; Fig. 3A), however, was not as large as obtained with BNF (1000- to 3000-fold; Fig. 2A). Induction changes in protein levels for CYP 1A1 were also detected by Western blot (Fig. 3B, day 1).

DISCUSSION

Currently, one of the most important limiting factors for the use of qRT-PCR to monitor changes in gene expression is the absence of data showing a clear correlation between qRT-PCR and conventional assays over a time course in an animal model. In this study, we have demonstrated that over the entire time course (0.5 to 8 days) persistent and larger fold changes in CYP induction could be measured by qRT-PCR, with changes either preceding or occurring simultaneously with changes in protein levels by conventional methods. In most cases, qRT-PCR was more sensitive than the conventional techniques with a large dynamic range (3 orders of magnitude for CYP 1A1). For example, basal expression of CYP 1A1 could be readily measured by qRT-PCR but not by Western blots.

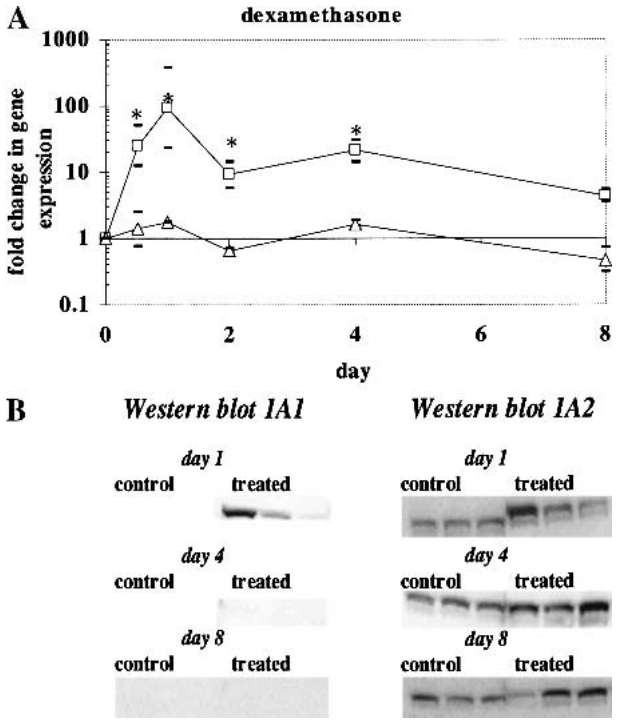

Fig. 3. Time course of gene expression and Western blots for CYP 1A1 and CYP 1A2 subtypes following treatment with dexamethasone. This figure shows the average and standard error for the individual results corresponding to liver tissue isolated from three rats in each group. A, qRT-PCR of CYP 1A1 (□) and CYP 1A2 (△). *$p <$ 0.05. B, Western blots of CYP 1A1 and CYP 1A2. Photographs of the original Western blot strips for each individual animal are shown for days 1, 4, and 8.

The dynamic range of Western blots and activity assays was only 1 order of magnitude in our studies using optimal doses of model inducers to elicit maximal responses. This larger dynamic range is most likely related to the ability of qRT-PCR to consistently measure low basal levels of CYP genes. qRT-PCR has a wide dynamic range, as can be shown by diluting a standard as is conventionally done to determine PCR efficiency. For example, from a C_T of 8 to a C_T of 38, there is a change of 2^{30} in copy number. However, not all biologic systems will exhibit changes in gene expression over such a wide range.

A significant finding of this study was that maximal changes in gene expression in the time course occurred by the first time point evaluated (0.5 days). This may have been a result of the high doses of model inducers intentionally used in this study to elicit maximal changes in protein levels for Western blot and enzyme activity analyses. We did not expect to see a point-by-point match in the shape of the induction profile or an exact match in magnitude of signal among the three techniques because they measure different entities. Hence, further investigation of lower doses and earlier time points may better characterize CYP induction profiles and determine the lower limit of sensitivity for qRT-PCR using samples from an *in vivo* model system.

An assay with enhanced sensitivity could potentially provide early identification of drugs that show induction by conventional methods only after a long period of exposure, i.e., show no induction in an 8-day dosing regimen but do show induction after 30-days of dosing. The quantitative gene expression methodology could potentially find utility as a key confirmatory tool in studies where a conventional analysis (Western blot and/or activity assay) does not provide a clearly discernible result. For example, an ambiguous marginal change observed after 8-days by conventional techniques may likely be readily resolved as a real change by monitoring gene expression changes

The real value of this technique (qRT-PCR) lies in its ability to preempt potential induction issues that may arise later in toxicology studies because typical early induction studies in discovery are conducted at lower doses. At lower doses, potential issues may not become evident based on marginal changes using conventional assays (Western blots and activity assays) but could readily be ascertained as real induction changes by qRT-PCR.

In addition to the improved sensitivity, quantitative RT-PCR may also provide enhanced specificity over conventional techniques such as Western blots, enzyme assays, and gene reporter assays. An advantage of qRT-PCR is that specific primers and probes can be designed to distinguish between different CYP subtypes. Achieving such subtype-specific CYP specificity can often be difficult with antibodies and enzyme substrates. For example, qRT-PCR for CYP 1A1 and CYP 1A2 demonstrate the specificity of qRT-PCR. BNF induced a several-thousandfold increase in CYP 1A1 gene expression but only a ~50-fold increase in CYP 1A2 gene expression. Also, DEX induced a ~10 fold increase in CYP 1A1 gene expression but did not induce CYP 1A2.

Another positive feature of the qRT-PCR methodology is that assay development can be quite rapid if the DNA sequence of a gene of interest is known. The fast turnaround of qRT-PCR assay development would be helpful in the retrospective analysis of samples from toxicology studies. Such

retrospective needs are commonly encountered in drug development programs and are often suggested and accepted by regulatory agencies as supportive data.

The most powerful impact of rapid, sensitive, specific, and accurate enzyme induction measurements such as these on drug development timelines is in the development of rapid high-throughput screening methods for candidate prioritization. There have been several recent publications [for a review see Worboys and Carlile (19)] showing the application of quantitative gene expression to the measurement of CYP enzyme induction in stable cell lines (20), primary hepatocytes (6,7,21), and liver slices (22). Interestingly, fold changes reported in these studies are usually lower than those observed in our *in vivo* study. The results shown here demonstrate the *in vivo* correlation between quantitative gene expression and currently accepted methods for assessing CYP induction. This work complements a previous study, which reported a correlation between mRNA expression and enzyme activity in human liver for several P450s (CYP 1A1, CYP 1A2, CYP 3A4, CYP 2D6, and CYP 2B6) in the absence of induction (23).

Although high-throughput testing for CYP induction in cells or liver slices is likely to become the standard early in the drug development process, confirmation of CYP induction in the rat model is also likely to continue to be applied at later stages in development where sufficient compound is available for this purpose. Testing for induced RNA levels in the rat model will help validate the predictions from *in vitro* high-throughput induction screens. Quantitative gene expression measurements of CYP induction such as those presented here provide a standard method with which induction results from a high-throughput format can be directly compared to those obtained from an *in vivo* model.

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