EFFECT OF THE ADRENAL 11-ß-HYDROXYLASE INHIBITOR METYRAPHONE ON HUMAN HEPATIC CYTOCHROME P-450 EXPRESSION: INDUCTION OF CYTOCHROME P-450 3A4

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(Received June 16, 1999; accepted September 23, 1999)

This paper is available online at http://www.dmd.org

ABSTRACT:
The drug metyrapone in the presence of glucocorticoid has been shown to induce the expression of rat hepatic cytochrome P-450 (CYP) 1A1 mRNA in vivo and in vitro through disruption of endogenous CYP1A1 regulator homeostasis and without either compound’s binding to the aryl hydrocarbon receptor. Addition of metyrapone to human liver cancer cell cultures, with or without dexamethasone, did not induce CYP1A1 mRNA, in contrast to the aryl hydrocarbon receptor ligand β-naphthoflavone. Addition of metyrapone to primary cultures of human hepatocytes also failed to induce detectable levels of CYP1A1 mRNA or CYP1A1 protein in two separate preparations, whereas the treatment with 2,3,7,8-tetrachlorodibenzo-p-dioxin or omeprazole induced detectable levels of CYP1A1 mRNA in one preparation and CYP1A1 protein in both preparations. Addition of metyrapone to human hepatocyte cultures resulted in the induction of CYP3A4 expression. The pregnane X receptor (PXR), which has recently been shown to mediate the transcriptional induction of CYP3A4 expression in response to rifampicin, was activated by metyrapone in CV-1 cells transiently cotransfected with an expression vector encoding the human PXR and a reporter construct containing the everted repeat sequence that confers CYP3A4 induction responsiveness to inducers within its promoter. Metyrapone activated the human PXR at concentrations that also resulted in the induction of CYP3A4 in human cultured hepatocytes. Metyrapone treatment is therefore unlikely to result in the induction of CYP1A1 but may induce the expression of CYP3A4 in humans.

Cytochrome P-450s (CYP)2 are heme-thiolate proteins that play a prominent role in the metabolism of both endogenous and exogenous compounds (Nelson et al., 1996) and are widely acknowledged to be primary determinants in the toxicity and/or carcinogenicity of exogenous compounds such as drugs (Paine, 1995). Metyrapone [2-methyl-1,2-bis-(3-pyridyl)-1-propanone] is used clinically to block adrenal CYP11β hydroxylase activity (CYP11B1), which functions to convert deoxycortisol to cortisol (Dominiguez and Samuels, 1963). Metyrapone administration is used as a treatment for Cushing’s syndrome (Verhelst et al., 1991); as a treatment for depression (Mitchell and O’Keane, 1998); to test for hypothalamic-pituitary-adrenal axis function (Deguchi et al., 1998); to prevent tumor metastasis after surgery (Deguchi et al., 1998); to test for hypothalamic-pituitary-adrenal axis function (Fiad et al., 1994); and may be of clinical value in reducing brain damage as a result of hypoxia-ischemia (Kruegers et al., 1998).

This work was supported in part by a short-term fellowship from the European Science Foundation (to J.L.H.).

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2 Abbreviations used are: CYP, cytochrome P-450; AhR, aryl hydrocarbon receptor; β-NE, β-naphthoflavone; CAT, chloramphenicol acetyl transferase; ER6, everted repeat; Gra-PDH, glyceraldehyde phosphate dehydrogenase; PXR, pregnane X receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; XRE, xenobiotic response element; TBS-T, Tris-buffered saline containing 0.05% Tween 20; DMEM, Dulbecco’s modified Eagle’s medium.

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Work by this group has demonstrated that metyrapone is an inducer of rat hepatic CYP1A1 and CYP3A gene expression (Wright et al., 1994, 1996; Harvey et al., 1998). CYP1A1 is prominent in the metabolism and activation of many polyaromatic hydrocarbons to genotoxic intermediates (Shimada et al., 1992), and its polymorphic inducibility in humans has been shown to be linked to lung cancer (Kawaijiri et al., 1995). CYP1A1 has been shown to be inducible in human gut (Buchthal et al., 1995), and therefore the oral administration of metyrapone for long periods may increase the risk of developing cancer in the alimentary canal in particular, as well as in other tissues. The CYP3A subfamily of CYPs are expressed constitutively at a high level in human gut and liver and metabolize a broad range of structurally diverse drugs and xenobiotics (Wrighton and Stevens, 1992). Administration of metyrapone may therefore affect the metabolism of other drugs.

Human liver cell culture is a convenient model system to examine the ability of xenobiotics to modulate drug-metabolizing enzyme expression (Curi-Pedrosa et al., 1994). In view of the effects of metyrapone on rat hepatic CYP1A1 and CYP3A expression, the effect of metyrapone on human CYP1A1 and CYP3A expression has been examined.

Materials and Methods

The human liver cancer cell lines HepG2 and Hep3B and the monkey kidney fibroblast CV-1 cell line were purchased from the European Collection of Animal Cell Cultures (Salisbury, UK). A plasmid containing the human glyceraldehyde phosphate dehydrogenase (Gra-PDH) cDNA was obtained from Dr. P. Fort (Fort et al., 1985). Oligonucleotides were synthesized by Amersham Pharmacia Biotech (Amersham, Bucks, UK). [α-32P]dCTP and [γ-32P]ATP (>3000 Ci/mol) were purchased from Amersham Pharmacia.
Biotech. Goat anti-rat CYP1A anti-sera was purchased from Daiichi Pure Chemicals (Tokyo, Japan), and microsomes from human B lymphoblastoid cells stably expressing human CYP1A1 were purchased from Gentest Corp. (Woburn, MA). Antibodies raised to C-terminal peptide sequences present in CYP3A4/CYP3A7 and CYP3A5 were provided by Dr. Rob Edwards (Royal Postgraduate Medical School, London, UK) (Hakkola et al., 1996). Metryrapone and rifampicin were purchased from Sigma Chemical Co. (Poole, Dorset, UK). 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and omeprazole were generous gifts from Dr. A. Smith (University of Leicester, Leicester, UK) and Astra (Molndal, Sweden), respectively. The minimal dioxin-responsive reporter vector pTIX1/xin was provided by Dr. L. Poellinger (Berghard et al., 1993); the pBLHA1CAT reporter plasmid was constructed by polymerase chain reaction amplification of the human CYP1A1 S′-flanking sequence (~1560 to +88, relative to the transcription start site), which was then fused to the promoter-enhancerless plBlvector (Luckow and Schutz, 1987), as previously outlined (Daujat et al., 1996); and the pSV-Gal plasmid was purchased from Promega (Southampton, UK) (Lehmann et al., 1998). All other chemicals were of the highest purity available from local commercial sources.

**Cell Culture.** HepG2 and Hep3B and cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% v/v heat-inactivated fetal calf serum (Life Technologies, Inc., Rockville, MD), 1% v/v nonessential amino acids (Life Technologies, Inc.), 0.3% amphotericin B (Fungizone), and 1% v/v penicillin-streptomycin. CV-1 cells were cultured in DMEM supplemented with 10% fetal calf serum (Life Technologies, Inc.) and 1% penicillin-streptomycin. The cells were cultured in a humidified atmosphere of 5% CO2/95% air at 37°C and routinely passaged by trypsinization when they were approximately 90% confluent.

Human hepatocytes were isolated from discarded surgical material by collagenase perfusion as described previously (Diaz et al., 1990), after hepatectomies from patients with secondary hepatic tumors. The use of this tissue in these studies was approved by the French Ethics Committee. Hepatocyte culture FT122 was prepared from liver taken from a 61-year-old female with a primary hepatocellular carcinoma; hepatocyte culture FT123 was prepared from liver taken from a 66-year-old male subjected to a left hepatectomy; and hepatocyte culture FT123 was prepared from liver taken from a 66-year-old man after metastasis of a colon primary tumor. Hepatocytes routinely had a viability of 90%, as determined by trypan blue exclusion, and were cultured at a density of 10 million cells/ml of culture medium on collagen-coated (95–98% type 1), 100-mm diameter plates (ICN Pharmaceuticals, Paisley, Scotland). Hepatocytes were cultured for the first 4 h in 1:1 (v/v) Ham’s F12/Williams’ medium. Hepatocytes were harvested, extracts prepared by freeze-thaw, and normalized CAT activities determined as described (Daujat et al., 1996).

**Northern Blotting.** Total RNA samples were denatured and size fractionated through a denaturing formaldehyde/agarose gel (1.3% w/v agarose gel) and transferred from the gel to a nylon membrane (Hybond-N, Amersham, UK) as described previously (Wright et al., 1996). The CYP1A1 and Gra-PDH cDNA probes were labeled by random priming and oligonucleotides designed to hybridize to 28S rRNA (5′-AACGTACAGGATGTGATTTACC-3′) and CYP3A7 mRNA (5′-AATCTACCTCCCCAGCACTA-3′) and CYP3A5 mRNA (5′-AATCTACCTCCCCAGCACTA-3′) by 5′-end labeled with [γ-32P]ATP and polynucleotide kinase using kits from Promega. Radiolabeled probes were hybridized to immobilized RNA in Quickhyb solution (Stratagene, Southampton, UK) under the conditions recommended by the manufacturers. After washing, membranes were wrapped in Saran wrap and exposed to autoradiographic film at −70°C.

**Western Blotting.** Hepatocytes were washed with PBS, scraped in 1 ml of PBS, and centrifuged at 500 g for 3 min to pellet the cells. The supernatant was removed, and the pellet was resuspended in 20 mM Tris-HCl buffer, pH 7.5, containing 250 mM sucrose, trurrax homogenized, and an aliquot of the cell extract homogenate was removed for protein analysis (Lowry et al., 1951) using BSA as standard. Cell extracts were examined for CYP expression by Western blotting after separation of proteins on 9% w/v separating/4% stacking discontinuous SDS-polyacrylamide gels under reducing conditions. Proteins were transferred to nitrocellulose (Schleicher & Schuell, Anderman & Co., Kingston, Surrey, UK), and nonspecific binding sites were blocked by incubation with 3% BSA in 20 mM Tris-Cl-200 mM NaCl buffer, pH 7.4 (TBS) containing 0.3% (v/v) Tween 20 overnight at 4°C. Membranes were incubated in TBS containing 0.05% Tween 20 (TBS-T) with 0.3% w/v BSA with either goat anti-rat CYP1A, which cross-reacts with human CYP1A; sheep anti-human CYP3A4 (Dalet-Beluche et al., 1992); rabbit anti-TSGA, an antibody raised to the C terminus peptide sequence present in CYP3A4 and CYP3A7 (Hakkola et al., 1996); or rabbit anti-TLSGE, an antibody raised to the C terminal peptide sequence of CYP3A5 (Hakkola et al., 1996). Membranes were then washed in TBS-T, incubated with the appropriate horseradish peroxide-conjugated anti-IgG antibody in TBS-T with 0.3% w/v BSA, and extensively washed with TBS-T. Immunoreactive proteins were detected by exposing X-ray film to chemiluminescence generated from a reaction mediated by the antibody-conjugated horseradish peroxidase, using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech).
mRNA expression in both cell lines (Fig. 1). Fig. 1 also indicates that addition of dexamethasone to β-NF-treated cells did not further increase the levels of induction of CYP1A1 mRNA induced by β-NF.

Figure 2A shows that CYP1A1 mRNA was undetectable in hepatocytes isolated from two human livers and that CYP1A1 mRNA was induced in cultured hepatocytes by the potent AhR ligand TCDD in preparation FT123 but not FT122. The induction of CYP3A4 in preparation FT122 (Fig. 3) indicates that the absence of CYP1A1 induction by TCDD is likely to be associated with a specific defect in CYP1A1 gene expression and not associated with a nonspecific response. FT123 hepatocyte CYP1A1 mRNA was also induced by the addition of omeprazole, which has been predicted to be metabolized to a novel class of AhR ligand and agonist (Dzeletovic et al., 1997). Despite the presence of dexamethasone in the basal culture medium for human hepatocytes (at 100 nM), addition of metyrapone did not result in the detectable induction of CYP1A1 mRNA in human hepatocyte FT122 and FT123 cultures (Fig. 2A). In rat hepatocyte culture, metyrapone-dependent induction of CYP1A1 requires the presence of a glucocorticoid at levels that activate the glucocorticoid receptor (Wright et al., 1996). However, the protein synthesis inhibitor cycloheximide may substitute for the permissive role of dexamethasone with respect to metyrapone-dependent CYP1A1 mRNA induction (Harvey et al., 1998). Both TCDD-dependent and omeprazole-dependent induction of CYP1A1 mRNA in preparation FT123 was further increased by the addition of the protein synthesis inhibitor cycloheximide (Fig. 2A). However, the presence of cycloheximide in culture medium containing metyrapone had no effect on the refractivity of FT122 and FT123 hepatocytes to CYP1A1 mRNA induction by metyrapone (Fig. 2A).

Figure 2B shows that whole-cell extracts prepared from FT122 and FT123 isolated cells contain readily detectable levels of CYP1A protein, likely associated entirely with the expression of the constitutively expressed CYP1A2 (Forrester et al., 1992). The levels of CYP1A fall to undetectable levels in culture FT122 in control and metyrapone-containing cultures, whereas TCDD-treated hepatocytes had increased levels of CYP1A protein (Fig. 2B). In culture FT123, CYP1A levels decrease in control cells but remain detectable. Addition of metyrapone resulted in higher levels of CYP1A protein than were detectable in control cultures. However, the levels remained less than those present in the isolated cells from which the culture was made. In contrast, both TCDD and omeprazole induced CYP1A to greater levels than were present in the isolated cells (Fig. 2B).

**Effect of Metyrapone and Dexamethasone on Xenobiotic Response Element (XRE) and Human CYP1A1 5′-Flanking Region-Driven Reporter Gene Expression in Transiently Transfected HepG2 Cells.** Table 1 shows that pTX1X/Inv reporter gene expression was not induced in response to metyrapone or dexamethasone treatment and was weakly induced, 1.5-fold, when these compounds were coadministered. Addition of the AhR agonist TCDD markedly increased pTX1X/Inv reporter gene expression, 8.6-fold (Table 1). Coaddition of dexamethasone with TCDD did not significantly affect the levels of pTX1X/Inv reporter gene expression (Table 1). pBLh1AICAT reporter gene expression also was not induced in response to metyrapone or dexamethasone treatment but was significantly induced, 2.1-fold, when these compounds were coadministered (Table 1). Addition of the AhR agonist TCDD increased pBLh1AICAT reporter gene expression 4.9-fold, although, in contrast to pTX1X/Inv, coadministration of dexamethasone with TCDD further increases the levels of pBLh1AICAT reporter gene expression (Table 1).

**Effect of Metyrapone on CYP3A Expression in HepG2 and Human Hepatocyte Culture.** Metyrapone has been demonstrated to be a transcriptional inducer of the rat CYP3A1/CYP3A23 genes (Wright et al., 1996). Figure 3 indicates that CYP3A proteins were readily inducible in human hepatocytes from preparations FT122 and HTL98 in response to the addition of the potent human CYP3A inducer rifampicin (Pichard et al., 1990) and metyrapone to culture media. The response is also observed when extracts are probed with an antibody that immunoreacts with the major expressed CYP3A4 and the fetal expressed CYP3A7. CYP3A7 is polymorphically, lowly expressed in adult liver, and expression of the protein is not induced in response to rifampicin treatment in adult human liver (Greu et al., 1996). This suggests that the protein induced in response to metyrapone treatment in human liver cells is CYP3A4. The HepG2 cell line has been shown to induce the expression of CYP3A7 mRNA in

A Northern blot of total RNA (20 μg/lane) from hepatocytes (lanes 1–7, culture FT122; lanes 8–16, culture FT123) cultured for 24 h with treatments: lane 1, isolated cells (IC); lane 2, vehicle solvent only (CONTROL); lane 3, 500 μM metyrapone (MET); lane 4, 1 nM TCDD; lane 5, 8 μM cycloheximide (CYCLO); lane 6, 8 μM cycloheximide and 500 μM metyrapone (CYCLO/MET); lane 7, 8 μM cycloheximide and 1 nM TCDD (CYCLO/TCDD); lane 8, isolated cells (IC); lane 9, vehicle solvent only (CONTROL); lane 10, 500 μM metyrapone (MET); lane 11, 1 nM TCDD; lane 12, 20 μM omeprazole (OM); lane 13, 8 μM cycloheximide (CYCLO); lane 14, 8 μM cycloheximide and 500 μM metyrapone (CYCLO/MET); lane 15, 8 μM cycloheximide and 1 nM TCDD (CYCLO/TCDD); lane 16, 8 μM cycloheximide and 20 μM omeprazole (CYCLO/OM). Note, 100 nM dexamethasone was present in basal culture medium. Upper panel was probed with radiolabeled CYP3A1 cDNA, lower panel was probed with radiolabeled 28S rRNA oligonucleotide probe. B, effect of metyrapone on the levels of CYP1A protein in human hepatocyte cultures. Western blot of total cell protein (15 μg/lane) from hepatocytes cultured for 4 days: lanes 1–7, culture FT122; lanes 8–12, culture FT123. Lane 1, isolated cells (IC); lane 2, vehicle solvent only (CONTROL); lane 3, 2.5 μM metyrapone; lanes 4–7, culture FT122; lanes 8–12, culture FT123. Lane 1, 1 μg of liver microsomal protein from three methylcolanthrene-treated rats; lane 2, 2.5 μg of human liver (FT61) microsomal protein; lane 3, 2.5 μg of human liver (FT61) microsomal protein; lane 4, isolated cells (IC); lane 5, vehicle solvent only (CONTROL); lane 6, 500 μM metyrapone (MET); lane 7, 1 nM TCDD; lane 8, isolated cells (IC); lane 9, vehicle solvent only (CONTROL); lane 10, 500 μM metyrapone (MET); lane 11, 1 nM TCDD; lane 12, 20 μM omeprazole (OM); lane 13, 0.1 pmol of CYP1A1; lane 14, 0.2 pmol of CYP1A1; lane 15, 0.6 pmol of CYP1A1 (lanes 13–15, microsomes from human B lymphoblastoid cell line AHH-1 stably transfected with human CYP1A1 (AHH)). The blot was probed with anti-rat CYP1A antisera.

response to rifampicin but does not express CYP3A4 and CYP3A5 mRNAs (Schuetz et al., 1993). In our hands, CYP3A7 mRNA levels in HepG2 cells were at the limit of detection by Northern blotting, and treatment daily for 3 days with metyrapone did not result in an induction of CYP3A7 mRNA levels (data not shown). This observation therefore supports the suggestion that metyrapone induces the expression of CYP3A4 but not CYP3A7 proteins.

Figure 4 demonstrates that the induction of CYP3A4 protein by metyrapone in cultured human hepatocytes was dependent on the medium concentration of metyrapone. A metyrapone concentration of 50 μM resulted in a low but measurable increase in CYP3A4 protein compared with control cells. However, a concentration of metyrapone of 500 μM was required to induce a level of CYP3A4 protein similar to that induced by 20 μM rifampicin (Fig. 4).

Table 2 indicates that metyrapone is an activator of the human PXR and that the concentrations required to activate the human PXR as judged by reporter gene expression are similar to the concentrations of metyrapone required to induce the de novo synthesis of CYP3A4 in human hepatocytes (Fig. 4). Figure 4 indicates also, as previously published, that rifampicin and dexamethasone are also activators of the human PXR (Lehmann et al., 1998).
that it is possible to stimulate reporter gene expression regulated by response to metyrapone and glucocorticoid in human liver, we found AhR. Despite the absence of genomic CYP1A1 gene induction in a, 1998). Our previous work with rats suggests that metyrapone does not compete with TCDD for binding to the rat AhR (Harvey et al., 1995). Metyrapone and all of result in increased transcription of the CYP1A1 gene (Denison et al., 1998; Fischer et al., 1990; Hankinson, 1995). Metyrapone and all of


The expression of CYP3A4 may be modulated by many drugs through activation of a recently identified orphan nuclear receptor entitled the PXR (Blumberg et al., 1998; Lehmann et al., 1998). Transcriptional induction of the CYP3A4 gene in response to inducers, including metyrapone, has been mapped to the ER6 DNA sequence present in the 5′ upstream region of the CYP3A4 gene (Barwick et al., 1996; Ogg et al., 1999). Inducers of CYP3A4 activate the PXR to bind in a complex with the retinoid X receptor to the ER6 DNA sequence (Blumberg et al., 1998; Lehmann et al., 1998) and cells transiently transfected with PXR expression vectors up-regulate ER6-reporter gene construct expression in response to inducer treatment (Blumberg et al., 1998; Lehmann et al., 1998). Data presented in this article indicate the first time that metyrapone is an inducer of CYP3A4 expression and an activator of the human PXR [CYP3A5 was undetectable in preparation FT122, FT123, and HTL98 (data not shown)]. Therefore, it is likely that metyrapone induced the CYP3A4 protein in preparations FT122 and HTL98, because it has been shown that CYP3A7 protein, but not mRNA, is refractive to induction by rifampicin in adult human hepatocyte culture (Gruet et al., 1996)). Interestingly, CYP3A4 is not induced by either rifampicin or metyrapone (implying a shared induction mechanism) in preparation FT123 hepatocyte cultures (although CYP3A1 mRNA and CYP1A protein are inducible in response to TCDD and omeprazole), and this lack of induction may be associated with the low response in some humans to CYP3A4 induction. Although relatively high concentrations of metyrapone compared with rifampicin are required to induce CYP3A4 in human hepatocytes, it should be noted that metyrapone may be coadministered drugs or endogenous steroids. The nature of any metabolism of numerous drugs as well as endogenous compounds such as steroids. The induction of CYP3A4 by metyrapone could therefore result in a significant alteration in the metabolism of any coadministered drugs or endogenous steroids. The nature of any drug-steroid metabolism interaction is likely to be complicated by a period of CYP inhibition, because metyrapone is also an inhibitor of many CYP reactions.

This article indicates that metyrapone induces the expression of
CYP3A4 likely through its activation of the human PXR in human hepatocytes. Although metyrapone induces the expression of rat liver CYP1A1 through a disruption in the homeostasis of an endogenous regulator, metyrapone does not induce a comparable level of induction of CYP1A1 in human liver. This species difference in the response of the CYP1A1 gene to induction by disruption in the homeostasis of an endogenous regulator may be specific to human liver and may be related to a relatively low responsiveness of this gene in the liver to induction by numerous inducers compared with rodents. The induction of CYP1A1 by metyrapone in human liver is likely to pose a greater problem to long-term treatment with metyrapone and is as yet unknown.

References


