Pergamon

0960-0760(93)E004-Q

J. Steroid Biochem. Molec. Biol. Vol. 48, No. 2/3, pp. 271–276, 1994

0960-0760/94 $6.00 + 0.00

Induction of Rat Hepatic Glucocorticoid-inducible Cytochrome *P*450 3A by Metyrapone

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The bipyridyl compound metyrapone is a potent inhibitor of cytochromes *P*450, a gene superfamily of haemoproteins involved in the metabolism of many xenobiotics as well as endogenous compounds such as steroid hormones. Administration of metyrapone to male rats induces the expression of the cytochrome *P*450 sub-family 3A (CYP3A). In order to determine whether metyrapone was causing the induction of CYP3A by blocking endogenous glucocorticoid metabolism, CYP3A levels were examined in rat hepatocytes cultured in serum-free medium supplemented with hydrocortisone 21-hemisuccinate plus or minus metyrapone. Western blotting indicated that metyrapone alone induces CYP3A and that hydrocortisone 21-hemisuccinate is ineffective. However, hydrocortisone 21-hemisuccinate enhanced the levels of CYP3A induced by metyrapone. In contrast, glucocorticoid-inducible tyrosine aminotransferase (TAT) activity was unaffected by metyrapone but metyrapone enhanced the levels induced by hydrocortisone 21-hemisuccinate. An examination of the metabolism of hydrocortisone by rat hepatocytes *in vitro* indicated that metyrapone perturbed the catabolism of hydrocortisone under conditions which give rise to an enhancement of hydrocortisone 21-hemisuccinate and hydrocortisone-dependent TAT induction. However, evidence is presented to suggest that such a perturbation of hydrocortisone metabolism could not account for the glucocorticoid potency amplifying property of metyrapone. Thus the induction of CYP3A and the enhancement of glucocorticoid-mediated TAT induction appears not to be associated with any perturbation in glucocorticoid metabolism but with some other as yet undefined mechanism(s).

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INTRODUCTION

The term "cytochromes *P*450" (CYP450) describes a gene superfamily of haemoproteins which mediate the metabolism of many xenobiotics and certain endogenous compounds [1]. High levels exist within the liver as a defence against ingested xenobiotics and are a major determinant in xenobiotic toxicity/carcinogenicity [2]. Many forms are inducible by xenobiotics although endogenous compounds regulate their expression. For example the major constitutively-expressed form in male rat liver is CYP2C11 and its expression is regulated by growth hormone [3]. In contrast CYP1A1 is not constitutively expressed but potently induced by the environmental toxin TCCD [4]. Little is known about the mechanisms which regulate members of the CYP3 family which constitute the majority of CYP450 in human liver [5].

The CYP3A sub-family in rats contains at least two forms—CYP3A1 and CYP3A2—which share 89% amino acid sequence similarity [6]. CYP3A2 is constitutively expressed in adult rat liver whereas CYP3A1 is not constitutively expressed but induced by dexamethasone [6] and the anti-glucocorticoid pregnenolone 16α carbonitrile [7]. Both forms are induced by the classical CYP2B inducer, phenobarbitone [6], a sub-family also induced by metyrapone [8]. A protein of greater electrophoretic mobility which is recognized by polyclonal sera raised to rat CYP3A1 has been observed and is elevated by CYP3A1 inducers although its full amino acid sequence was not reported [9] but may correspond to the recent addition to this sub-family named CYP3A9 (sequence unpublished [10]).

We have examined the effect of metyrapone on the expression of rat hepatic CYP3A since metyrapone has

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Abbreviations: Cytochromes *P*450, CYP450; glucocorticoid potency amplifier, GPA; tyrosine aminotransferase, TAT; 2,3,7,8-tetrachlorodibenzo-p-dioxin, TCCD. The cytochrome *P*450 nomenclature system employed is that recommended by Nelson *et al.* [8].
Received 1 June 1993; accepted 1 Oct. 1993.

been proposed to be a "glucocorticoid potency amplifier" (GPA) [11] and because CYP3A1 is reported to be induced by glucocorticoids although through a mechanism that is distinct from the glucocorticoid receptor [7].

EXPERIMENTAL

Materials

Anti-sera to rat CYP3A and purified CYP3A2 were gifts from Dr D. Waxman (Harvard Medical School, Boston, MA) and Dr J. Halpert (University of Arizona, AZ), respectively. [4-^{14}C]Hydrocortisone (55.0–58.5 mCi/mmol) was purchased from NEN Research Products (Stevenage, Herts, England). All other chemicals were of the highest purity available from commercial sources.

Animal treatment and cell culture

Male Sprague–Dawley CD rats (250–300 g) were used in all experiments. Administration of metyrapone (100 mg/kg body wt), the preparation of cytosolic and microsomal liver fractions, and Western blotting were performed essentially as described previously [8] except that enhanced chemiluminescence (ECL kit, Amersham International, England) was used for immunodetection on Western blots. The chemiluminescence generated by a range of purified CYP3A2 amounts was used to ensure that fold changes in immunodetectable CYP3A protein were within the linear range of the detection system.

Hepatocytes were isolated by collagenase perfusion [8] and cultured in William's Medium E supplemented with 2 nM insulin, 10 nM glucagon and 50 μg/ml gentamycin in a humidified atmosphere of 5% carbon dioxide in air at 37°C on Lux™ 150 mm diameter plates (18 million cells/plate). Where indicated, hydrocortisone 21-hemisuccinate and metyrapone were dissolved directly in the medium.

Tyrosine aminotransferase (TAT) induction in vitro

Hepatocytes were isolated and cultured with additions as indicated above except that insulin was omitted since it has been demonstrated to depress TAT induction in cultured rat hepatocytes [12]. Other additions were made from ethanol solvated stock solutions. After 20 h of treatment the medium was aspirated and the cells scraped in 80 mM *N*-tris (hydroxymethyl) methyl 2-aminoethanesulphonic acid buffer pH 7.3 containing 0.04% (v/v) Triton X-100. The cells were lysed by rapid freeze/thawing in liquid nitrogen and vigorous mixing. TAT activity was measured in 3000 *g* lysed-cell supernatants by the method of Diamondstone [13].

Hydrocortisone metabolism

Hepatocytes were cultured under the basal conditions used for the induction of TAT except that fibronectin (1 μg/ml) was included to promote cell attachment since the medium was replaced after 2 h of culture and fresh medium returned without fibronectin but containing additionally 100 μM hydrocortisone, approx. 50 nCi/ml [^{14}C]4-hydrocortisone (55.0–58.5 mCi/mmol) and where indicated 0.5 mM metyrapone. Cells were incubated for up to 20 h. Medium was removed at various times and frozen at −70°C until analysis by thin layer chromatography according to the method of Nienstedt [14]. Organically extracted metabolites were identified using authentic standards. Aqueous and organically extracted metabolites accounted for 97% of the radioactivity with the remaining as yet unknown contaminants of the tracer stock.

Protein assay

Samples were diluted with water and protein concentration estimated by the method of Lowry *et al.* [15] using bovine serum albumin as standard. At the dilutions employed, Triton X-100 did not interfere with the assay.

RESULTS

Metyrapone and CYP3A

CYP3A1 and CYP3A2 have similar electrophoretic properties which prevent them from being separated by normal electrophoretic techniques [6] and hence immunodetectable protein is referred to as CYP3A. Figure 1 indicates that the administration of metyrapone to male rats significantly increases the levels of immunodetectable CYP3A to around 4-fold the levels present in untreated animals. Metyrapone treatment did not affect the levels of cytosolic TAT activity examined in the same liver samples (data not shown) suggesting that the mechanism of induction of CYP3A by metyrapone is distinct from the glucocorticoid receptor.

In order to determine whether metyrapone exerts its affects on the liver *in vivo* by perturbing the metab-

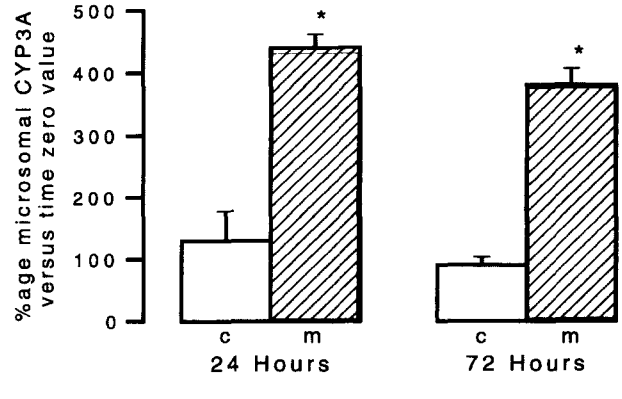

Fig. 1. Effect of metyrapone administration to male rats on the immunodetectable levels of the CYP3A sub-family. Data are the mean and standard deviation of three animals for each timepoint/treatment. c, control saline vehicle; m, 100 mg metyrapone/kg body wt. *Significantly different at 5% level (two tailed) from control values using Student's *t*-test.

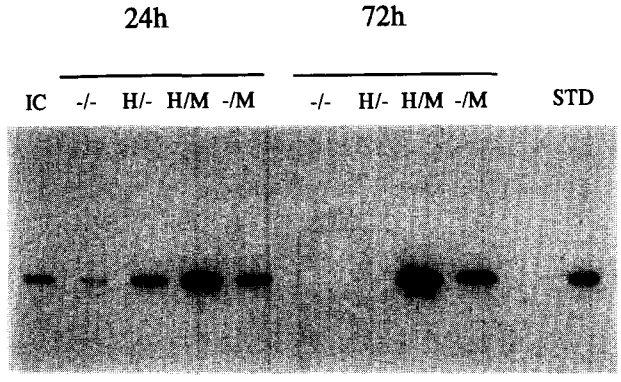

Fig. 2a. Western blot of microsomes (0.6 μg protein/lane) isolated from cultured hepatocytes and probed with anti-CYP3A serum. Cells were cultured as outlined in the Experimental section with medium supplementation where indicated: 100 μM hydrocortisone 21-hemisuccinate, HC; 0.5 mM metyrapone, M. IC, microsomes from freshly isolated cells; STD, 0.2 pmol purified CYP3A2.

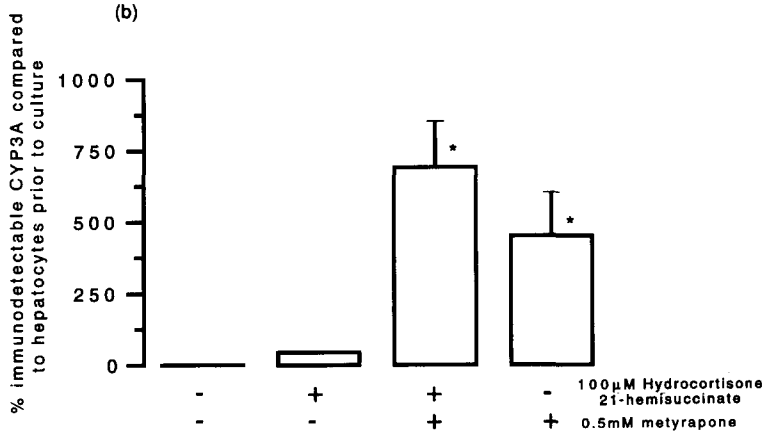

Fig. 2b. Effect of hydrocortisone 21-hemisuccinate and metyrapone on the CYP3A sub-family levels in rat hepatocytes cultured for 72 h. Data are the mean and standard deviation of three separate experiments. *Significantly different at 5% level (two tailed) from control values using Student's *t*-test.

olism of glucocorticoids, the effect of metyrapone and the glucocorticoid hydrocortisone 21-succinate on CYP3A levels were examined in rat hepatocytes cultured in a defined (serum-free) medium. Figure 2a indicates that CYP3A levels rapidly fell in culture and that hydrocortisone 21-hemisuccinate may only slow this loss. In contrast, metyrapone elevates the levels of CYP3A such that after 72 h of culture, the levels are similar to the levels observed in metyrapone treated animals (Fig. 2b). The addition of hydrocortisone 21-hemisuccinate to the culture medium significantly enhances the levels of CYP3A induced by metyrapone 1.6- ± 0.27-fold (mean fold increase from 3 separate experiments, significantly greater at the 5% level using the paired Students' *t*-test) and results in the expression of an immunoreactive protein of greater electrophoretic mobility after 72 h of culture as evidenced by a single distinct band on Western blots (Fig. 2a).

TAT induction in vitro

TAT is a liver-specific glucocorticoid inducible enzyme catalysing the rate limiting step in tyrosine catabolism [16]. Figure 3 indicates that metyrapone potentiates the induction of TAT by hydrocortisone 21-hemisuccinate without inducing TAT itself, a similar effect previously observed with dexamethasone [11].

A change in enzyme catalytic activity may be mediated by the presence of effectors although none have been reported for TAT [16]. With respect to TAT, metyrapone does not affect catalytic activity in liver cytosol when added at a concentration of 100 μM and may inhibit activity at millimolar concentrations although there is considerable interference with the spectrophotometric measurement of hydroxybenzaldehyde (data not shown). However, it is clear that metyrapone does not act as a positive effector for TAT activity and that the increase in specific TAT activity in cells induced in the presence of metyrapone is likely to be associated with an increase in TAT levels.

Effect of metyrapone on hydrocortisone metabolism

Many steroids are catabolized by hepatic CYP450 [3], and so metyrapone-dependent GPA may be caused by a block in glucocorticoid degradation which would effectively prolong the half life of the active hormone. We therefore examined the effect of metyrapone on the metabolism of hydrocortisone under conditions in which metyrapone-dependent GPA is observed.

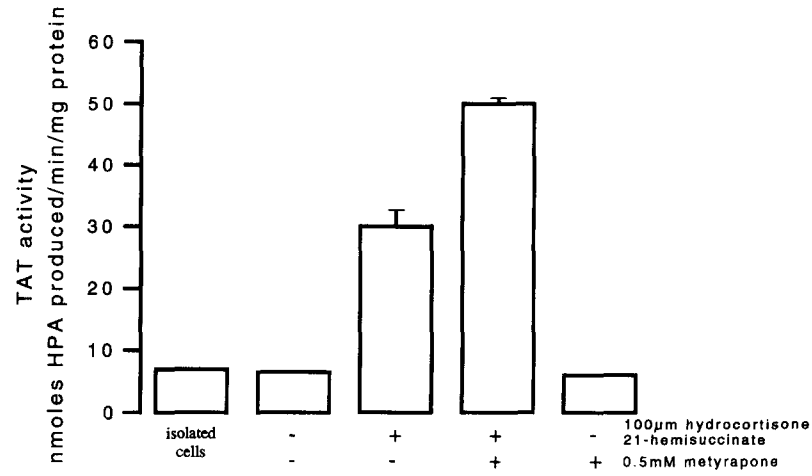

Fig. 3. Effect of metyrapone on the induction of TAT activity by hydrocortisone 21-hemisuccinate in cultured rat hepatocytes. Data are the mean and standard deviation of three separate determinations from the same experiment. One experiment typical of five.

Figure 4 indicates that the disappearance of hydrocortisone is unaffected by metyrapone. Hydroxylated metabolites of hydrocortisone (i.e. potentially CYP450 mediated metabolites) were not observed although the timecourse data suggested that metyrapone reduces the flux of 3α-ketosteroid reduction to 3α-hydroxysteroid. Thus, during the period (0–20 h) examined, the concentration of the metabolites dihydrocortisone and dihydrocortisol were observed to be significantly increased when incubations were performed in the presence of metyrapone at 4 h (Fig. 5a). The levels of water soluble (probably conjugated) metabolites were significantly reduced when incubations were performed in the presence of metyrapone at 20 h (Fig. 5b). The latter observation is consistent with a reduced flux of 3α-ketosteroid reduction to 3α-hydroxysteroids since

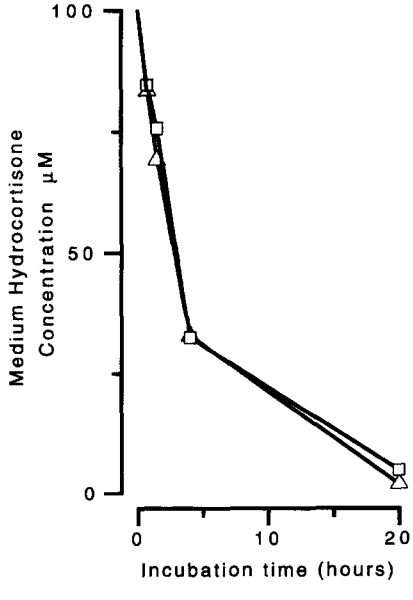

Fig. 4. Medium hydrocortisone levels in rat hepatocyte culture, effect of metyrapone. Data are typical of three separate experiments. Cells cultured in absence of metyrapone (△); cells cultured in presence of 0.5 mM metyrapone (□).

3α-hydroxysteroids are the most actively conjugated metabolites [17]. The effects of metyrapone on hydrocortisone metabolism may be due to competition for 3α-hydroxysteroid dehydrogenase activity since metyrapone is reported to be reduced to metyrapol by a similar activity in human liver [18].

Hydrocortisone metabolites and TAT induction

Figure 6 indicates that hydrocortisone 21-hemisuccinate, hydrocortisone and cortisone induce TAT activity in cultured rat hepatocytes and that the hydrocortisone metabolites that were observed in culture and sulphate-conjugates of the active glucocorticoids are ineffective. Since 100 μM concentrations of 3α-ketosteroids (i.e. maximum possible concentration that could be produced from 100 μM glucocorticoid) do not stimulate TAT induction and the levels of TAT-inducing glucocorticoids are unaffected by the presence of metyrapone, the ability of metyrapone to potentiate the effects of hydrocortisone appear to be unrelated to its perturbation of hydrocortisone metabolism.

DISCUSSION

Metyrapone is an inhibitor of CYP450, a feature which has been used clinically to test for pituitary function and in the treatment of Cushing's Syndrome by virtue of its inhibition of adrenal steroid 11β-hydroxylase (CYP11B1) [19]. Metyrapone also inhibits hepatic CYP450-dependent drug metabolism [20]. The mechanism of inhibition of CYP450 by metyrapone is presumably associated with its ability to ligand the haem prosthetic group [2].

The elevation of CYP3A by metyrapone *in vivo* may therefore be associated with a reduction in glucocorticoid/mineralocorticoid synthesis or be due to a reduction in hepatic-mediated degradation of a repressor. However, the induction of CYP3A by metyrapone may be reproduced in hepatocytes cultured in a

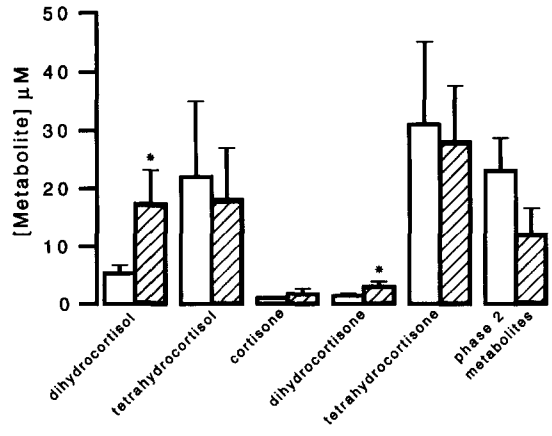

Fig. 5a. Hydrocortisone metabolite levels at 4 h of culture, effect of metyrapone. Data are the mean and standard deviation of three separate experiments, hatched bars represent incubations made in the presence of metyrapone. *Significantly different from incubations in the absence of metyrapone at the 5% level (two tailed) using Student's *t*-test.

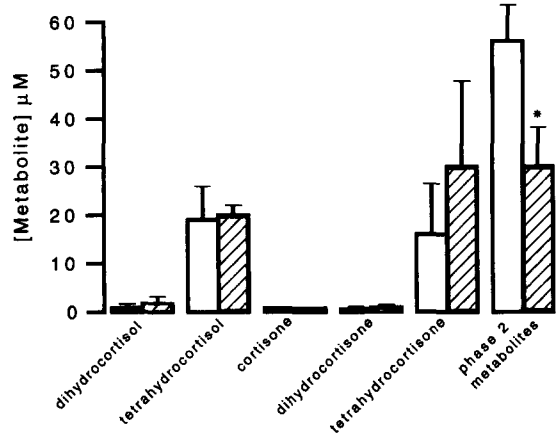

Fig. 5b. Hydrocortisone metabolite levels at 20 h of culture, effect of metyrapone. Data are the mean and standard deviation of three separate experiments, hatched bars represent incubations made in the presence of metyrapone. *Significantly different from incubations in the absence of metyrapone at the 5% level (two tailed) using Student's *t*-test.

fully-defined medium, suggesting that metyrapone acts directly on the liver. Metyrapone also induces mRNA which hybridizes to CYP3A1 cDNA under conditions which give no detectable signal in liver and therefore does not hybridize to the constitutive CYP3A2 mRNA transcript [21]. Therefore, these data together suggest that metyrapone induces the expression of a glucocorticoid-inducible protein at a pretranslational level and that the mechanism of induction is not due entirely to an inhibition in its degradation as reported for macrolide antibiotics [22]. Furthermore, in these studies an anti-CYP3A immunoreactive protein of greater electrophoretic mobility was also induced *in vitro* which may be identical to the dexamethasone-inducible form observed recently [9].

The classical CYP450 inducer phenobarbitone induces the expression of CYP2B [1] and both CYP3A1 and CYP3A2 [6]. Since metyrapone induces CYP2B [8], metyrapone may also affect the CYP3A sub-family in the same manner as phenobarbitone. CYP3A form specific oligonucleotides have been described previously [6] and the effect of metyrapone on the expression of individual CYP3A mRNAs is presently in progress.

The report that metyrapone potentiates dexamethasone-dependent TAT induction was shown to correlate with an inhibition of a CYP450-mediated activity and the authors concluded that a block in dexamethasone metabolism was responsible for the potentiating effects of metyrapone [11]. Little work has been done specifically on the effects of metyrapone on hepatic steroid metabolism although metyrapone has been shown to qualitatively affect rat hepatic xenobiotic metabolism the same way as the steroid hydroxylase inhibitor SU-10'603 [23]. Hydrocortisone has been shown to be metabolized by a CYP450 in human liver [24] but we were unable to detect a metabolite of hydrocortisone that could be generated by a CYP450 under conditions in which GPA is observed. Although hepatic CYP450 may metabolize glucocorticoids, metyrapone may not *a priori* inhibit their metabolism since it is not a universal inhibitor of CYP450 in contrast with another haem ligand, carbon monoxide [25]. Indeed, with respect to one constitutively expressed rat hepatic form, metyrapone enhances its activity [26].

Evidence presented within this report suggests that the ability of metyrapone to act as a GPA with respect to TAT is associated with an increase in enzyme synthesis and analysis of such an effect by Western blotting will confirm or refute this suggestion. However, the apparent potentiation of glucocorticoid-dependent induction is demonstrated to be independent of a perturbation in glucocorticoid metabolism and must therefore also be by a direct interaction of the potentiating factor with for example, a receptor in liver cells.

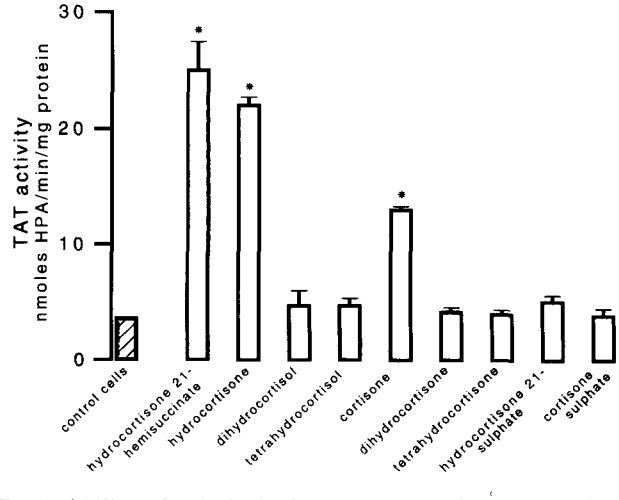

Fig. 6. Ability of 100 μM hydrocortisone and 100 μM hydrocortisone metabolites to induce TAT activity in rat hepatocyte culture. Data are one experiment typical of three. *Significantly different from control cells at the 5% level (two tailed) using Student's *t*-test.

The concentrations of hydrocortisone 21-hemisuccinate and metyrapone which demonstrate a GPA effect on TAT induction also effect a potentiation of CYP3A induction although the inducer and potentiating factor are reversed to metyrapone and hydrocortisone 21-hemisuccinate, respectively, suggesting that metyrapone may be operating through a common mechanism when acting as inducer and potentiator. A consideration of both may help to elucidate a novel glucocorticoid-dependent mechanism of gene regulation which may regulate the major constitutively expressed xenobiotic-metabolizing CYP450 in humans.

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