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Analysis of Rat Cytochrome P450 Isoenzyme Expression Using Semi-Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT–PCR)

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ABSTRACT. A method was developed using reverse transcriptase-polymerase chain reaction (RT–PCR) to selectively detect and qualitatively determine the levels of mRNA expression of the major isoenzymes of cytochrome P450 (P450 1A1, 1A2, 2B1/2, 2C11, 2E1, 3A1, 3A2, and 4A1) and fatty acyl-CoA oxidase (FACO) in the rat. Total liver RNA was isolated from male Sprague–Dawley rats treated with various inducers of cytochrome P450 (P450) and analyzed for the presence and relative quantities of each P450 isoenzyme mRNA using this technique. The specificity of the oligonucleotide primers used in the detection of each P450 mRNA was tested and confirmed through the simultaneous analysis of liver microsomal protein preparations for the presence of constitutive or inducible P450 apoprotein and enzyme activities using western immunoblotting and specific enzyme activity measures, respectively. This method of P450 expression analysis is proven to be highly specific and readily applicable for the assessment of P450 enzyme induction and down-regulation in the rat during routine toxicology studies when expression of the gene product is regulated by transcriptional activation and/or mRNA stabilization. BIOCHEM PHARMACOL 52;5:781–792, 1996.

KEY WORDS. RT–PCR; P450; rat; enzyme; liver

The P450† MFOs are a group of enzymes that are expressed predominantly within the liver, kidney, lung, and intestine of mammalian species where they play an important role in the oxidative metabolism of both endogenous and exogenous (xenobiotic) compounds. The role of the various members of this enzyme superfamily in the metabolism of drugs and chemicals, as well as their potential role in the generation of toxic metabolites and chemical-induced carcinogenesis, has been reviewed extensively [1–8].

Several approaches have been used to monitor the regulation of P450 enzymes following exposure to xenobiotics. The approach most often employed is to measure the enzymatic profiles of microsomal protein fractions using enzyme selective substrates [9–14]. Although this technique is essential for the study of substate specificities, enzyme kinetics, and metabolism of new chemical entities, there are several disadvantages that can limit the usefulness of this technique in assessing the biochemical regulation of these enzymes. Two important disadvantages to consider are that P450 enzyme activities, as opposed to quantitation of mRNA or apoprotein levels, require additional cofactors (e.g. requirement for the presence of heme) and can show non-selectivity for, or be inhibited by (e.g. ketoconazole and MET), certain chemical substrates, potentially resulting in misleading or inaccurate assessments of enzyme expression.

More recently, polyclonal and monoclonal antibodies have been generated against the various isoenzymes of P450 found in the rat, thereby allowing for a more selective and defined analysis of P450 expression [15]. This approach to monitoring changes in P450 isoenzymes has many advantages over those which monitor enzyme activity, particularly with regard to enzymes that are regulated post-translationally (e.g. CYP2E1). However, the success of this approach is dependent on the quality and availability of reagents (e.g. polyclonal versus monoclonal antibodies) and may lack the specificity for determining enzyme subtype expression (e.g. CYP3A1 and CYP3A2). Moreover, the generation of antibodies and the measurement of protein using western immunoblot analysis are both labor intensive and time consuming and cannot be readily implemented when new enzymes are identified.

With current advances in molecular biology and the

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† *Abbreviations:* P450, cytochrome P450; RT–PCR, reverse transcriptase–polymerase chain reaction; FACO, fatty acyl-CoA oxidase; MFO, mixed-function oxidase; BNF, β-naphthoflavone; PB, phenobarbital; MET, metyrapone; DEX, dexamethasone; CLO, clofibrate; CO, corn oil; mRNA, messenger ribonucleic acid; CYC, cyclophilin; cDNA, complementary DNA; EROD, ethoxyresorufin O-dealkylase; PROD, pentoxyresorufin O-dealkylase; pNPH, *p*-nitrophenol hydroxylase; LA-11-H, lauric acid-11-hydroxylase; and LA-12-H, lauric acid-12-hydroxylase.

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cloning and sequencing of many of the rat P450 genes, the expression of any P450 enzyme can be measured efficiently and selectively at the mRNA level. Using the technique known as RT–PCR, a profile of gene expression can be measured easily in any tissue. This technique is highly selective, is extremely sensitive, and can be automated for diagnostic applications.

The goal of the present study was to develop an RT–PCR method using specifically designed oligonucleotide probes that could be used to rapidly and qualitatively assess the levels of mRNA expression of the major P450 isoenzymes in the rat following exposure to drugs and chemicals. This technique is applicable in routine toxicology screening for the assessment of chemically induced changes in liver P450 enzyme expression.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats between the ages of 6 and 8 weeks and weighing 200–300 g were used in these studies. Food and water were available *ad lib*.

Chemicals and Reagents

PB, BNF, MET, DEX, CLO, and CO were purchased from the Sigma Chemical Co. (St. Louis, MO). Oligonucleotide primers were custom synthesized by Oligo Therapeutics, Inc. (Wilsonville, OR). Antibodies against specific P450 enzymes were obtained from the following sources: rabbit anti-rat CYP3A1 was purchased from Human Biologics, Inc. (Phoenix, AZ); goat anti-rat CYP4A1 was purchased from the Daiichi Pure Chemicals Co. (Tokyo, Japan) and monoclonal mouse anti-rat CYP1A1, monoclonal mouse anti-rat CYP2C11, goat anti-rat CYP2E1, and monoclonal mouse anti-rat CYP2B1 were all obtained from Oxford Biomedical Research, Inc. (Oxford, MI). Secondary antibodies (goat anti-rabbit IgG, rabbit anti-goat IgG and goat anti-mouse IgG) were all purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Administration of Chemicals and Tissue Collection

Animals were administered either PB (100 mg/kg), BNF (100 mg/kg), MET (100 mg/kg), DEX (200 mg/kg) or CLO (250 mg/kg) for 4 consecutive days via intraperitoneal injection following a dosing regimen similar to that used by Wang *et al.* [16]. Animals treated with H_2O and CO were used as controls. Two hours following the last injection (day 4), animals were killed, and the livers were removed. The livers were immediately frozen and stored at −70°.

Preparation of Total Liver RNA

Total RNA was prepared from frozen liver tissue using a modification of the method described by Xie and Rothblum [17]. Approximately 100–200 mg of liver tissue was homogenized in RNA extraction buffer [17] to isolate total RNA. The resulting RNA was reconstituted in diethylpyrocarbonate-treated water (DEPC-H_2O), quantitated spectrophotometrically at 260 nm, and adjusted to 100 μg/mL. Total RNA was stored in DEPC-H_2O at −70° until used. RNA in DEPC-H_2O can be stored for up to 1 year at −70 without any apparent degradation.

RT–PCR

For conversion of total RNA to cDNA, a 20-μL reaction mixture was prepared containing 1x reverse transcriptase (RT) buffer (Gibco BRL), 10 nM dithiothreitol, 0.5 nM dNTPs, 2.5 μM oligo $d(T)_{15}$ primer (Oligo Therapeutics, Inc.), 40 U RNasin (Promega, Madison, WI), 200 U RNase H-RT (Gibco BRL, Grand Island, NY) and 400 ng of total RNA (in DEPC-H_2O). The reaction was incubated for 1 hr at 37° followed by inactivation of the enzyme at 95° for 5 min. cDNA was stored at −20° until used.

For the PCR amplification of cDNA, a 10-μL reaction mixture was prepared containing 10x polymerase reaction buffer, 2 mM $MgCl_2$, 1 U *Taq* DNA polymerase (Perkin–Elmer Corp., Norwalk, CT), 20 ng cDNA, and a 200 nM concentration of the 5′ and 3′ specific PCR primers. Primers were designed to be selective for each isoenzyme through sequence alignments to identify non-conserved regions and to conform to the criteria of having a length of 18–24 bases and a G-C content of 50–60%.

PCR reactions were carried out in a Perkin–Elmer 9600 thermal cycler using melting, annealing, and extension cycling conditions of 94° for 30 sec, 56° for 1 min, and 72° for 1 min. All amplifications were carried out for 23 cycles. Under these conditions, all cDNA fragment amplifications were found to produce single products within a linear range of 20–26 cycles (data not shown). Amplified cDNA products were separated by PAGE using 5% native gels. Gels were stained with ethidium bromide and photographed on a transilluminator using Polaroid positive/negative film.

Preparation of Liver Microsomes

Liver microsomes were prepared from frozen livers as previously described [18]. Microsomes were resuspended in buffer containing 10 mM Tris–HCl, 1 mM EDTA, and 20% glycerol. Microsomes were snap-frozen in liquid nitrogen and stored at −80° until used. Total protein was measured using the bicinchoninic acid (BCA) method (Pierce, Rockville, IL) using BSA as a standard.

SDS–PAGE and Western Immunoblotting

Proteins were separated on 10% SDS–PAGE gels under reducing conditions and immunoblotted for detection of P450 isoenzymes using modifications of previously reported methods [19]. Proteins were loaded at 50 μg/lane and resolved under constant current (250 V) for approximately 4 hr at 2°. Proteins were transferred to nitrocellulose mem-

branes (Bio-Rad, Hercules, CA) in 15 mM Tris buffer containing 120 mM glycine and 20% (v/v) methanol. The nitrocellulose membranes were blocked with 2.5% BSA and immunoblotted for P450 isoenzymes, using primary monoclonal and polyclonal antibodies and secondary alkaline phosphatase conjugated anti-IgG. Immunoblots were developed with the Bio-Rad alkaline phosphatase substrate kit (Bio-Rad).

Quantitation of PCR Amplified cDNA Products and Western Immunoblots Using Scanning Laser Densitometry

The amount of each electrophoretically separated cDNA or microsomal protein product was quantitated densitometrically using a scanning laser densitometer (Molecular Dynamics, Sunnyvale, CA). Negative film images of gels or immunoblots were scanned directly on the densitometer, and each product was quantitated by outlining the respective bands with rectangular shaped regions. Background readings were taken from the outside perimeter of each rectangular region and subtracted prior to reading the density of each specific product band. Resulting data are reported as the O.D. of the signal detected within each rectangular region.

Microsomal Enzyme Activity Measures

The enzyme activities of liver microsomes obtained from these studies were determined in the laboratory of Dr. Andrew Parkinson (Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, Kansas City, KS). Detailed methods used in these analyses are described below. Additional enzyme activity parameters (i.e. cytochrome b_5, total cytochrome P450, and NADPH–cytochrome *c* reductase activities) were performed but are not presented as part of this investigation. All microsomal protein samples were compared to positive control microsomes with known enzymatic activities.

p-NITROPHENOL HYDROXYLATION. The hydroxylation of *p*-nitrophenol was measured spectrophotometrically as described by Koop [20]. The generation of 4-nitrocatechol was determined from the absorbance at 546 nm as based on an extinction coefficient of 9.52 mM^{-1} cm^{-1} [21]. A DW2C dual-beam spectrophotometer was used for these determinations.

EROD AND PROD ACTIVITIES. The O-dealkylation of 7-ethoxyresorufin and 7-pentoxyresorufin was measured fluorometrically with minor modifications [11] of the methods described by Burke *et al.* [9]. The amount of resorufin generated was measured fluorometrically ($\lambda_{ex} \approx 535$ nm, $\lambda_{em} \approx 585$ nm) using a Shimadzu RF 540 spectrofluorometer.

TESTOSTERONE OXIDATION. The oxidation of testosterone was determined by reverse-phase HPLC as described by Wood *et al.* [22], Sonderfan *et al.* [23] and Sonderfan and Parkinson [24] using a Shimadzu LC-6A binary gradient HPLC system (Shimadzu Scientific Instruments, Columbia, MD). Testosterone, androstenedione, 6-dehydrotestosterone and 12-hydroxytestosterone isomers (i.e. 6α-, 15β-, 6β-, 15α-, 7α-, 16α-, 16β-, 1β-, 18-, 11β-, 2α-, and 2β-hydroxytestosterone) were resolved on a Supelcosil LC-18 octyldecylsilane (C_{18}) column and quantitated by integration of peak areas using a Shimadzu C-R3A recording data processor.

LAURIC ACID HYDROXYLATION. The rate of lauric acid hydroxylation was determined by a combination of the radiometric partition method of Giera and van Lier [25] and the radiometric HPLC method of Romano *et al.* [26]. The rate of lauric acid hydroxylation was measured as the rate of conversion of [^{14}C]lauric acid to 11- and 12-hydroxylauric acid.

RESULTS

The oligonucleotide PCR primers used to amplify rat P450 1A1, 1A2, 2B1/2, 2C11, 2E1, 3A1, 3A2, and 4A1, FACO, and CYC cDNA are presented in Table 1. The housekeeping gene, CYC [27], was used to determine the constitutive level of gene transcription and to control for variations in RNA recoveries from each liver specimen. Normalization of the data was accomplished by quantitating the amount of

TABLE 1. Oligonucleotide PCR primers for rat P450 isoenzymes and FACO

P450 isoenzyme	5′ Sense primer (5′→3′)	3′ Antisense Primer (5′→3′)	Fragment size (bp)
1A1	CTGGTTCTGGATACCCAGCTG	CCTAGGGTTGGTTACCAGG	331
1A2	GTCACCTCAGGGAATGCTGTG	GTTGACAATCTTCTCCTGAGG	236
2B1/2	GAGTTCTTCTCTGGGTTCCTG	ACTGTGGGTCATGGAGAGCTG	549
2C11	CTGCTGCTGCTGAAACACGTG	GGATGACAGCGATACTATCAC	248
2E1	CTCCTCGTCATATCCATCTG	GCAGCCAATCAGAAATGTGG	473
3A1	ATCCGATATGGAGATCAC	GAAGAAGTCCTTGTCTGC	579
3A2	CGACTTGGAACCCATAGAC	GGCTTAGGGAGATTTGACATG	116
4A1	GGTGACAAAGAACTACAGC	AGAGGAGTCTTGACCTGCCAG	344
FACO	CTGTTATGATGCTGCAGACAGC	ACACAGGTTCCTCAGCACAG	403
CYC	CTTCGACATCACGGCTGATGG	CAGGACCTGTATGCTTCAGG	265

amplified cDNA products by scanning laser densitometry and calculating the ratio of the amount of each enzyme cDNA relative to the amount of CYC cDNA (i.e. the enzyme cDNA O.D.:CYC cDNA O.D.). This enzyme:CYC ratio was generated for each P450 isoenzyme and used to compare the relative amounts of P450 isoenzyme mRNA in each liver (see Materials and Methods).

Figure 1 illustrates the results obtained using RT–PCR to assess the levels of P450 mRNA in the livers of male rats exposed to various chemical inducers of P450. Results are presented in a series of graphs (see Fig. 3) that normalize the results obtained for each P450 gene (and FACO) to that of CYC (i.e. P450:CYC ratio). In the sections that follow, comparisons are made between the results obtained using RT–PCR and those obtained using western immunoblotting and substrate-specific enzyme activity for measurement of P450 expression (Figs. 2 and 3).

Analysis of CYP1A1 and CYP1A2 Expression

The expression of CYP1A1 and CYP1A2 mRNA following exposure to various inducers of P450 is presented in Figs. 1 and 3a–d. Under linear amplification conditions, expression of the CYP1A1 and CYP1A2 mRNA was not detected in the livers of H_2O- and CO-treated control rats. The expression of both genes was found to be increased markedly following exposure of rats to the prototypical CYP1A inducer, BNF. One of three rats appeared to have a slight increase in CYP1A2 expression in response to DEX in this experiment. No other treatments were found to change the expression of CYP1A1 or CYP1A2 at the mRNA level.

Figures 2 and 3c illustrate the detection of the CYP1A1 protein by western immunoblotting of microsomal protein fractions isolated from these livers. These results demonstrate close agreement in the detection of *CYP1A1* gene expression by the two techniques. CYP1A2 protein expression was not measured as part of this study. A slight increase in CYP1A1 protein was observed in the livers of the PB-treated rats (Fig. 3c). This increase in CYP1A1 expression was confirmed by an increase in microsomal EROD activity as described below (Fig. 3d). Since an increase in CYP1A1 mRNA was not detected in any of the PB-exposed animals, these data suggest that a stabilization of the CYP1A1 protein by PB may be occurring.

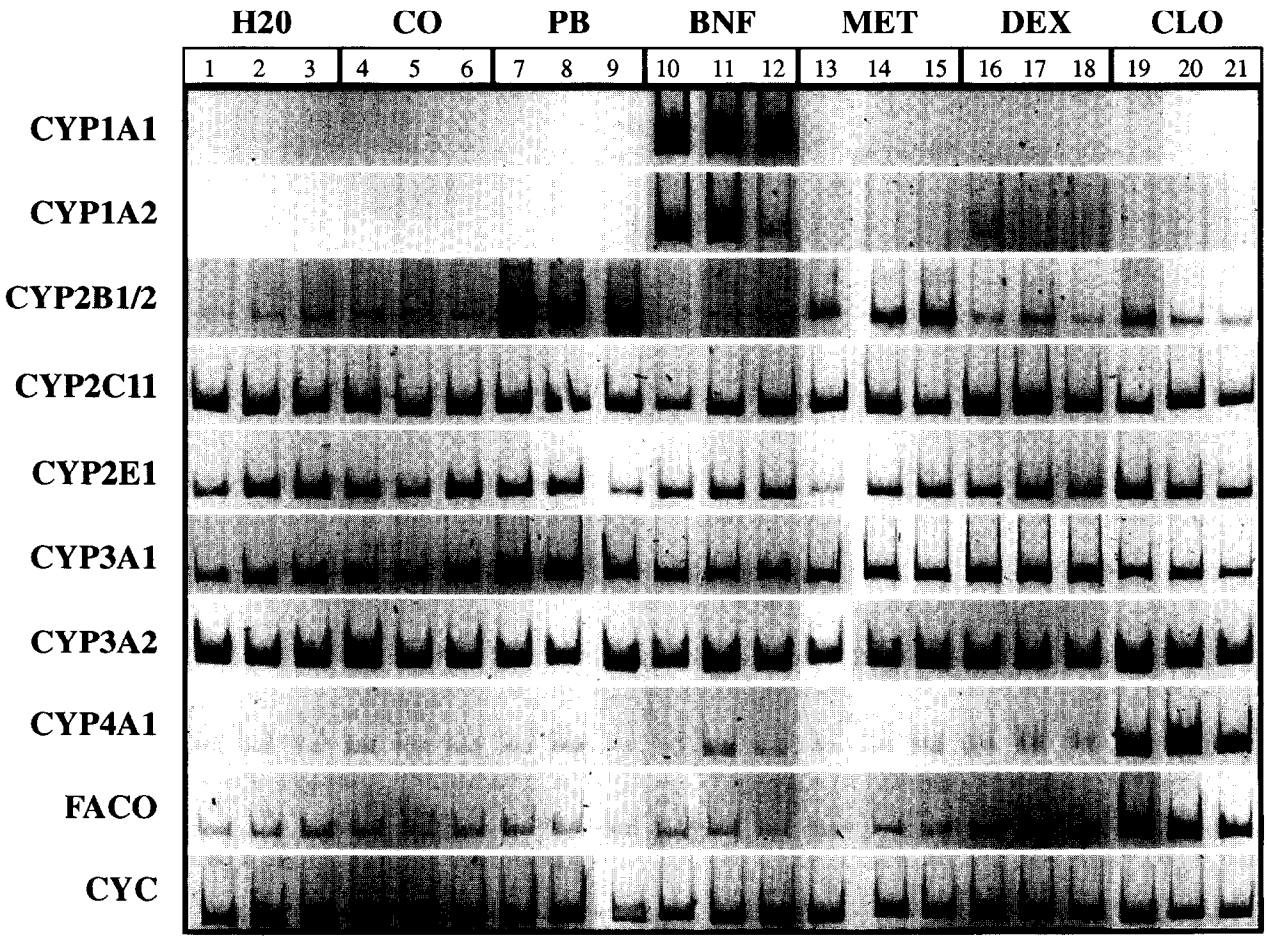


FIG. 1. Analysis of rat liver P450 expression by RT–PCR. Total liver RNA was isolated from rats exposed to vehicles, H_2O (lanes 1–3) and CO (lanes 4–6), and prototypical P450 enzyme inducers, PB (lanes 7–9), BNF (lanes 10–12), MET (lanes 13–15), DEX (lanes 16–18) and CLO (lanes 19–21), and analyzed for the expression of P450 1A1, 1A2, 2B1/2, 2C11, 2E1, 3A1, 3A2, and 4A1 and FACO using RT–PCR (N = 3 rats/treatment group). PCR products were separated on 5% native PAGE gels. The housekeeping gene, CYC, was used as a loading control.

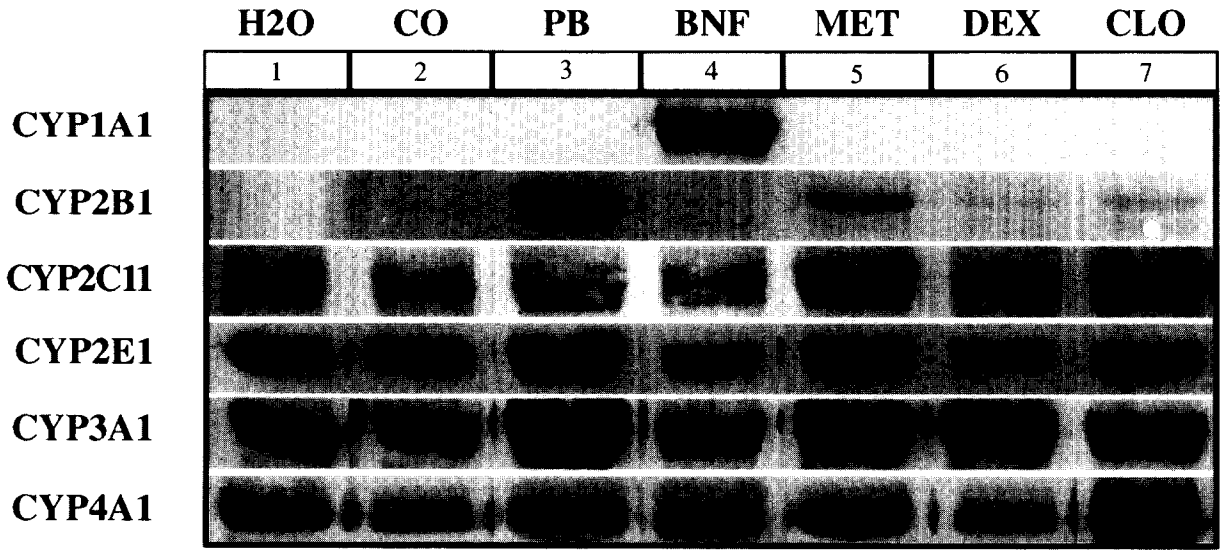


FIG. 2. Analysis of rat liver P450 expression by western immunoblotting. Microsomes were prepared from the livers of rats exposed to vehicles, H_2O (lane 1) and CO (lane 2), and prototypical P450 enzyme inducers, PB (lane 3), BNF (lane 4), MET (lane 5), DEX (lane 6) and CLO (lane 7), and analyzed for the expression of P450 1A1, 2B1, 2C11, 2E1, 3A1, and 4A1 using western immunoblotting (N = 1 of 2 rats/treatment group blotted for each enzyme). Microsomal protein (50 μg/lane) was separated on 10% SDS–PAGE under reducing conditions and transferred to nitrocellulose membranes. Membranes were immunoblotted using polyclonal or monoclonal antibodies selective for each P450 isoenzyme as described in Materials and Methods.

Results of the analysis of microsomal EROD activity in the livers of exposed rats confirmed the induction of CYP1A1 by BNF at both the mRNA and protein levels and the induction of CYP1A1 by PB at the protein level (Fig. 3d). No other chemical treatments were found to cause an increase in liver EROD activity over control levels.

Analysis of CYP2B1/2 Expression

The combined expression of CYP2B1 and CYP2B2 mRNA in male rat liver following exposure to the various P450 inducers is presented in Figs. 1 and 3e–h. These two enzymes have a 97% sequence identity and are coregulated by chemical inducers of this P450 subfamily [28–35]. The oligonucleotide primers used in this study do not distinguish between the two CYP2B isoenzymes.

CYP2B1/2 mRNA was found to be constitutively present in control (H_2O and CO treated) rat livers (Figs. 1 and 3e). Following exposure of rats to PB, a prototypical CYP2B inducer, expression of CYP2B1/2 mRNA was found to increase markedly relative to controls. MET and CLO were also found to cause an increase in the expression of CYP2B1/2 mRNA relative to their respective controls.

Similar results were obtained using western immunoblotting for expression of the CYP2B1 apoprotein in rat liver microsomal protein fractions (Fig. 3f). The CYP2B1 apoprotein was found to be constitutively expressed at a relatively low level in control rat livers and inducibly increased following exposure to PB. Likewise, MET and CLO (to a lesser extent) were also found to cause an increase in the expression of the CYP2B1 apoprotein.

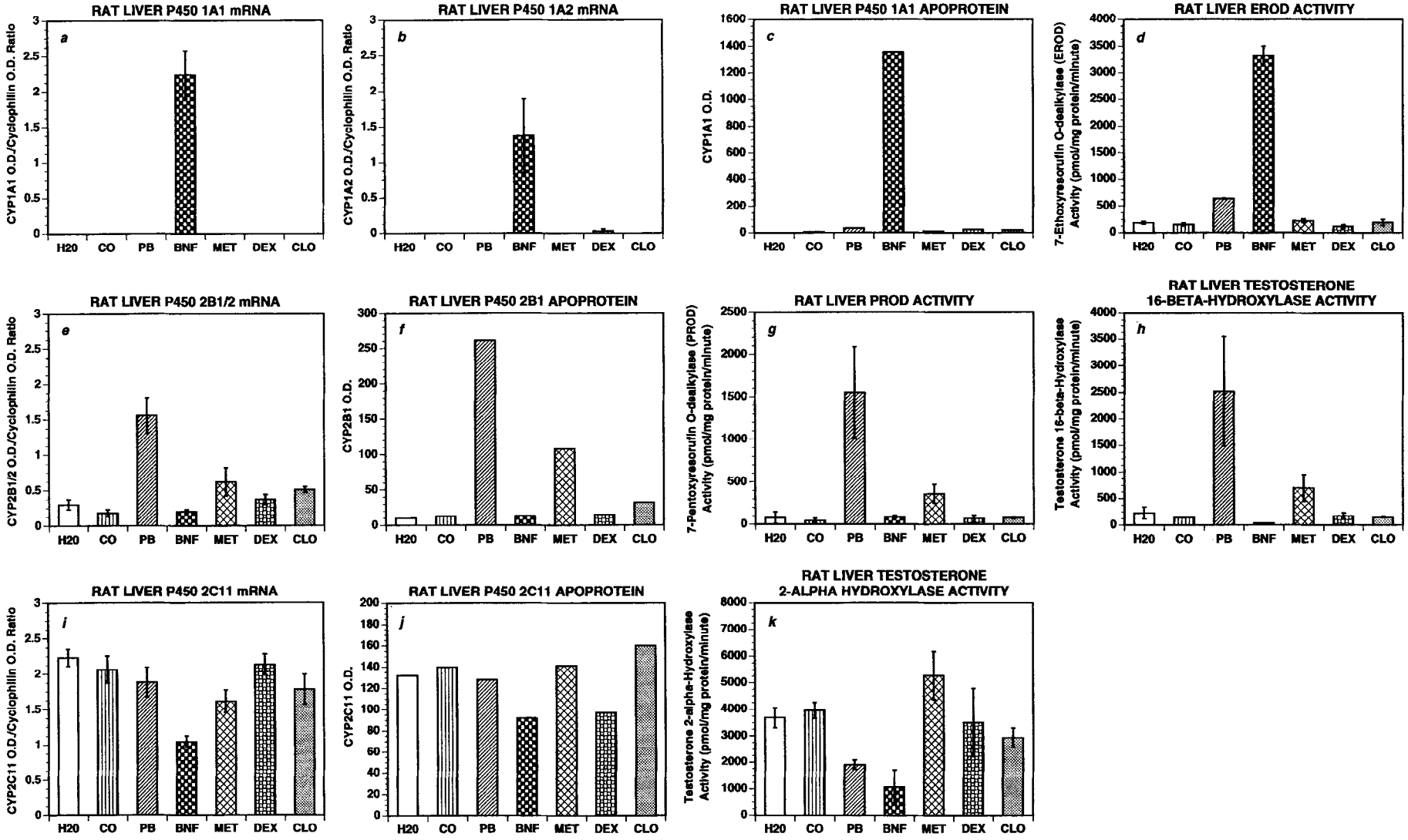
These findings were further confirmed by analysis of microsomal PROD and testosterone 16-β-hydroxylase activity in the livers of chemically exposed rats (Figs. 3g and h). Increases in PROD and testosterone 16β-hydroxylase activity were detected in both the PB- and MET-treated livers. CLO was not found to increase microsomal PROD activity in these livers. However, the induction of CYP2B at the mRNA and protein levels was found to be extremely weak and likely to be below the level of detection by enzymatic assay techniques.

Analysis of CYP2C11 Expression

High levels of mRNA expression of the adult male specific P450 isoenzyme [36, 37], CYP2C11, were detected in control (H_2O and CO) rat livers (Figs. 1 and 3i). Following exposure of rats to BNF (and MET to a lesser extent), a decreased expression of CYP2C11 mRNA was observed. None of the chemical treatments used in these studies was found to increase CYP2C11 mRNA, confirming the high level constitutive expression of this enzyme in the male rat [37].

Western immunoblot analysis of microsomal protein fractions for CYP2C11 apoprotein expression also demonstrated the high level constitutive expression of this enzyme in control rats and a decreased expression following exposure of animals to BNF (Fig. 3j). DEX was also found to have a slight effect on CYP2C11 protein. As indicated above, this effect of DEX was not observed at the mRNA level.

Analysis of microsomal testosterone 2α-hydroxylase (T2aH) activity also showed considerable CYP2C11 en-


RAT LIVER P450 1A1 mRNA
a
CYP1A1 O.D./Cyclophilin O.D. Ratio
RAT LIVER P450 1A2 mRNA
b
CYP1A2 O.D./Cyclophilin O.D. Ratio
RAT LIVER P450 1A1 APOPROTEIN
c
CYP1A1 O.D.
RAT LIVER EROD ACTIVITY
d
7-Ethoxyresorufin O-dealkylase (EROD) Activity (pmol/mg protein/minute)
RAT LIVER P450 2B1/2 mRNA
e
CYP2B1/2 O.D./Cyclophilin O.D. Ratio
RAT LIVER P450 2B1 APOPROTEIN
f
CYP2B1 O.D.
RAT LIVER PROD ACTIVITY
g
7-Pentoxyresorufin O-dealkylase (PROD) Activity (pmol/mg protein/minute)
RAT LIVER TESTOSTERONE 16-BETA-HYDROXYLASE ACTIVITY
h
Testosterone 16-beta-Hydroxylase Activity (pmol/mg protein/minute)
RAT LIVER P450 2C11 mRNA
i
CYP2C11 O.D./Cyclophilin O.D. Ratio
RAT LIVER P450 2C11 APOPROTEIN
j
CYP2C11 O.D.
RAT LIVER TESTOSTERONE 2-ALPHA HYDROXYLASE ACTIVITY
k
Testosterone 2-alpha-Hydroxylase Activity (pmol/mg protein/minute)
H20
CO
PB
BNF
MET
DEX
CLO

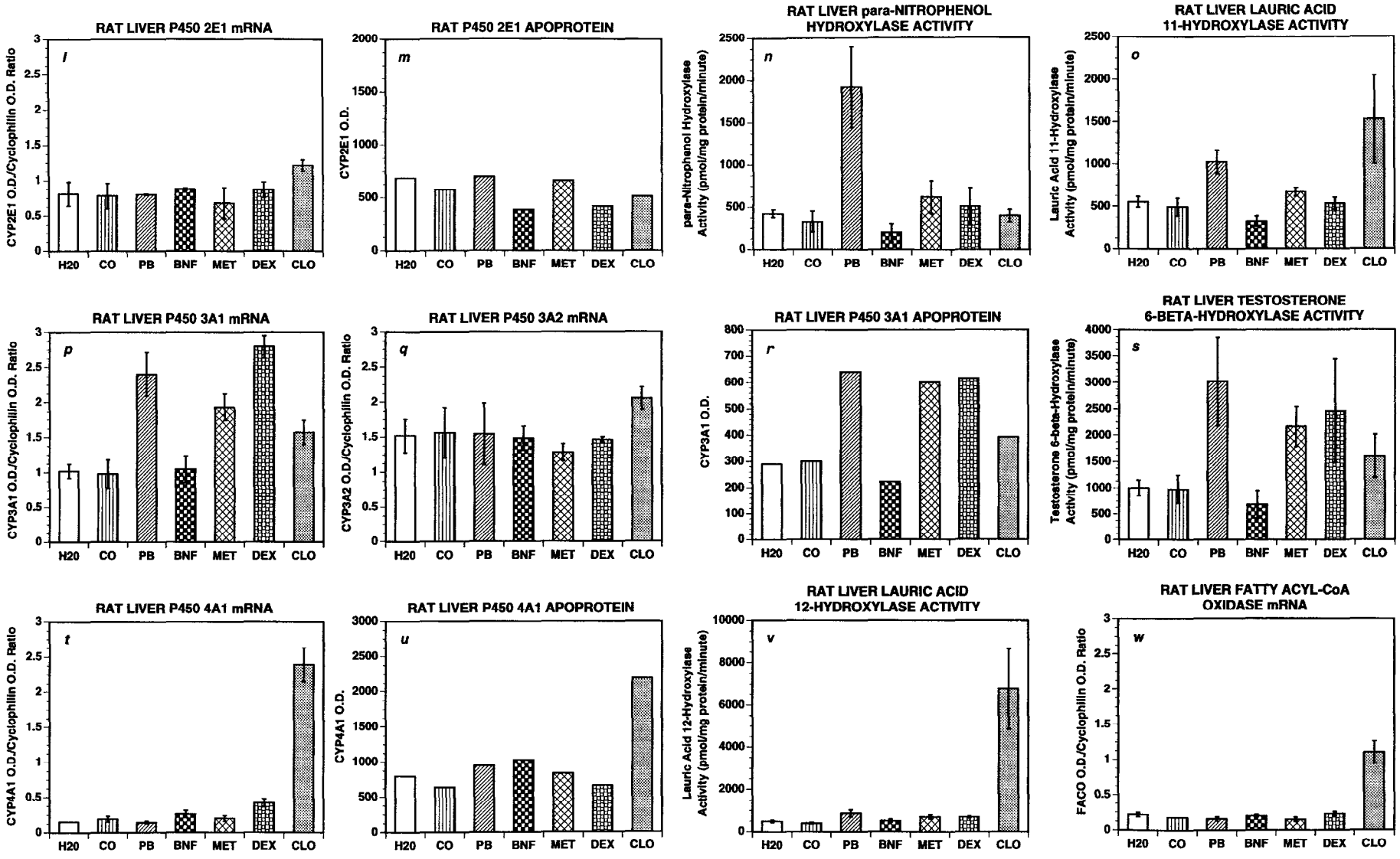


FIG. 3. Comparison of RT–PCR (mRNA), western immunoblotting (apoprotein), and enzyme activity measures for the analysis of P450 expression in rat liver. Data represent the results obtained from the analysis of rat liver P450 expression by RT–PCR, immunoblotting, and enzyme activity measures following exposure to prototypical P450 enzyme inducers. The effects of P450 enzyme inducers on rat liver P450 1A1/1A2 (a–d), 2B1/2 (e–h), 2C11 (i–k), 2E1 (l–o), 3A1/3A2 (p–s), and 4A1/FACO (t–w) are compared. Data are presented as the mean ± SD of the values obtained from N = 3 rats per treatment group for the mRNA and enzyme activity measurements; values are given as means (N = 2) for the apoprotein measurements.

zyme activity present in control rat livers which was decreased following exposure to BNF (Fig. 3k). A slight decrease was also observed in some of the rats exposed to DEX, confirming the decrease observed in apoprotein in these livers. In contrast to both the mRNA and protein determinations, T2aH activity was also found to be decreased in the livers of animals exposed to PB. The mechanism for this effect, however, remains unclear.

Analysis of CYP2E1 Expression

Analysis of CYP2E1 mRNA demonstrated a high level constitutive expression in control male rat livers (Figs. 1 and 3l). Following exposure to various inducers, only CLO was found to cause a consistent increase in the expression of the CYP2E1 mRNA. In some experiments, CLO was found to cause a 2- to 3-fold increase in CYP2E1 mRNA expression (data not shown).

As with mRNA, high levels of CYP2E1 apoprotein were found to be expressed in control rat liver (Figs. 2 and 3m). However, none of the compound treatments, including CLO, caused a consistent effect on the levels of CYP2E1 apoprotein. BNF was found to cause a slight, but consistent, reduction in the levels of CYP2E1 apoprotein.

Two microsomal enzyme activities were monitored to assess the levels of CYP2E1 activity, pNPH and LA-11-H (Fig 3, n and o). Although they are each indicative of CYP2E1 activity, neither is specific for the CYP2E1 enzyme [21, 38, 39]. Keeping this in mind, pNPH was found to closely parallel the findings observed with CYP2E1 protein, where PB and MET treatments both caused increases in enzyme activity. In addition, BNF was found to cause a marked reduction in pNPH activity. LA-11-H activity was also found to be increased by PB and decreased by BNF treatments but was not found to be changed following exposure to MET. LA-11-H was also found to be increased by CLO exposure, which reflects the ability of this compound to induce the CYP4A subfamily of enzymes (see below). Based on mRNA and apoprotein expression, these data suggest that pNPH activity is more accurate in the assessment of CYP2E1 enzyme expression than LA-11-H. The significance of the apparent induction of CYP2E1 mRNA by CLO is presently unclear.

Analysis of CYP3A1 and CYP3A2 Expression

Both CYP3A1 and CYP3A2 mRNA were found to be constitutively expressed in control male rat liver (Figs. 1 and 3p–s). Following exposure to PB, MET, DEX, and CLO, CYP3A1 mRNA was found to be increased relative to control, whereas CYP3A2 mRNA expression was only increased in CLO-exposed livers.

Western immunoblot analysis of liver microsomal protein fractions for CYP3A1 apoprotein showed a similar profile of expression for this enzyme (Fig. 3r). As indicated by the expression of CYP3A1 mRNA, PB, MET, and DEX exposures all resulted in marked increases in the apparent levels of the CYP3A1 apoprotein. CYP3A2 apoprotein expression was not determined as part of this study.

Analysis of liver testosterone 6β-hydroxylase (T6bH) activity supports the changes in CYP3A mRNA and apoprotein expression (Fig. 3s). Although CLO was not found to cause an apparent increase in the levels of liver CYP3A1 protein in this experiment, the induction of CYP3A1 mRNA was confirmed by an increase in T6bH enzyme activity. These data suggest that the induction of CYP3A enzyme activity may be due to a combined increase in both CYP3A1 and CYP3A2 in exposed rats.

Analysis of CYP4A1 and FACO Expression

CYP4A1 and FACO mRNA were both found to be constitutively expressed in control male rat livers (Figs. 1 and 3t and w). Following exposure to CLO, a prototypical CYP4A1 inducer, both CYP4A1 and FACO mRNA were increased. DEX was also found to cause a slight increase in CYP4A1 mRNA expression in this experiment but not in other experiments or with any other inducers tested (Figs. 1 and 3t). DEX was not found to affect the expression of FACO in any of the experiments.

A similar profile of CYP4A1 expression was found using western immunoblot analysis of microsomal protein fractions where CLO was found to cause a marked increase in the expression of the CYP4A1 apoprotein (Fig. 3u). DEX was not found to cause any increase in the CYP4A1 apoprotein. Increased CYP4A1 expression by CLO was further demonstrated by an increase in LA-12-H activity in liver microsomal protein fractions from exposed rats (Fig. 3v). Analysis of FACO protein and enzyme activity were not determined as part of this study.

DISCUSSION

The P450 MFOs represent one of the body's most important mechanisms of defense against chemical-induced toxicity. This superfamily of metabolic enzymes oxidizes both endogenous and exogenous (xenobiotic) compounds, converting them to hydrophilic metabolites that can be removed readily from the body. In some instances however, metabolism of chemicals by the P450 enzymes may be undesirable or detrimental to the body. Metabolism of certain compounds can lead to toxic or reactive intermediates, resulting in target organ toxicity and/or carcinogenic insult [3]. Additionally, several classes of P450 inducers represented by chemical agents such as PB, CLO, and the polychlorinated dioxins, have been shown to be rodent liver tumor promoters, establishing a possible link between P450 induction and tumor promotion. With chemical-based therapeutics, induction of P450 isoenzymes can lead to metabolism and accelerated removal of drugs, which may limit their desired pharmacological actions. This phenomenon, known as "autoinduction," can be problematic in drug discovery and an obstacle to the development of adequate therapeutics. Finally, species differences in the pharmaco-

dynamic properties of certain chemical therapeutics suggest that responses in some test species are not relevant for humans because of differential metabolic capabilities. Since P450 enzyme induction can aid in establishing the potential for toxicity and/or carcinogenesis, it is extremely important to monitor P450 expression profiles in response to new chemical entities in the early stages of drug discovery, to assist in the development of safe and effective therapeutics.

Whereas most of the P450 enzymes have been shown to be constitutively expressed in rat liver, many are increased markedly in expression upon exposure to various chemicals. For example, in adult male rats, expression of CYP2C11 has been shown to be constitutively expressed and noninducible in the liver, whereas the expression of P450 1A1, 2B1, 2E1, and 4A1 are all selectively induced following exposure to various chemicals [1, 2, 5, 40]. This increase in P450 enzyme expression can occur via a number of mechanisms, including increased transcription, stabilization of mRNA, and increased protein synthesis.

The objective of the present investigation was to develop a rapid, high throughput, screening assay for the assessment of P450 expression in the rat, which could be used for routine toxicology applications. Using the technique known as RT–PCR, the approach used was to measure changes in P450 gene expression (i.e. mRNA) as a surrogate to changes in apoprotein expression and substrate-specific enzymatic activity in the livers of chemically exposed rats. This approach has been used previously by various investigators to measure P450 expression in various tissues of the rat [41–46].

In the present investigation, semi-quantitative measurements of gene expression were obtained by comparing the relative amounts of mRNA from each specific gene of interest with that of the housekeeping gene, CYC. Although this method is useful in determining qualitative (i.e. fold) changes in gene expression, more quantitative RT–PCR methodologies can be applied to determine more accurately the number of mRNA transcripts generated from a given gene [41, 47, 48]. Quantitative RT–PCR methods are based on the use of "engineered" cDNA sequences that are used as internal standards or competitive templates that provide a means for determining the actual number of amplified fragments generated during the PCR and thus the number of mRNA transcripts in the original sample(s).

To test the validity of the RT–PCR approach to measure changes in P450 isoenzyme expression, we selected several prototypical inducers of liver P450 enzymes to determine the association between changes in mRNA, protein, and enzymatic activity and to assess the feasibility of using mRNA as a reliable endpoint for induction of P450 isoenzyme expression. The inducers used in these studies included BNF as an inducer of the CYP1A subfamily, PB as an inducer of the CYP2B subfamily, DEX as an inducer of the CYP3A subfamily, and CLO as an inducer of the CYP4A subfamily and fatty acyl-CoA oxidase. In addition, MET was included in these studies to determine the effects of an enzyme activity inhibitor on the expression of P450 isoenzymes.

In all experiments conducted as part of this investigation, almost complete agreement was found between the changes observed in the expression of P450 enzymes at the mRNA and protein levels (Fig. 3). Independent of the mechanisms responsible for the changes in mRNA expression (i.e. transcriptional versus stabilization), these results strongly suggest that P450 enzyme expression, in general, is regulated primarily at the mRNA level. This is particularly evident in cases where certain of the compounds tested (e.g. BNF) were found to coordinately reduce the expression of P450 as measured at the mRNA, apoprotein, and enzyme activity levels. The expression of the CYP2E1 isoenzyme, which is increased generally via protein stabilization, is a known exception to this mechanism of P450 enzyme regulation [49, 50]. In a similar fashion, it was determined from these studies that for most of the P450 enzymes (i.e. P450 1A1, 2B1/2, 3A1, and 4A1) coordinate changes were observed in the levels of mRNA and apoprotein expression and that of enzymatic activity against selective substrates (Fig. 3). Minor differences were identified for the CYP2C11 and CYP2E1 isoenzymes, where the enzymatic substrates are known to be relatively less specific.

Treatment of rats with BNF was found to cause increases in the expression of both CYP1A1 and CYP1A2 mRNA, CYP1A1 apoprotein, and corresponding EROD activity. PB was also found to cause a slight increase in both CYP1A1 apoprotein and EROD activity, but not in either CYP1A1 or CYP1A2 mRNA. An increase in EROD activity by PB has been observed previously and likely reflects a lack of complete selectivity of the CYP1A1 enzyme for this substrate [10, 14, 51]. Alternatively, this finding may suggest that PB increases CYP1A activity through stabilization of protein, although further studies are needed to confirm this effect. Wortelboer *et al.* [52] previously reported an increase in microsomal EROD activity without an increase in CYP1A apoprotein levels following PB exposure in Wistar rats. Although an associated increase in the CYP1A apoprotein was not detected by these investigators, the source of the antibody reagents used was different, potentially resulting in a lower sensitivity for detection by western blot analysis. This explanation is further suggested by the low levels of CYP1A1 protein that were consistently detected in the present investigation (Fig. 1).

Treatment of rats with BNF was also found to cause a marked decrease in the expression of CYP2C11. This male rat specific P450 isoenzyme has been shown to be regulated by androgenic hormones that, in turn, regulate the release of growth hormone from the pituitary [53–57]. The pulsatile pattern of growth hormone secretion appears to be an important factor in the maintenance of constitutive *CYP2C11* gene expression in the adult male rat. The suppression of CYP2C11 expression has been observed previously with other xenobiotic agents [58], and has often been

referred to as the "feminization" of P450 expression because of a disruption in the circulating levels of androgenic hormones. Collectively, these findings would suggest that BNF exposure in the rat has either a direct effect on gonadal or pituitary function or an indirect effect on the levels of certain endocrine hormones in the peripheral circulation. The effect of BNF on CYP2C11 was particularly prominent at the mRNA level, suggesting that P450 mRNA expression may be useful in identifying the potential effects of xenobiotics on certain endocrine functions (e.g. synthesis of androgenic steroids). This concept is further supported by the fact that constitutive expression of many of the P450 enzymes is regulated or co-regulated by endocrine hormones [2, 5, 59–61].

Aside from the weak induction of CYP1A1 described above, PB treatment was found to result in the prototypical increase in CYP2B and CYP3A1 expression [32, 62–64]. CYP3A2 was not induced by PB in these studies, although this effect has been reported [62]. MET was also found to be an inducer of the CYP2B and CYP3A1 enzymes, suggesting a common structure–activity relationship between these compounds for chemically induced gene expression. The effect of MET treatment on the expression of CYP2B1/2 and CYP3A1 protein levels in rat liver has been investigated previously [65, 66]. These findings indicate that inhibitors of P450 enzyme activity have no readily detectable effect on their capacity to cause enzyme induction at either the mRNA or apoprotein levels.

DEX treatment was also found to cause a prototypical pattern of P450 expression in rat liver, resulting in a selective increase in the expression of CYP3A1 [62, 67–69]. As in the case of PB, DEX was not found to induce the expression of the CYP3A2 enzyme, demonstrating that these two isoenzymes of P450 are differentially regulated in the rat liver.

CLO, a hypolipidemic agent, was found to be a broad spectrum inducer of P450 in the rat liver, inducing an increase in the expression of P450 3A1, 3A2, and 4A1, and FACO using all three methods of detection. These findings demonstrate that, in addition to peroxisome proliferator activity, CLO causes an increase in the expression of multiple P450 enzymes. Moreover, CLO was found to consistently induce the expression of CYP2E1 at both the mRNA and apoprotein levels in our studies. Although CYP2E1 was not increased markedly in the present study, we (and others [52]) have found up to a 3-fold induction of this enzyme by CLO in previous experiments (data not shown). This finding suggests that CLO can regulate the expression of CYP2E1 at the mRNA level, distinguishing it from prototypical inducers (e.g. ethanol, acetone, and isoniazid) that increase the expression of this enzyme via a mechanism involving protein stabilization [49].

In conclusion, the findings of this study clearly demonstrate that mRNA expression as measured by RT–PCR can be used to monitor the changes in expression of most P450 isoenzymes in rat liver following exposure to xenobiotics. Given the advantages of selectivity, sensitivity, and speed of this versus traditional methods, these studies demonstrate the utility of the RT–PCR method for analysis of P450 expression in the rat during routine toxicology studies.

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