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Glucocorticoid Receptor-Independent Transcriptional Induction of Cytochrome P450 3A1 by Metyrapone and its Potentiation by Glucocorticoid

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SUMMARY

Metyrapone administration to 21- and 90-day-old male rats causes a transcriptional induction of the hepatic glucocorticoid-inducible *CYP3A1* gene within an hour as determined by nuclear run-on experiments. Analyses performed 24 hr after metyrapone administration in both ages of rat demonstrate that the transcriptional induction of *CYP3A1* gene expression is followed by significant increases in CYP3A1 mRNA, CYP3A-immunoreactive microsomal protein and total microsomal cytochrome P450 (CYP). In 21-day-old rats, there is a significant increase in microsomal CYP3A-dependent steroid 6β-hydroxylase activity but not in 90-day-old rats, possibly because of a slower clearance of this drug, which inhibits CYP activities. In hepatocytes cultured in serum- and glucocorticoid hormone-free medium, metyrapone alone induces CYP3A1 mRNA expression, which demonstrates that metyrapone transcriptionally induces hepatic CYP3A1 by a direct interaction with the liver. Metyrapone does not compete with the binding of the synthetic glucocorticoid and potent transcriptional CYP3A1 inducer dexamethasone to the glucocorticoid receptor (GR) in soluble fractions from liver. This suggests that metyrapone is not a ligand for the GR and induces CYP3A1 by a mechanism independent of the GR. Addition of glucocorticoid to cultured hepatocytes at levels that induce GR-dependent genes potentiate CYP3A1 mRNA induction by metyrapone without inducing CYP3A1 mRNA alone. A GR-dependent mechanism may therefore mediate the potentiation of CYP3A1 transcriptional induction by metyrapone. The CYP3A1 transcriptional inducer and glucocorticoid antagonist pregnenolone 16α-carbonitrile at 100 μM blocks dexamethasone binding to the GR in 21-day-old rat liver soluble fractions but is less effective in 90-day-old rat liver soluble fractions in contrast with 10 μM glucocorticoid antagonist RU486, which is equally effective at blocking dexamethasone binding to the GR. The inability of pregnenolone 16α-carbonitrile to fully compete with dexamethasone for cytosolic binding in adult animals suggests that there may exist variant receptors with different affinities for dexamethasone and pregnenolone 16α-carbonitrile and may explain the mechanism by which low concentrations of dexamethasone potentiate the transcriptional induction of CYP3A1 mediated by high concentrations of pregnenolone 16α-carbonitrile [*J. Biol. Chem.* **270**:28917–28923 (1995)]. Examination of membrane-bound dexamethasone binding activity, with which other steroidal and nonsteroidal CYP3A inducers have been shown to compete, indicates that binding activity is detectable in 90- but not 21-day-old rat liver microsomes. The absence of membrane-bound glucocorticoid binding site activity and the presence of a functional CYP3A1 transcriptional response in 21-day-old rats suggest that membrane-bound glucocorticoid binding site activity is not involved in the transcriptional activation of CYP3A1 expression. These data suggest that both glucocorticoids and nonsteroidal compounds may trigger the transcriptional induction of CYP3A1 by a GR-independent mechanism that may be potentiated by a GR-dependent mechanism.

Cytochrome P450s (CYP)[1] are heme-thiolate proteins that are expressed in both prokaryotes and eukaryotes (1). A characteristic reaction catalyzed by CYPs is hydroxylation, which is directed to both endogenous and exogenous compounds (1). CYPs are widely acknowledged to be of major importance in the metabolism and therefore the determination of toxicity in xenobiotics (2). An understanding of their regulation may explain the effect of modulators of CYP expression and altered responses to xenobiotics. CYP3A is a highly expressed subfamily in both rodents and humans and

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[1] The nomenclature used for CYPs is that recommended by Nelson *et al.* (1). However, CYP3A1 should be taken to refer to both PCN1 (4) and cDEX/RL33 (12, 13) mRNAs which code for similar proteins. cDEX and RL33 have been designated CYP3A23 according to the directory of CYP-containing systems, IGCEB (http://www.icgeb.trieste.it/). However, both CYP3A1 and CYP3A23 appear to be co-ordinately regulated; CYP3A refers to both CYP3A1 and CYP3A2 proteins since they are inseparable by standard electrophoretic techniques

ABBREVIATIONS: CYP, cytochrome P450; GR, glucocorticoid receptor/soluble liver fraction dexamethasone saturable binding site; PR, progesterone receptor; RU486, mefepristone/17β-hydroxy-11β-(4-dimethylaminophenyl) 17α-1-propynyl-estra-4,9-dien-3-one; metyrapone, 2 methyl-1,2-bis(3-pyridyl)-1-propanone.

has been demonstrated to have a prominent role in the metabolism of xenobiotics in humans (3). However, the mechanisms of regulation of CYP3A gene expression are not well understood in any species.

The *CYP3A* gene subfamily in rats is known to contain at least 2 distinct genes, *CYP3A1* and *CYP3A2* (1). The isolation of the first cDNAs (PCN1 and PCN2, respectively) indicated that they share 90% nucleotide and 89% deduced amino-acid sequence homology (4, 5). Protein studies indicate the presence of further CYP3A forms, which suggests additional *CYP3A* genes (6–9). More recently, cDNA and genomic clones have been isolated that bear high sequence homology to either *CYP3A1* or *CYP3A2* genes (i.e., PCN1 and PCN2 respectively) (10–14, 15). cDNA clones—cDEX and RL33—have been isolated that have 26 coding nucleotide changes (resulting in a deduced 11 amino acid changes) and a 2-amino-acid deletion compared with PCN1 (12, 13). Both clones have been isolated from the outbred Sprague-Dawley strain.

CYP3A1 is constitutively expressed at low levels (5, 16) although some reports have demonstrated that there is a transient induction in expression in immature rats (11, 15, 17, 18). Dexamethasone is a potent glucocorticoid that, when bound to the GR, effects a change in transcription of genes that contain specific responsive elements (glucocorticoid response elements) in the locus (19). Dexamethasone transcriptionally activates expression of CYP3A1 (20), although there is no classical glucocorticoid response element within the region upstream of the gene, which is demonstrated to confer inducibility by both dexamethasone and the GR-antagonist pregnenolone 16α-carbonitrile (21). The response of cultured hepatocytes to dexamethasone indicate that higher concentrations are required to induce CYP3A1 compared with the classical GR-dependent gene tyrosine aminotransferase (*TAT*) (22, 23). Induction of CYP3A1 by dexamethasone therefore may not operate through a classical GR-dependent mechanism. In addition, a solely GR-mediated transcriptional mechanism of induction of CYP3A1 is unlikely because the antiglucocorticoid pregnenolone 16α-carbonitrile as well as structurally unrelated compounds (such as phenobarbitone, rifampicin, clotrimazole, and organochlorine pesticides) all induce CYP3A1 by a direct effect on the liver (23–25). The high concentrations of dexamethasone required to induce CYP3A1 may be consistent with the existence of a lower affinity receptor for dexamethasone activating *CYP3A1* gene expression. In the present study, the expression of membrane-bound dexamethasone binding activity is examined in relation to the induction of CYP3A1 because this binding site has a 20-fold lower affinity for dexamethasone compared with the GR and also binds other CYP3A inducers that are not bound by the GR, such as phenobarbitone (26, 27). An examination of CYP3A1 induction response and age is also assessed to rationalize the age-dependent changes in transcriptional/post-transcriptional mechanisms of CYP3A1 induction recently described (28).

Experimental Procedures

Materials. Metyrapone was purchased from Sigma Chemical (Poole, Dorset, UK). [^{3}H]dexamethasone (38–83 Ci/mmol), [4-^{14}C]androst-4-ene-3,17-dione (57 mCi/mmol), [α-^{32}P]dCTP (3000 Ci/mmol), [γ-^{32}P]ATP (3000 Ci/mmol) and [α-^{32}P]UTP (800 Ci/mmol) were purchased from Amersham (Buckinghamshire, UK). 6β-hydroxyandrostenedione and 7α-hydroxyandrostenedione standards were obtained from Steraloids (London, UK). Rabbit anti-rat CYP3A antibody and purified CYP3A2 were generously provided by David Waxman (Harvard Medical School, Boston, MA) and James Halpert (University of Arizona, Tucson, AZ) respectively. RU486 was supplied by Roussel-Uclaf (Romainville, France). The albumin cDNA was from rat-pRSA8 (29) and the CYP1A1 cDNA was provided by John Fagan (Maharishi International University, Fairfield, IA) (30). Oligonucleotides were purchased from Pharmacia (St. Albans, Herts, UK). All other chemicals were of the highest purity available from commercial sources.

Animals and dosing. Males bred from Sprague-Dawley rats supplied by Charles River (Margate, Kent, UK) were used in all studies. Rats were maintained on diet number 1 (SDS, Witham, Essex, UK) and water *ad libitum*. Groups of 3 rats per treatment group were treated intraperitoneally at 21 days old (weaning) and 90 days old with 100 mg of metyrapone/kg of body weight dissolved in saline vehicle. Control groups received saline.

Rat hepatocytes were prepared from 90-day-old male rats by collagenase perfusion as previously described (31) and cultured on 150-mm diameter collagen (Vitrogen-100; GIBCO, Paisley, Scotland)-coated plastic plates in William's medium E supplemented with 2 nM insulin, 10 nM glucagon and 50 μg/ml gentamycin with further additions as indicated. Hepatocytes were cultured at a density of 18 million cells/plate in 20 ml of medium at 37° in an humidified incubator gassed with 5% CO_2 in air.

Preparation of liver cell fractions. Liver soluble (100,000 × *g* supernatant) and washed microsomal (100,000 × *g* pellet) fractions were prepared essentially as described previously (26) using ice-cooled 10 mM Tris buffer, pH 7.5, containing 250 mM sucrose. Protein analysis was performed by the method of Lowry *et al.* (32). Microsomes for the analysis of androstenedione hydroxylase activity were resuspended in 10 mM potassium phosphate buffer, pH 7.2, containing 20% v/v glycerol and 1 mM EDTA and stored at −80° until analysis. Analysis of dexamethasone binding activity and spectrophotometric CYP (31) was performed on freshly prepared fractions only.

Nuclei for run-on transcription assays were prepared essentially as previously described (15). After preparation and counting, nuclei were frozen in aliquots at −80° until required.

Run-on transcription assay. Liver nuclei were defrosted and run-on transcription was performed as previously described (15) except that 2.5 × 10^6 nuclei were used in each assay. The RNA was purified and the relative levels of *in vitro* synthesized CYP3A1 and albumin mRNAs were determined by autoradiography after hybridization of a fixed amount of purified, synthesized run-on RNA to a fixed excess of immobilized probes—0.9-Kb cosCYP/3A1 intronic sequence (15), albumin cDNA pRSA8 (29) and pBluescript II SK$^+$ phagemid DNA (Stratagene, Cambridge, UK).

Analysis of dexamethasone binding in rat liver cell fractions. Incubation of soluble liver fractions with radiolabeled [^{3}H]dexamethasone (83 Ci/mmol) for analysis of GR binding activity was performed as described (26) at 0° after dilution with buffer supplemented with 10 mM sodium molybdate, 10 mM sodium citrate, and 5 mM dithiothreitol. Incubation of soluble fractions with 25 nM radiolabeled dexamethasone [10 × K_D for the GR (26)] was used throughout with a 1000-fold molar excess of unlabeled dexamethasone used to determine nonspecific binding.

Microsomes were diluted in buffer supplemented with 5 mM dithiothreitol and incubated with [^{3}H]dexamethasone (38 Ci/mmol). For analysis of potential binding competitors, a fixed radiolabeled dexamethasone concentration of 100 nM was used and a 1000-fold molar excess of unlabeled dexamethasone was used to determine nonspecific binding. For the determination of the microsomal dexamethasone binding parameters—K_D and R_{max}—a range of dexamethasone concentrations (5–500 nM) were used and binding data fitted by least squares nonlinear regression using the Enzfitter program (RJ Leatherbarrow; Elsevier Bioscience, Cambridge, UK).

Slot, Northern and Southern blotting. Total liver RNA was prepared using RNAzol (Biogenesis, Bournemouth, UK). For analysis of mRNA expression, RNA was either electrophoresed on 1.5% w/v denaturing (formaldehyde) agarose gels and transferred to nylon membrane (Hybond N; Amersham) as previously described (33) or subjected to slot blot analysis as described (15). For examination of CYP3A1 expression, the oligonucleotide 5′-TGTGCGGGTCCCA-AATCCGT complementary to residues 149–168 of CYP3A1 (PCN1) (5) and cDEX/RL33 (12, 13) mRNAs was employed. The 28-S rRNA probe had the sequence 5′-AACGATCAGAGTAGTGGTATTTCACC (34). Oligonucleotides were 5′ end-labeled with [γ-^{32}P]ATP and polynucleotide kinase. Albumin and CYP1A1 mRNA expression was determined using randomly primed cDNAs radiolabeled with [α-^{32}P]dCTP.

Genomic DNA was isolated for Southern blotting from Wistar and Sprague-Dawley rats by treatment of tissue with RNase and proteinase k followed by salt precipitation of DNA using a kit supplied by Stratagene. DNA was digested with restriction endonucleases before subjecting fragments to agarose electrophoresis and blotting onto nylon membranes for hybridization with randomly primed [α-^{32}P]dCTP-labeled cosCYP/3A1 fragment as previously outlined (15).

Western blotting. Western blotting was performed after sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions using a MiniP2 electrophoresis apparatus (BioRad, Hemel Hempstead, UK). Protein was transferred onto nitrocellulose and blocked overnight with 3% (w/v) bovine serum albumin/0.3% (w/v) Tween 20. After incubation with the primary antibody, the blots were incubated with the appropriate species-specific horseradish peroxidase-conjugated anti-IgG antibody. Detection was accomplished using chemiluminescence (ECL kit; Amersham).

Steroid 6β-hydroxylase assay. Microsomal steroid hydroxylase activities were determined as previously described (35) using [4-^{14}C]androstenedione. After incubation, the metabolites were separated by thin layer chromatography and located by autoradiography. Metabolites were identified by co-migration of authentic standards. Metabolite formation was quantitated by scintillation counting after scraping metabolites from the plate.

Results

Effect of metyrapone administration to 21- and 90-day-old rats on CYP. The effect of metyrapone on *CYP3A1* gene transcription was assessed by nuclear run-on analysis using the cosCYP/3A1 intronic sequence present as a single copy in Wistar rats (15). Fig. 1 confirms the presence of this same intronic sequence as a single copy in Sprague Dawley rats used in this study. Fig. 2 indicates that administration of metyrapone to rats leads to the transcriptional activation of CYP3A1 premRNA expression within an hour in both 21- and 90-day-old rats. Transcription of the constitutively expressed albumin mRNA is apparently depressed by metyrapone administration in both 21- and 90-day-old rats and may be associated with a competition for nucleotides and/or transcription factors on activation of gene expression by metyrapone. Because albumin mRNA levels are unaffected as determined 24 hr after metyrapone administration in 21-day-old rats (Fig. 3A) and 90-day-old rats (data not shown), the depression of albumin mRNA synthesis by metyrapone after 1 hr would appear to be a transient effect. There is a ~3-fold greater rate of *CYP3A1* gene transcription in nuclei from metyrapone-treated 21-day-old rats (relative to the rate of transcription of albumin in nuclei from control animals of the same age) compared with nuclei from 90-day-old rats suggesting that the transcriptional component of CYP3A1 expression is either greater or more rapid in response in younger versus older animals as suggested by previous studies with glucocorticoid inducers of CYP3A1 (15, 28).

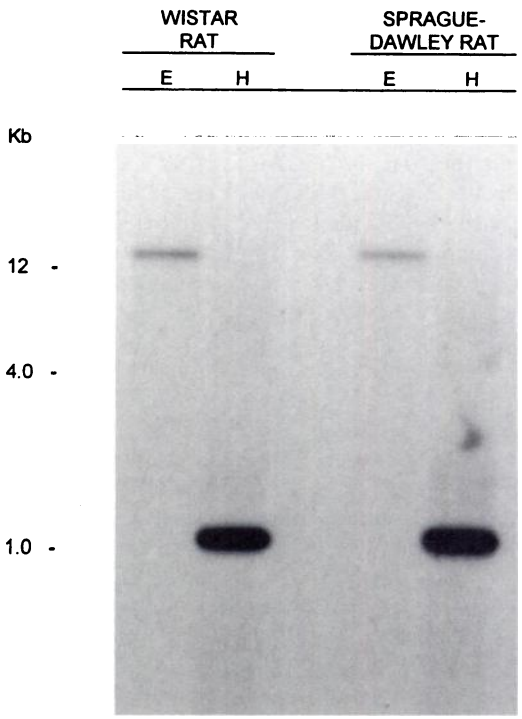

Fig. 1. Southern blot analysis of Wistar and Sprague Dawley DNA (10 μg of DNA/lane) digested with *Eco*RI (*E*) or *Hin*dIII (*H*) restriction endonucleases and probed with ^{32}P-labeled cosCYP/3A1 fragment.

Fig. 3B indicates that in both 21-day-old and 90-day-old rats, metyrapone administration leads to an increase in hepatic CYP3A1 mRNA expression 24 hr after administration. The level of CYP3A1 mRNA induced by metyrapone in 21-day-old rats is similar to the levels maximally induced by other inducers, such as dexamethasone and pregnenolone 16α-carbonitrile (data not shown). However, the levels of CYP3A1 mRNA induced by metyrapone treatment are greater in 21- than in 90-day-old rats, which is compatible with an apparent greater rate of transcription of the *CYP3A1* gene in 21-day-old rats.

Figs. 4 and 5 indicate that 24 hr after administration of metyrapone there are similar significant increases in total CYP3A immunoreactive protein in liver microsomes from 21- and 90-day-old rats. The anti-CYP3A antibody is immunoreactive toward CYP3A1, CYP3A2, and related forms; it can be seen from Fig. 5 that the antibody also detects CYP3A-immunoreactive protein in adult female rats, which are reported to express negligible levels of both CYP3A1 and the male-specific CYP3A2 in the adult (16). The levels of microsomal immunodetectable CYP3A in 21- and 90-day-old male control animals are approximately the same; this is confirmed by similar levels of the CYP3A-dependent (4, 36) steroid 6β-hydroxylase enzyme activity (Table 1). Table 1 indicates that metyrapone only induces a significant increase in steroid 6β-hydroxylase activity in 21-day-old animals despite similar increases in immunoreactive CYP3A protein (Fig. 5) and increases in spectrophotometric CYP (indicative that the increased levels of CYP also contain the prosthetic heme required for activity) in 21- and 90-day-old metyrapone-treated rats. Table 1 also indicates that the levels of

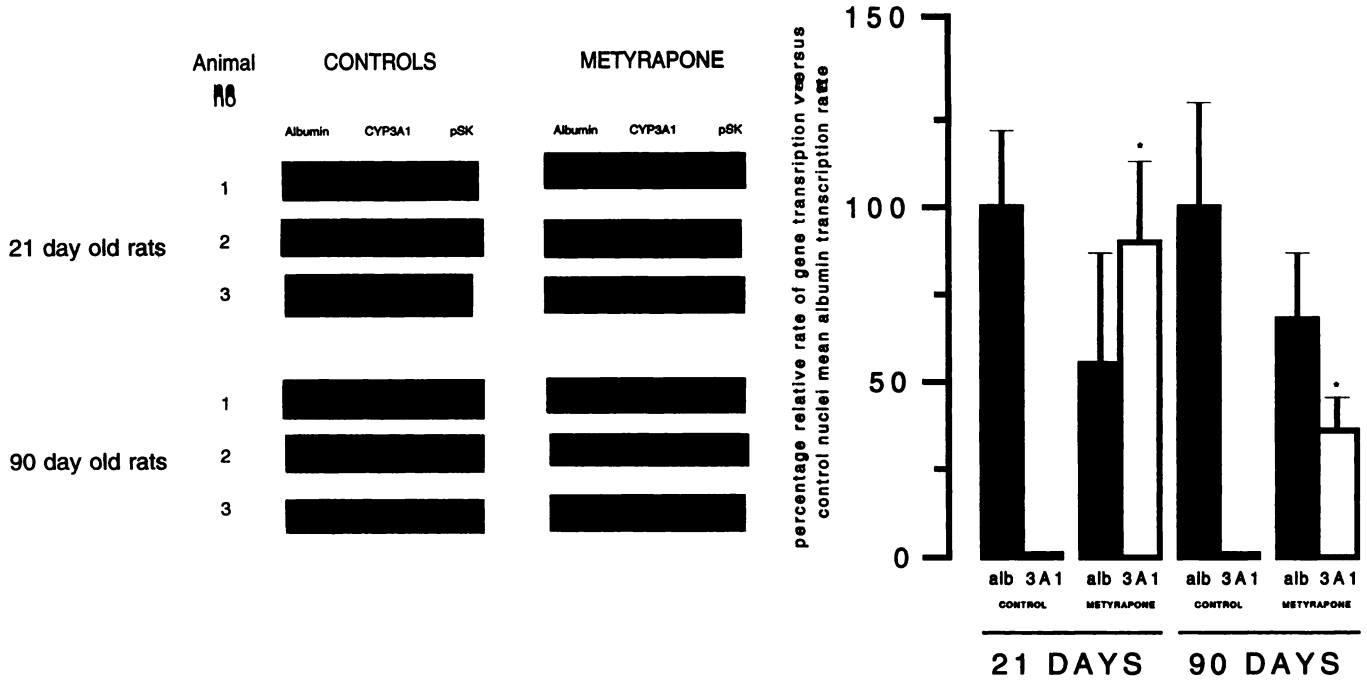

Fig. 2. Transcriptional induction of CYP3A1 premRNA expression by metyrapone. Liver nuclei were prepared from male rats (3 animals/treatment) 1 hr after administration of metyrapone or vehicle control and initiated transcripts elongated *in vitro* for 20 min, incorporating radiolabeled UTP precursor. Purified radiolabeled *in vitro* synthesized RNA (1.5×10^6 cpm) [98 ± 5.4% (n = 12) specifically labeled as determined by percentage of radioactivity bound to Whatman DE81 ion-exchange filters after extensive washing] was hybridised to 0.25 pmol of each probe and the membrane washed (*pSK*, pBluescript II SK^+ phagemid DNA; used as negative control). After autoradiography, results were analyzed by laser densitometry; band intensities (transcription rate) were determined using Phoretix software and expressed as a percentage of the mean transcription rate of albumin in control nuclei. *, significantly different: $p > 95\%$ transcription rate versus control using Student's t test (two tailed).

hepatic CYP2A1-dependent steroid 7α-hydroxylase activity show an age-dependent decrease in levels in agreement with previous studies (37). However, metyrapone administration affects neither the levels of steroid 7α-hydroxylase activity (Table 1) nor the levels of CYP2A immunoreactive protein (data not shown) in either 21- or 90-day-old rats. This suggests that metyrapone treatment does not lead to a nonspecific loss of CYP-dependent metabolism in 90-day-old rats, because there is no decrease in 7α-hydroxylase activity in metyrapone treated rats compared with controls. The lack of an increase in steroid 6β-hydroxylase activity by metyrapone administration to mature rats is in agreement with previous studies that metyrapone treatment does not increase CYP3A-dependent erythromycin demethylase activity (38, 39).

Induction of CYP3A1 in cultured hepatocytes. Fig. 6 indicates that the addition of metyrapone to primary cultures of rat hepatocytes induces the expression of CYP3A1 mRNA in the absence of any glucocorticoids. Co-incubation of 100-μM hydrocortisone 21-hemisuccinate with metyrapone further enhances the levels of CYP3A1 mRNA induced by metyrapone without inducing CYP3A1 mRNA alone. Induction of CYP1A1 also occurs when hydrocortisone 21-hemisuccinate and metyrapone are co-administered as previously observed (33). The mechanism of induction of CYP1A1 *in vitro* is distinct from classical aryl hydrocarbon receptor ligand induction in terms of time course[2] and may be independent of direct binding of metyrapone to the aryl hydrocarbon receptor. Fig. 6 also indicates that the protein synthesis inhibitor cycloheximide blocks the induction of CYP3A1 stimulated by metyrapone. The apparent de-repression of CYP1A1 mRNA expression by cycloheximide in the presence of metyrapone suggests that inhibition of CYP3A1 mRNA transcription by cycloheximide is not caused by a generalized inhibition of mRNA transcription.

Age-dependent changes in dexamethasone binding activity in rat liver. Table 2 indicates that in 21-day-old rats, microsomal dexamethasone binding activity is undetectable (using up to 500 nM radiolabeled dexamethasone) but is readily detectable at 14 pmol/mg of microsomal protein (K_D, 52 ± 17 nm; mean and mean ± standard deviation of three determinations) in 90-day-old rats. RU486 is relatively ineffective at competing for dexamethasone binding in microsomes (Table 2), which distinguishes this binding site from the GR. Table 2 also indicates that metyrapone competes with dexamethasone binding in microsomes as previously noted (26); however, the CYP1A inducer β-naphthoflavone is also a relatively weak competitor of dexamethasone binding in microsomes.

Analysis of glucocorticoid binding in rat liver soluble liver fractions indicate that there is a 2-fold increase in specific binding activity between 21- and 90-day-old rats (Table 3). Scatchard analysis suggests that this is not caused by a difference in affinity of dexamethasone for the GR (data not shown). Table 3 indicates that metyrapone at a concentration of 1 mM (4×10^4-fold molar excess) does not compete with dexamethasone for binding to the GR in soluble fractions from both 21- and 90-day-old rats, which suggests that it is not a ligand for the GR. Co-incubation of the glucocorticoid antagonists pregnenolone 16α-carbonitrile and RU486 (23, 40) demonstrate that both these compounds compete for dexamethasone binding to the GR, although pregnenolone

[2] J. Harvey, A. Paine and M. Wright, unpublished observations.

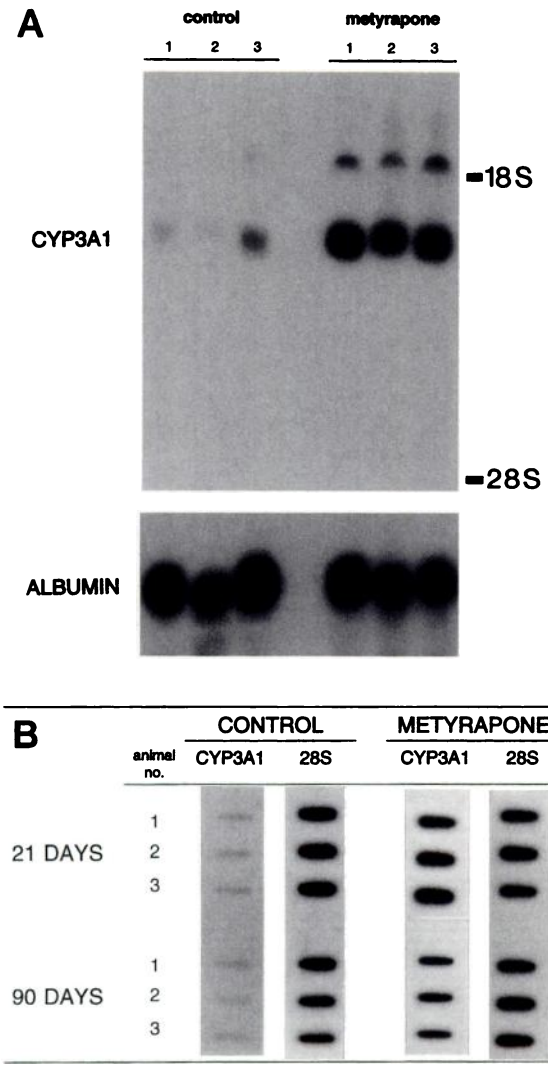

Fig. 3. Effect of metyrapone administration on hepatic CYP3A1 mRNA expression in 21- and 90-day-old male rats. Total RNA was isolated from rats (3 animals/treatment) 24 hr after administration of metyrapone or vehicle control. A, Northern blot of 21-day-old rats treated with metyrapone or saline control, 20 μg of hepatic RNA/lane probed for CYP3A1 (*top*) and albumin (*bottom*). B, Slot blots were performed on total RNA samples over a 3-fold dilution and probed for CYP3A1 or 28-S rRNA. Results presented are 2.5 μg of RNA/slot.

16α-carbonitrile is a less potent competitor in 90-day-old rat soluble fractions than 21-day-old rat soluble fractions. This is in contrast with RU486, which is an equipotent competitor in both fractions (Table 3). Examination of the concentration dependent competition of pregnenolone 16α-carbonitrile for dexamethasone binding to the GR in both 21- and 90-day-old rat liver soluble fractions (Fig. 7) indicate that pregnenolone 16α-carbonitrile has an IC_{50} concentration that is ~10-fold lower in 21-day-old hepatic soluble fractions. It is also apparent that in 90-day-old rat liver soluble fractions, pregnenolone 16α-carbonitrile does not fully displace the specific binding of dexamethasone as suggested in Table 3.

Discussion

The results presented here indicate that metyrapone administration to rats leads to a transcriptional activation of

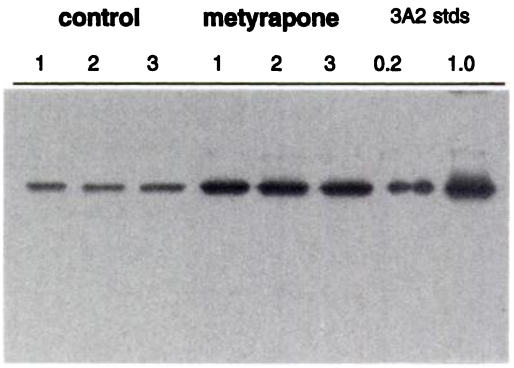

Fig. 4. Effect of metyrapone administration on hepatic microsomal CYP3A expression in 21-day-old male rats. Liver microsomal fractions were isolated from rats (3 animals/treatment) 24 hr after metyrapone or vehicle control administration. *Lanes 1–3*, control animals (1 μg of microsomal protein/lane); *lanes 4–6*, metyrapone-treated animals (1 μg of microsomal protein/lane); *lane 7*, 0.2 pmol of purified CYP3A2; *lane 8*, 1 pmol purified CYP3A2.

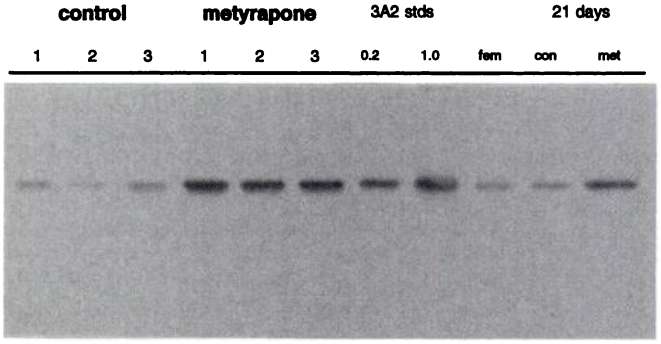

Fig. 5. Effect of metyrapone administration on hepatic microsomal CYP3A expression in 90-day-old rats. Liver microsomal fractions were isolated from male rats (3 animals/treatment) 24 hr after metyrapone or vehicle control administration. *Lanes 1–3*, control animals (1 μg of microsomal protein/lane); *lanes 4–6*, metyrapone treated animals (1 μg of microsomal protein/lane); *lane 7*, 0.2 pmol of purified CYP3A2; *lane 8*, 1 pmol of purified CYP3A2; *lane 9*, 90-day-old female rat liver microsomes (1 μg of microsomal protein/lane); *lane 10*, 21-day-old control rat liver microsomes (1 μg of microsomal protein/lane); *lane 11*, 21-day-old metyrapone-treated rat liver microsomes (1 μg of microsomal protein/lane).

CYP3A1 gene expression in the liver within 1 hr, which leads to increases in the levels of CYP3A1 mRNA, microsomal CYP3A protein and total heme-containing-CYP levels. The results unequivocally demonstrate that a substituted pyridine—metyrapone—is a transcriptional inducer of CYP3A1 and increases the list of structurally unrelated compounds that are transcriptional inducers of this gene in the rat, including glucocorticoids, imidazole antimycotic drugs, and barbiturates (25).

The transcriptional induction of CYP3A1 is a direct effect of metyrapone on the liver, because metyrapone induces CYP3A1 mRNA levels in hepatocytes cultured in a serum- and glucocorticoid-free culture medium. The effects of metyrapone observed *in vivo* are therefore not related to its effects on glucocorticoid and mineralocorticoid synthesis in the adrenals (41). The induction of CYP3A1 by metyrapone does not appear to be mediated by the GR, because metyrapone does not bind to the GR and did not induce tyrosine aminotransferase activity *in vitro*, in contrast with hydrocortisone 21-hemisuccinate (data not shown). However, hydro-

TABLE 1

Effect of metyrapone administration to 21 day and 90 day old male rats on the levels of microsomal androstenedione hydroxylase activity and total cytochrome P450

Rats were treated i.p. with metyrapone as outlined in experimental procedures section and hepatic microsomes prepared 24 hours post injection for analysis. Data are mean ± standard deviation determinations in microsomes from 3 separate animals.

TREATMENT	21 DAYS OLD			90 DAYS OLD		
	total CYP450 pmoles/mg protein	metabolite	activity pmoles/min/mg protein	total CYP450 pmoles/mg protein	metabolite	activity pmoles/min/mg protein
CONTROL	460 ± 52	6β	330 ± 80	700 ± 65	6β	450 ± 100
		7α	380 ± 140		7α	130 ± 10
METYRAPONE	900 ± 66*	6β	600 ± 30*	1020 ± 69*	6β	440 ± 100
		7α	390 ± 60		7α	130 ± 10

* Significantly different from control P > 95% using Student's T test (two tailed).

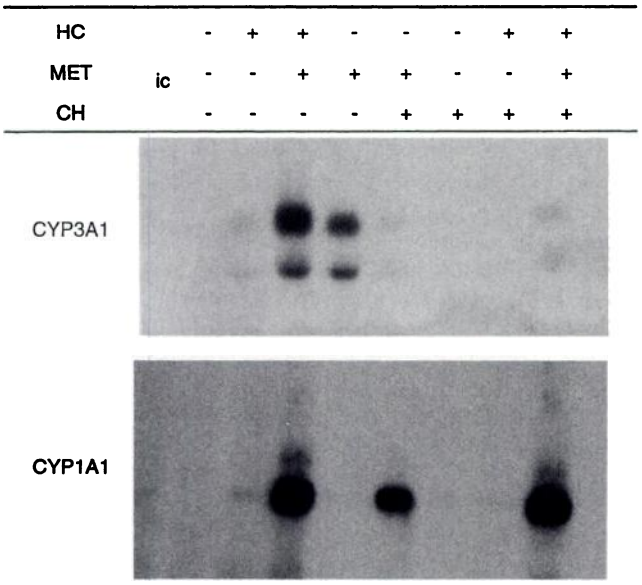

Fig. 6. Effect of metyrapone on CYP3A1 expression in cultured male rat hepatocytes, potentiation by glucocorticoid, and inhibition by cycloheximide. Total RNA was isolated from hepatocytes seeded in medium supplemented with 1 μg/ml fibronection for 2 hr and then treated with medium without fibronectin for 24 hr, additionally containing 0.5 mM metyrapone (*MET*), 100 μM hydrocortisone 21-hemisuccinate (*HC*) and 10 μM cycloheximide (*CH*) alone or in combination as indicated. *ic*, Isolated cells at time of seeding. RNA (20 μg/lane) was subjected to Northern blotting and probed with CYP3A1 oligo or CYP1A1 cDNA. Results are one experiment typical of three.

TABLE 2

Specific levels of microsomal steroid binding activity in 21 and 90 day old male rats

Microsomal fractions were prepared from 21 day and 90 day old rats and incubated with 100nM [^{3}H]-dexamethasone (38Ci/mmol) for 24 hours at 0°C. PCN, pregnenolone 16α-carbonitrile; MET, metyrapone. Data are the mean ± standard deviation determined in microsomes from 3 separate animals.

	21 Days Old	90 Days Old
	Levels of Expression R_{max} (pmoles/mg microsomal protein)	
	n/detect	14 ± 2.4
		Effect of Competitors (percentage of control binding)
RU486 10μM	—	94 ± 28.1
PCN 100μM	—	55 ± 8.3*
β-NF 100μM	—	79 ± 2.2*
MET 1mM	—	23 ± 1.4*

n/detect = not detectable.
* Significantly different from control P > 95% using Student's T test (two tailed).

TABLE 3

Specific levels of cytosolic glucocorticoid receptor in 21 day and 90 day old male rats

Cytosolic fractions were prepared from 21 day and 90 day old rats and incubated with 25nM [^{3}H]-dexamethasone (83Ci/mmol) for 24 hours at 0°C. PCN, pregnenolone 16α-carbonitrile; MET, metyrapone. Data are the mean ± standard deviation determined in soluble fractions from 3 separate animals.

	21 Days Old	90 Days Old
	Levels of Expression R_{max} (fmoles/mg cytosolic protein)	
	160 ± 24	330 ± 33
	Effect of Competitors (percentage of control binding)	
RU486 10μM	n/detect*	n/detect*
PCN 100μM	1 ± 0.5*	40 ± 5.9*
β-NF 100μM	97 ± 14.5	95 ± 3.1
MET 1mM	95 ± 5.4	97 ± 2.2

n/detect = not detectable.
* Significantly different from control P > 95% using Student's T test (two tailed).

cortisone 21-hemisuccinate potentiated the induction of CYP3A1 by metyrapone, which suggests that the GR may mediate the potentiation of CYP3A1 when activated by agonists. Such a mechanism may also apply in human liver because metyrapone induces CYP3A protein in human hepatocytes (42).

The transcriptional induction of CYP3A1 by metyrapone is inhibited by cycloheximide as observed *in vivo* using glucocorticoid inducers (28), which suggests that the transcriptional induction of CYP3A1 requires ongoing protein synthesis. However, these data are in contrast with the superinducing effects of cycloheximide on dexamethasone-mediated CYP3A1 mRNA induction in cultured hepatocytes (18).

Assuming that glucocorticoid induction of CYP3A1 is driven by a GR-independent mechanism, metyrapone is a useful competitor in receptor-ligand binding studies to identify potential proteins involved in CYP3A1 regulation because it is not a glucocorticoid, does not compete for dexamethasone binding to the GR, and does not induce genes under GR control (43). Therefore, it may be possible to distinguish between dexamethasone binding to the GR and other possible sites of dexamethasone-protein interaction. A specific binding site that is shared by both dexamethasone

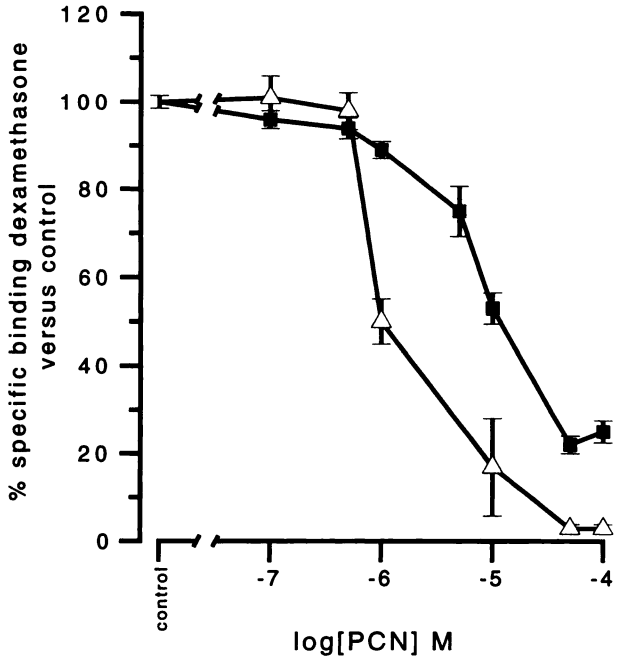

Fig. 7. Effect of pregnenolone 16α-carbonitrile on the specific binding of dexamethasone to male rat liver soluble fractions from 21- (△) and 90-day-old (■) rats. Soluble fractions were prepared as outlined in Experimental Procedures and incubated with 25 nM [^{3}H]dexamethasone and with the indicated concentration of pregnenolone 16α-carbonitrile for 24 hr at 0°. Free ligand was removed by dextran/charcoal adsorption and nonspecific binding determined by co-incubation with excess unlabeled dexamethasone. Data are mean ± standard deviation of three separate incubations from the same preparation and are typical of at least three separate experiments.

and metyrapone and other CYP3A1 inducers, such as pregnenolone 16α-carbonitrile, phenobarbitone (26), and clotrimazole,[3] is that which is found in liver microsomal membrane preparations (26, 27). However, the absence of detectable membrane-bound dexamethasone binding activity in 21-day-old rat liver suggests that this binding activity is not involved solely in the transcriptional regulation of CYP3A1 expression. The equivalent -fold induction of CYP3A protein induced by metyrapone in 21- and 90-day-old rats (Fig. 4) coupled with a lower level of CYP3A1 premRNA transcription and CYP3A1 mRNA expression in 90-day-old rats (Figs. 1 and 2), suggests that post-transcriptional mechanisms of CYP3A1 regulation are operating in 90-day-old rats as indicated by others (15, 20, 25). The membrane-associated binding site for CYP3A inducers may therefore have a post-transcriptional regulatory role in CYP3A1 expression. Although steroids have been studied for their transcriptional effects on gene expression, they have also been demonstrated to have epigenetic effects in cells (44). For example, estrogen transcriptionally activates vitellogenin gene expression in *Xenopus laevis* hepatocytes but also stabilizes vitellogenin mRNA in the cytoplasm (45). Steroids have also been demonstrated to affect Ca^{2+} channel activity (46, 47) and to stimulate phosphatidylcholine-specific phospholipase C activity (48). Therefore, the presence of a steroid/CYP3A inducer binding protein in the cellular compartment where CYP3A1 is synthesized may be associated with a mechanism for increasing steroid/CYP3A1 mRNA stabilization and/or mRNA translation efficiency.

Data presented in this report indicate that there are age-dependent changes in the dexamethasone binding activity of rat liver cytosol. The apparent increase in specific binding activity in adult animals is accompanied by an increase in IC_{50} levels for pregnenolone 16α-carbonitrile competition for total dexamethasone binding. These data suggest that in adult animals, there is expression of a binding site that shows a markedly differential selectivity for dexamethasone over pregnenolone 16α-carbonitrile compared with that observed in young animals. The existence of a putative dexamethasone-specific GR variant may rationalize the observation that dexamethasone synergistically increases pregnenolone 16α-carbonitrile-mediated induction of CYP3A1 (21, 49) despite the fact that pregnenolone 16α-carbonitrile should antagonize dexamethasone effects mediated by the GR. In many species, two isoforms of the PR have been shown to exist (50–52). In humans, there are 114-kDa (PR-A) and 94-kDa (PR-B) forms. The PR-A form contains an additional amino-terminal 164-amino-acid fragment to the PR-B form; otherwise, they are identical and arise either as a result of alternate initiation of translation from the same mRNA or by transcription from alternate promoters within the same gene (51). A further PR isoform of around 45–50 kDa has also been postulated to be expressed (53). Therefore it is possible that a GR or other steroid receptor variant expressed from a single gene is responsible for mediating the transcription of CYP3A1. Indeed, alternate splicing of the human GR premRNA has been reported to code for a highly homologous protein, which may potentially act as an inhibitor of glucocorticoid action (54).

The data presented in this report suggest that the mechanism for transcriptional induction of CYP3A1 by glucocorticoids and structurally unrelated inducers could be mediated by a single GR-independent mechanism that may be potentiated by glucocorticoid agonists through the GR.

[3] M. Wright, unpublished observations.

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