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CYTOCHROME P4502A5 EXPRESSION AND INDUCIBILITY BY PHENOBARBITAL IS MODULATED BY cAMP IN MOUSE PRIMARY HEPATOCYTES

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SUMMARY: Factors involved in CYP2A5 expression were studied in mouse liver primary hepatocytes in culture. CYP2A5-mediated coumarin 7-hydroxylase (COH) activity was retained in simple culture conditions for at least 96 hours and the activity was inducible up to 33-fold by phenobarbital (PB). The constitutive activity and inducibility of COH was totally blocked by treatment of hepatocytes with actinomycin D, and short initial treatment with cycloheximide caused superinducibility when co-administered with PB. Treatment of hepatocytes with inhibitors of protein kinase C, tyrosine kinases and a generator of nitric oxide did not affect COH basal activity or inducibility. Administration of dibutyryl cAMP, forskolin, and 3-isobutyl-1-methyl-xanthine (IBMX) enhanced both basal and PB-induced COH activities and CYP2A5 mRNA levels, indicating that cAMP plays a major role in CYP2A5 expression.

The cytochrome P450 (CYP) superfamily of genes encodes enzymes that mediate the metabolism of a vast array of xenobiotics and endogenous compounds (1). Coumarin 7-hydroxylase (COH) activity is catalyzed by the *Cyp2a-5* gene product (CYP2A5 enzyme) in mice (2). CYP2A5 expression is increased by number of structurally varying compounds, including several hepatotoxic agents and the classical CYP inducer phenobarbital (PB) (3).

PB increases the activity of liver cytochrome P4502A5 at both transcriptional and translational levels in the intact animal (4,5). The mechanisms of induction are still unclear, but may involve mRNA and protein stabilization.

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Abbreviations used: COH, coumarin 7-hydroxylase; CYP, cytochrome P450; db-cAMP, dibutyryl-cAMP; IBMX, 3-isobutyl-1-methyl-xanthine, PB, phenobarbital.

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It is well established that the expression of several CYP genes is regulated by intracellular second messenger pathways. This is especially true for CYP1A1, which is induced by a protein kinase C-dependent cascade involving the Ah receptor, the Arnt protein, and several cis-acting DNA elements in the promoter region of the gene (6). All CYP enzymes participating in steroid hormone synthesis are regulated by cAMP. The 5'-regulatory regions of these genes contain DNA elements that are targets to a number of transcriptional regulatory proteins (7).

The purpose of this study was two-fold: (i) To establish conditions in which primary mouse hepatocytes retain their constitutive and inducible CYP2A5 activity over several days in culture and (ii) to delineate the possible involvement of several second messenger factors in CYP2A5 expression.

Methods

Chemicals. Dexamethasone, dibutyryl cAMP (db-cAMP), forskolin, cycloheximide, actinomycin D, 3-isobutyl-1-methyl-xanthine (IBMX), lavendustin A, genistein, tetradecanoyl phorbol acetate (TPA), staurosporin, Williams E medium and ITS (insulin-transferrin-selenite media supplement) were obtained from Sigma (St. Louis, MO, USA). Collagenase was from Worthington Biochemical (New Jersey, USA).

Preparation of primary cultures of hepatocytes. Male DBA/2 mice, aged between 7-9 weeks, were used in this study. Mouse livers were subjected to collagenase perfusion according to established protocols. After filtration, centrifugation (50g 2 min) and two washes the isolated hepatocytes were dispersed in Williams medium E containing dexamethasone (20 ng/ml), ITS (insulin 5 mg/l, transferrin 5 mg/l, sodium selenite 5 μg/l), gentamicin (10 μg/ml) and 10% FCS at a density of 5 x 10^6 cells/90 mm uncoated dish (Falcon 3003). The cultures were maintained at 37°C in a humidified incubator. After a 2-h incubation, non-attached cells were discarded by aspiration, followed by changing the medium to Williams E without FCS. The chemicals to be tested were added at at least three different concentrations to the serum-free medium. PB was used at a concentration of 1.5 mM.

Enzyme activity assays. After 24 - 96 h in culture, cells were scraped, washed, suspended in a small volume of PBS (200 μl/dish) and sonicated. COH activity was measured as described (5) using 100 μM coumarin as substrate. Since enzyme activities varied substantially between different batches of isolated cells, the activities are given relative to the 24-hr control value in each batch, at which time-point the cells had stabilized in culture.

RNA blot analysis. Adherent cells were scraped and suspended in a small volume of isotonic buffer. mRNA was purified by QuickPrep Micro mRNA Purification Kit (Pharmacia, Uppsala, Sweden). About 3 μg of mRNA was separated on a 1% agarose gel and transferred to Qiabrane nylon membrane (Qiagen). RNA was fixed by UV-crosslinking in UV Stratalinker 1800 (Stratagene) and the membrane was hybridized with a [^{32}P]CTP-labelled ClaI-cut fragment of *Cyp2a5* cDNA (2) and rat GAPDH cDNA (8).

Results

A series of experiments were done to establish the optimal conditions for CYP2A5 induction in primary hepatocytes. The most important single factor proved to be

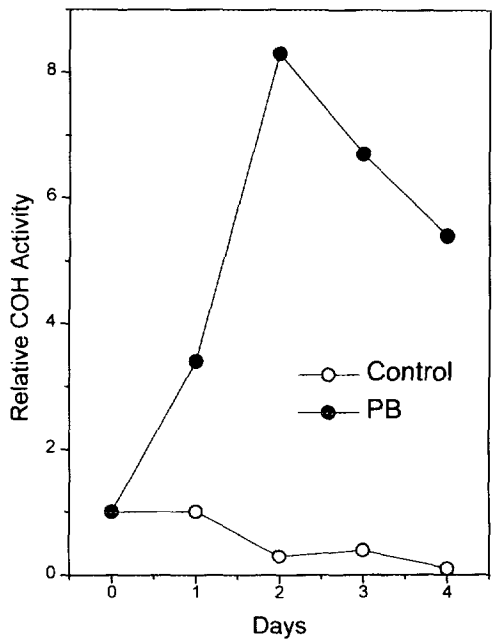

Figure 1. Time-course of COH induction by PB. Primary hepatocytes were plated and allowed to attach for 2 hours in medium containing serum. The medium was then changed to one without serum and containing phenobarbital or vehicle only. Cells were harvested at the indicated time-points. COH activities are given relative to the 24-h control value, which is normalized to 1. Means of two separate experiments are shown.

serum, which totally suppressed both COH constitutive activity and its induction by PB. Also coating of culture dishes with type I collagen decreased activity.

Basal COH activity in freshly isolated hepatocytes was 4 pmol/mg cellular protein/min and 7 pmol/mg cellular protein/min after 24 h in culture. Changes in constitutive activity over 96 h in culture and inducibility by PB are shown in Figure 1. The induction by PB was maximal after a 48-h exposure (8.3-fold over control value at 24 h and 33-fold over control value at 48 h), after which it declined gradually.

The effects of actinomycin D and cycloheximide on COH activity were studied by either exposing hepatocytes to the agents for the initial 1-h period just after attachment or by keeping them in the medium for the whole 48-h induction period (Table 1). Treatment with actinomycin D (10 μM) totally abolished both constitutive COH activity and its inducibility by PB. The short initial 1-h exposure to cycloheximide (10 μM) caused a 2-fold increase in basal COH activity and an apparent superinduction in combination with PB. Exposure of hepatocytes to cycloheximide for the whole 48-h induction period had no effect of induced COH activity (Table 1).

Various agents known to affect different second messenger processes were tested for their ability to influence COH activity. Since the actions of the agents used are

Table 1. Effect of actinomycin D and cycloheximide on COH activity

Treatment	Relative COH activity
Control 48 h	1.0
PB 48 h	9.4
ActD 1 h	0
ActD 1 h + PB 48 h	0
ActD 48 h + PB 48 h	0
CHX 1 h	2.1
CHX 1 h + PB 48 h	25.5
CHX 48 h + PB 48 h	11.9

COH activities are given relative to the control value at 48 h. Results of one representative experiment are shown.

short-lived, their effects on inducibility was studied at 24 h after treatment. 10 μM db-cAMP increased basal COH activity 4.5-fold and about 10-fold when co-administerd with PB (Figure 2). Inhibitors of protein kinase C, TPA and staurosporine, did not significantly affect either basal or PB-induced COH activities. The same was true for

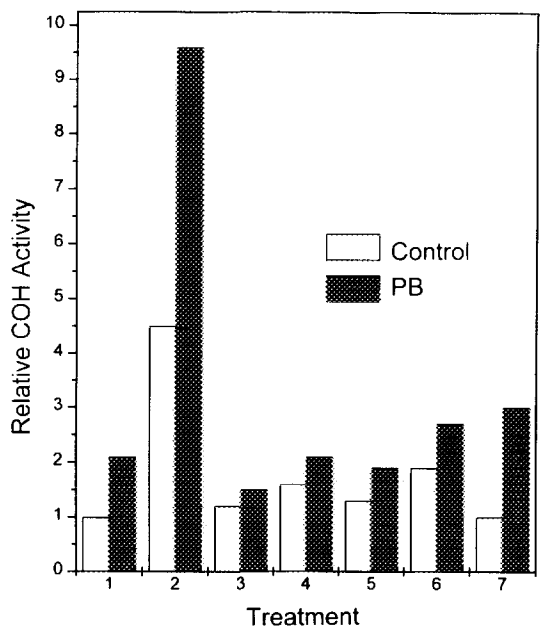

Figure 2. Effect of second messenger-modulating agents on COH activity. 1 - Control; 2 - db-cAMP 10 μM; 3 - TPA 200 nM; 4 - Staurosporine 200 nM; 5 - Genistein 10 mg/ml; 6 - Lavendustin 10 mM; 7 - Sodium nitrate 0.5 mM. Shaded bars indicate co-treatment with phenobarbital.

Table 2. Effect of administration of cAMP-modulating agents on induction by phenobarbital. The agents were allowed to act for 24 h. The values are means ± SD of at least two separate experiments.

Treatment	Relative COH activity
Control	1.0 ± 0.7
PB	2.1 ± 0.5
db-cAMP 10 μM	4.5 ± 1.0*
PB + db-cAMP 10 μM	9.6 ± 2.6*#
Forskolin 50 μM	4.7 ± 1.9*
PB + forskolin 50 μM	8.3 ± 3.4*#
IBMX 100 μM	2.7 ± 1.0*
PB + IBMX 100 μM	4.5 ± 1.3*#
Forskolin + IBMX	5.4 ± 0.9*
PB + Forskolin + IBMX	8.4 ± 1.7*#

* Significantly different from control (analysis of variance, $p<0.05$).
# Significantly different from values obtained without addition of PB.

the tyrosine protein kinase inhibitors genistein and lavendustin A and the nitric oxide (NO) source sodium nitrite.

The marked COH-stimulating effects elicited by db-cAMP were mimicked by agents that either stimulate cAMP synthesis or inhibit its breakdown (Table 2). Forskolin, an adenylyl cyclase activator, had a similar effect as db-cAMP on both basal and induced COH activity. IBMX acts as a phosphodiesterase inhibitor, thereby reducing the breakdown of cAMP. The effects of IBMX on COH activity was less marked than those of db-cAMP or forskolin, but combined treatment with forskolin and IBMX produced a marked 5.4-fold increase in basal COH activity. Inducibility by PB, however, was not increased further by forskolin/IBMX combination.

RNA blotting analysis was done to assess the effect of cAMP-modulating agents on CYP2A5 steady-state mRNA (Figure 3). PB, db-cAMP, forskolin and IBMX elicited 4-fold, 14-fold, 28-fold and 5-fold increases, respectively, in the amount of CYP2A5 mRNA. The amount of mRNA was uniformly increased to above these values in response to co-administration of PB and the cAMP-modulating agents, the peak being the 65-fold increase by the combination of PB, forskolin and IBMX.

Discussion

Cultivation of rat and mouse liver primary hepatocytes is now a well-established model for the study of xenobiotic metabolism. The maintenance of CYP constitutive activities and inducibility by PB in primary rat hepatocytes has turned out to be dependent on delicate nutritional factors, e.g. the presence of dexamethasone and absence of serum (9). This study shows that also mouse CYP2A5 activity and

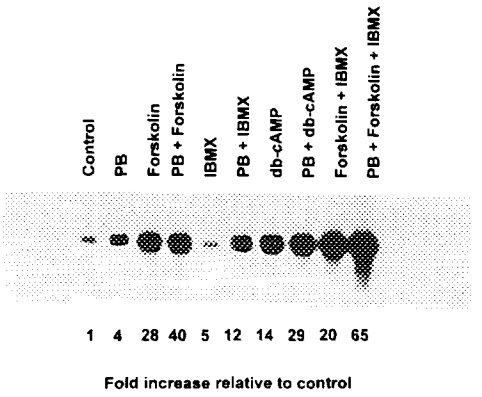

Figure 3. Effect of cAMP-modulating agents on CYP2A5 mRNA. Primary hepatocytes were treated with cAMP-modulating agents at doses indicated in Table 2. mRNA was isolated, electrophoresed, blotted and hybridized with *Cyp2a-5* and GAPDH cDNAs. Band densities were determined and the CYP2A5 signal was corrected against that of GAPDH. The magnitude of changes relative to control (density = 1.0) are shown.

inducibility is strongly suppressed by components in serum, most presumably growth hormone (10), and that coating of tissue culture dishes is not necessary for induction to occur.

When administered during the first 1 h in culture, cycloheximide caused an increase in basal COH activity and a superinduction in conjunction with PB. This is consistent with a model implicating a short-lived repressor protein being involved in basal COH activity and especially in the induction process elicited by PB. Such repressor proteins are clearly operating in the control of the *Bacillus megaterium* CYP102 gene (11), but their role the regulation of mammalian PB-inducible CYP genes is still elusive (9). The total abolishment of COH activity by actinomycin D suggests that *de novo* transcription rather than stabilization of pre-existing mRNA is needed for induction of COH activity by PB in primary hepatocytes.

It is well established that CYP1A1 induction, mediated by the Ah receptor and the Arnt protein, requires phosphorylation events catalyzed by different protein kinases, especially protein kinase C (6). Tyrosine kinases, in turn, play a role in the signal transduction pathways of several growth factors, affecting e.g. the epidermal growth factor receptor. In this study, inhibitors of protein kinase C and tyrosine kinase at high doses did not affect COH activity in mouse primary hepatocytes, suggesting that these kinases play a minor role in CYP2A5 expression. Nitric oxide (NO) was recently shown to inhibit heterologously expressed and endogenous rat CYP1A1 and CYP1A2 enzymes (12). In the present study, up to 1 mM concentrations of sodium nitrate failed to affect COH activity, suggesting that CYP2A5 enzyme is insensitive to the effects of NO.

Treatment with db-cAMP, forskolin, a specific activator for adenyl cyclase, or IBMX, an inhibitor of phosphodiesterase, enhanced both basal and PB-induced COH activities and CYP2A5 mRNA levels. cAMP is known to bind cooperatively to two sites on the regulatory subunit of protein kinase A releasing the active subunit. The active subunit phosphorylates a number of cellular proteins (13). Interestingly, a recent study shows that the cAMP-responsive sequence CRS1 of *CYP17* is the target for the homeodomain protein Pbx1 (14), implicating a role for this homeoprotein in sexual development.

A number of nuclear factors bind to DNA sequences present in the promoter regions of cAMP-inducible genes. The consensus cAMP-responsive element (CRE) is constituted by the palindromic sequence TGACGTCA. Variations from the consensus CRE are common (13). Examination of a 1900-bp strech in the 5'-upstream region of the *Cyp2a-5* gene (15) reveals one near-perfect CRE (TGACCTCA) and at least 4 CRE half-sites. It is thus likely that the induction of CYP2A5 by cAMP is mediated by mechanisms involving binding of one or several of the CRE-binding proteins to the CREs followed by increased transcription of the gene. Studies to directly prove this are currently underway in our laboratories.

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