Modulation of constitutive, benz[a]anthracene- and phenobarbital-inducible cytochromes P450 activities in rat hepatoma H4IIEC3/G⁻ cells by piperine

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Hepatoma cells, H4IIEC3/G⁻ in continuous culture were found to express both constitutive and benz[a]-anthracene (BA)- and phenobarbital (PB)-inducible monooxygenase activities. Renewal of medium from cultures increased arylhydrocarbon hydroxylase (AHH) activity 4-fold within 5 h which was blocked by cycloheximide. Piperine, a major alkaloidal ingredient of *Piper* species, impaired both the constitutive and dexamethasone (DEX)-inducible AHH activity equipotently, while it offered relatively poor sensitivity towards AHH from BA-treated cultures. Similarly, piperine offered differential inhibition of 7-methoxycoumarin demethlyase (MOCD) activity from cultures treated with or without PB. Exposure of cultures to 50 μM piperine evoked biphasic responses of AHH activity, an initial inhibitory followed by induction phase while the activity of MOCD was not affected after registering an early inhibition of 30%. The cells in culture appeared to mimic hepatic tissue in their response to piperine and may offer a system of choice for studying drug-interaction.

MAMMALIAN cells in culture are increasingly used for studying the mechanism of action and assessment of toxicity of drugs and chemicals. The capability of cells to inactivate or activate xenobiotics has inevitably been shown to depend upon the presence of cytochromes P450 (CYP450) and conjugases expressed in these cells[1, 2]. Since liver is the major site of drug metabolism, isolated hepatocytes from various species have been in use for several such studies. However, one serious drawback associated with the use of isolated hepatocytes in toxicology and pharmacology is the rapid loss of their differentiated functions, notably the activities of phenobarbital-inducible cytochrome P450s[3, 4]. In the past, hepatoma cells or their variants derived from the liver of human or rat have been shown to carry out monooxygenation and conjugation reactions and were found capable of activating procarcinogens and promutagens to cyto-cum-genotoxic metabolites[5-9]. The cells varied in their level of expression of differentiation and those expressing differentiated hepatocyte functions have been considered useful in supplanting hepatocytes. One such cell line H4IIEC3/G⁻ (H4/G⁻), which has been found phenotypically stable, retains drug-metabolizing enzymes and brings about activation and inactivation of hepatotrophic chemicals[10] and therefore, is used in this study to examine the influence of a pharmacologically important alkaloid piperine on catalytic activities of CYP4501A1 and CYP4502B1 in these cells. Piperine, which is a major ingredient of black and long peppers, has been reported to impair drug-metabolizing enzymes besides affecting several other biochemical functions[11, 12]. It caused non-competitive inhibition of major drug-metabolizing enzymes of hepatic[13] and pulmonary[14] tissues of rat and guinea pig. Besides, it was found to lower the level of UDP-glucuronic acid in intestinal cells[15] consequent to the inhibition of UDP-glucose dehydrogenase[12]. In this study the effect of piperine on the constitutive and polycylic aromatic hydrocarbon (PAH) or phenobarbital (PB) inducible CYP450 activities was examined in H4/G⁻ cells to compare piperine-mediated modulation of drug metabolising enzymes in cells with the reported inhibition in rat liver[13]. Such studies are a prerequisite for establishing the suitability of cells and specificity of inhibitor towards various CYP450s in predicting the modulation of pharmacokinetics and toxicity of test drugs *in vivo*.

Methods

Materials were purchased from the following sources: Dulbecco's Modified Eagle's Medium (DMEM), penicillin/streptomycin, trypsin/EDTA, phosphate buffer saline (PBS) from Seromed; fetal calf serum (FCS) from GIBCO; benzo (a) pyrene (BP), benz[a]anthracene (BA) and dexamethasone (DEX) from Sigma Chemical Co. (St. Louis, MO); phenobarbital (PB) from Merck and Co., Inc. (Rahway, NJ); 7-methoxycoumarin (MOC) from Aldrich Chemie, Steinheim and purified[16]; cycloheximide from Hi-Media, India and puromycin from Serva. Piperine from black pepper was purified[13] while other chemicals used were of analytical grade available locally.

Cells

Various hepatoma cells were obtained as a generous gift from Prof. Friedrich J. Wiebel (Institute of Toxicol-

ogy and Biochemistry, GSF, D-8042 Neuherberg, Germany) and grown in this laboratory or stored intermittently in liquid nitrogen. H4IIEC3/G$^-$ cells (H4/G$^-$) used in the study are descendants from rat Reuber hepatoma and their source and growth kinetics and status of differentiation were described earlier[9,10,17].

Cell culture, treatment and enzyme assays

Cells were routinely grown in 60 mm or 100 mm Falcon dishes in DMEM, instead of NCTC135 : Ham's F12 (ref. 10) containing 10% FCS, 100 U/ml pencillin and 100 µg/ml streptomycin in a humidified atmosphere containing 5% CO_2 in a carbon dioxide incubator (Hotpack, PA., USA). They were passaged once a week using a solution of 0.05% trypsin and 0.02% EDTA. Cultures were exposed to 2 µM DEX, 20 µM BA or 2 mM PB in dimethylsulphoxide (0.2%, v/v) as described under tables or figures. Medium was changed after every 48 h. For enzyme assays cells were washed twice with cold PBS and harvested by scraping with rubber policeman and centrifuged at 200 xg for 5 min. Cell pellets were stored at -40° and used within 7 days without significant loss of activity. At the time of assays, the cell pellets were suspended in 5 mM Tris/HCl, pH 7.4 and the activities of arylhydrocarbon hydoxylase (AHH)[18] and that of 7- methoxycoumarin demethylase (MOCD)[16] were determined. For MOCD assay, MOC was dissolved in 50% methanol and the solvent content in the assay was not more than 0.5% (v/v). Cellular protein was determined[19] using bovine serum albumin as standard.

Results

Presence and inducibility of CYP450 activities in H4/G$^-$ cells

The cells grown in culture were found to express both MOCD and AHH activities (Table 1). The level of AHH was almost 5-fold higher over MOCD activity in the cells. These activities were differentially inducible by the inducers used in the study. PB-induced MOCD activity by more than 2-fold, Dex alone by 6-fold while a very high induced activity of 9-fold was obtained with PB and DEX together. AHH activity, on the contrary, exhibited a remarkable induction of 7-, 32- and 44-fold in the presence of DEX, BA and DEX plus BA, respectively (Table 1).

Table 1. Expression and inducibility of cytochrome P450 mediated monooxygenase activities* in rat hepatoma H4IIEC3/G$^-$ cells

Treatment	MOCD	Induction (fold)	AHH	Induction (fold)
Untreated	1 59	–	8.3	–
DEX	9.17	5.8	63	7 5
PB	3.8	2.4	ND	–
BA	3 3	2.1	269	32
DEX + PB	14	9	ND	–
DEX + BA	7	4.4	363	44

Cells $5 \times 1\,\bar{0}$ were seeded in 100 mm plastic dishes. Treatment with 2 µM DEX or 2 mM PB was for 72 h each and with 20 µM BA for 18 h. The beginning of the treatment was staggered to harvest the cells at the same time. The cells were frozen-thawed before assay of enzyme activities. For each treatment two plates were used and activities assayed in duplicate with inter culture variation of less than 10%. Other conditions were the same as given in Materials and Methods. The untreated plates received the vehicle only.
*Specific activity = pmol per min per mg protein. ND = not determined.

Renewal of culture medium caused an initial rapid increase in AHH catalytic activity

Renewal of the used medium in cultures by a fresh one exerted a profound influence on the synthesis of CYP4501A1 catalytic activity. The activity of AHH showed exponential increase of 4 to 5-fold during the first 5 to 8 h of change of medium; thereafter, the activity was almost stabilized, although increase in protein content per culture after 24 h was not greater than 20%. Addition of cycloheximide, a potent inhibitor of protein synthesis, inhibited completely the monooxygenase activity. Puromycin which blocks synthesis by inhibiting translation was found to inhibit CYP450 synthesis only after five hours (Figure 1).

In vitro effect of piperine on AHH and MOCD activities of cells

Piperine caused concentration-related inhibition of both the constitutive and DEX-inducible AHH activity *in vitro*, equipotently (Figure 2). However, the AHH activity in cells treated with BA or BA and DEX showed poor sensitivity at lower concentrations. MOCD activity (Figure 2) in control cells registered a concentration-related inhibition while the inducer-treated cells altogether showed relatively poor sensitivity to piperine. PB-inducible activity was marginally inhibited while a higher magnitude of inhibition (50% at 30 µM) was observed in cells exposed to PB and DEX.

Influence of piperine on the monooxygenase activities in continuous cultures

Influence of piperine on the CYP450 activities of H4/G$^-$cells in culture was determined at different time intervals for five days. Piperine exerted biphasic influence on AHH activity, an initial inhibitory phase followed by the induction one. Addition of fresh medium to

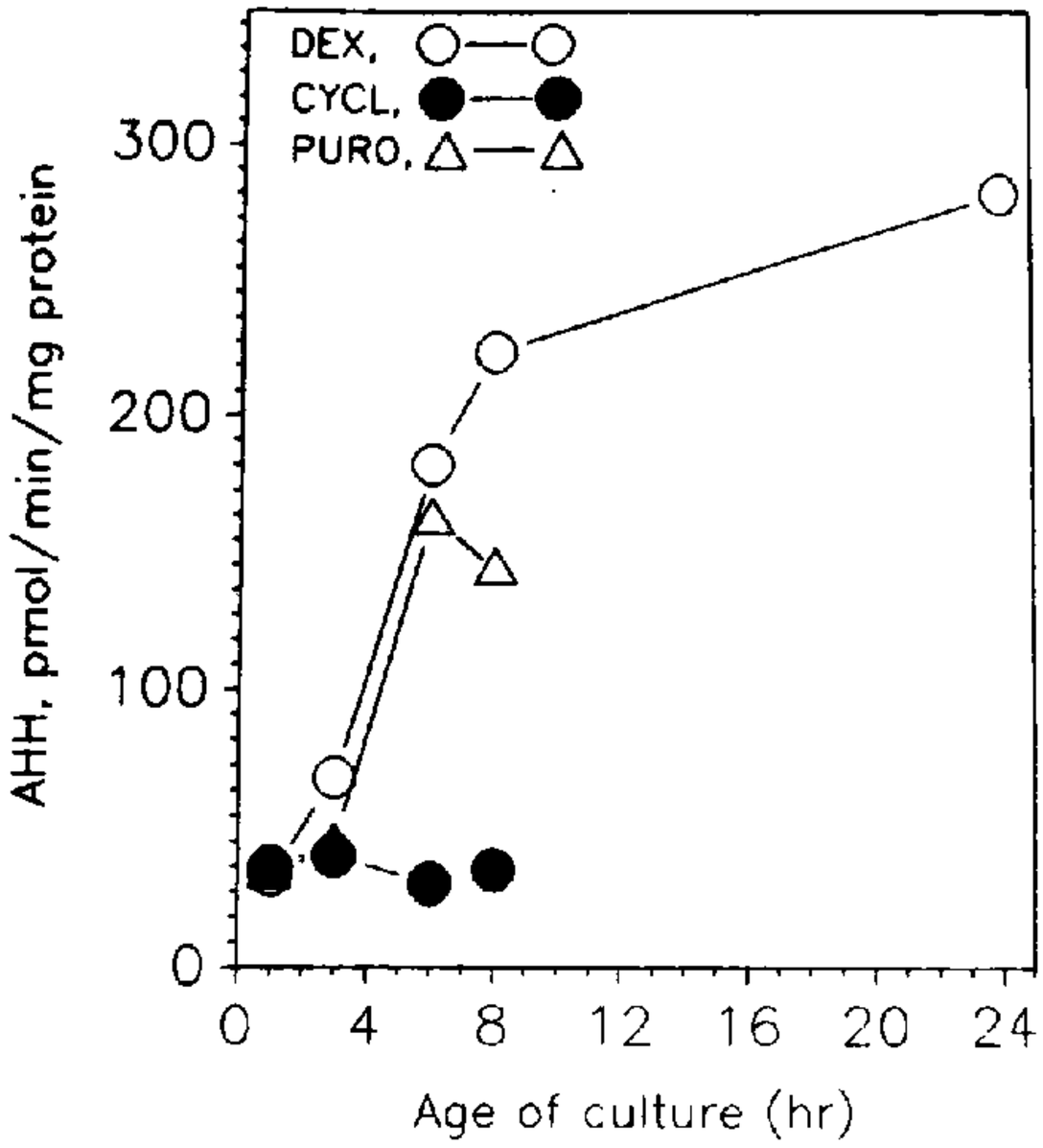

Figure 1. Influence of renewal of culture medium on AHH activity of H4/G⁻ cells in the presence and absence of inhibitors of protein synthesis. Cells were seeded in 60 mm plates and after 48 h medium was changed by fresh medium containing 2 μM DEX. Further at the end of 72 h, cells approaching confluency were given fresh DEX-medium containing 5 μM puromycin or 5 μM cycloheximide. The test compounds were delivered in 10 μl DMSO per 3 ml medium per plate. Data are mean values from three plates with coefficient of variation of less than 10%.

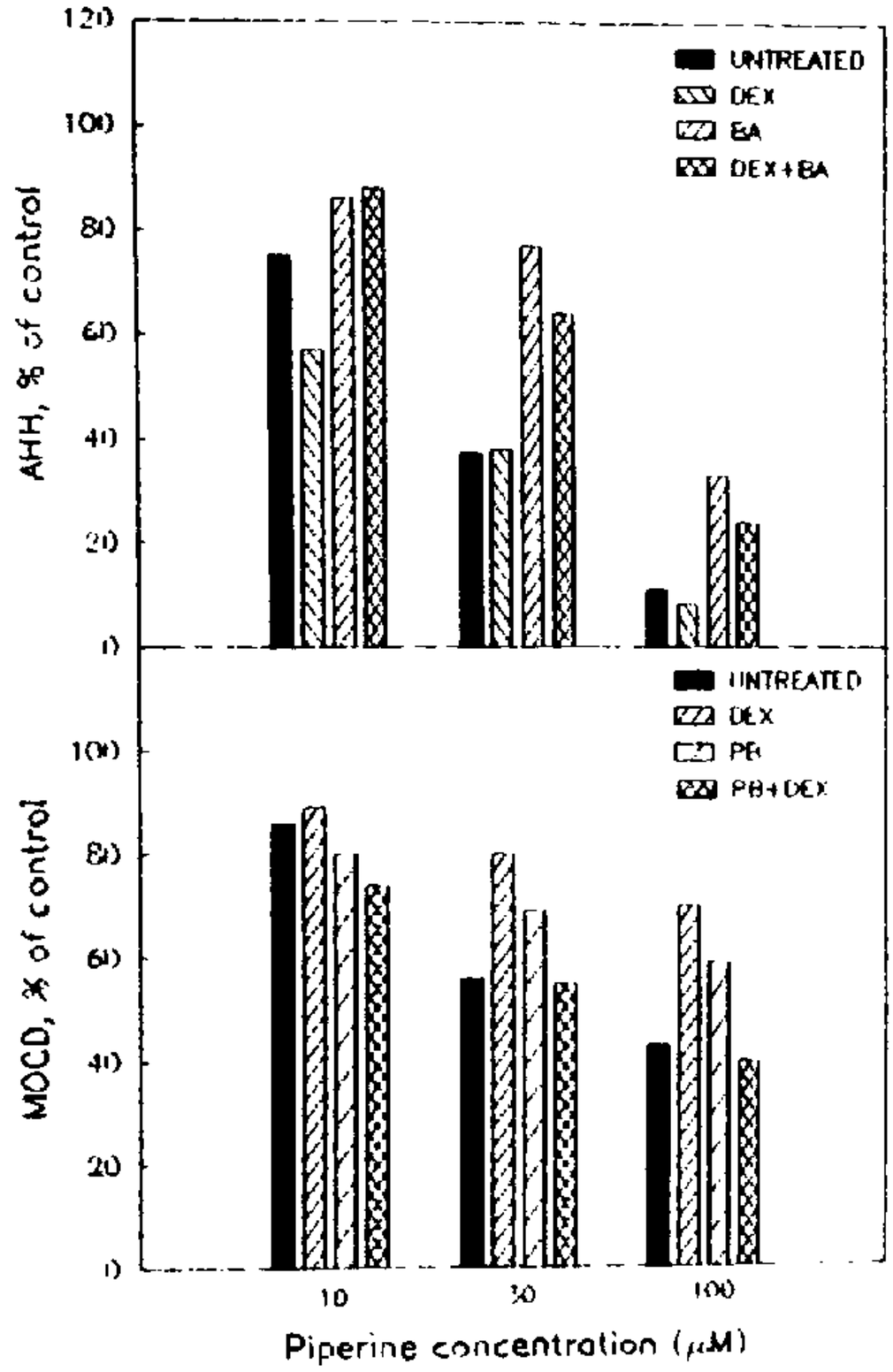

Figure 2. *In vitro* effect of different concentrations of piperine on CYP450 activities of untreated and inducers treated H4/G⁻ cells. Enzyme activities expressed as '% of control' represent values from each group determined in the absence of piperine, and taken as 100%. Piperine was added in 50% methanol (solvent < 1%, v/v). All other conditions were the same as given in Table 1.

cultures, after removal of the used one, increased AHH and MOCD activities which at the end of six hours were as high as 6- and 1.5-fold, respectively. In the presence of 50 μM piperine, the activities of AHH and MOCD (Figure 3) were consistently lower by 20–30% compared to control. Treatment of cells with piperine for a longer period of 1 to 5 days resulted in induction of AHH activity only while the activity of MOCD was not significantly altered. The activity of AHH was induced by 4- and 3-fold on day 3 and day 5 of treatment (Figure 4).

Discussion

Hepatoma cells H4IIEC3/G⁻ grown in DMEM were found to retain their reported expression of PB- and PAH-inducible CYP450 activities[7,8,10]. We assayed MOCD as a marker for various hepatic CYP450 isoforms because the oxidase is catalysed by both the constitutive and possibly by several PB-inducible rat liver CYP450s[16] while the activity of AHH is related to CYP4501A1 gene[20]. MOC is a good substrate for CYP4502B1 and possibly several constitutive and PB-inducible CYP450s and its dealkylation by PB-inducible CYP450s may be considered as one among several differentiated functions of liver. The constitutive activities which generally are low in these cells were raised several-fold, for instance AHH activity, by inducers. Dexamethasone appeared to transcriptionally activate and stabilize CYP450 genes for their catalytic activities towards MOC and B(a)P. This is evidenced by higher inducibility of AHH and MOCD in these cells. The synergestic interaction between inducers and DEX is considered to be a time-dependant process involving receptor synthesis[21] or by affecting the levels of regulatory factors[4]. However, in this study the rapid increase in CYP4501A1 occurred immediately after renewal of medium when the cultures were already maintained in DEX medium over 72 h. This suggested that the fresh medium has other putative regulatory

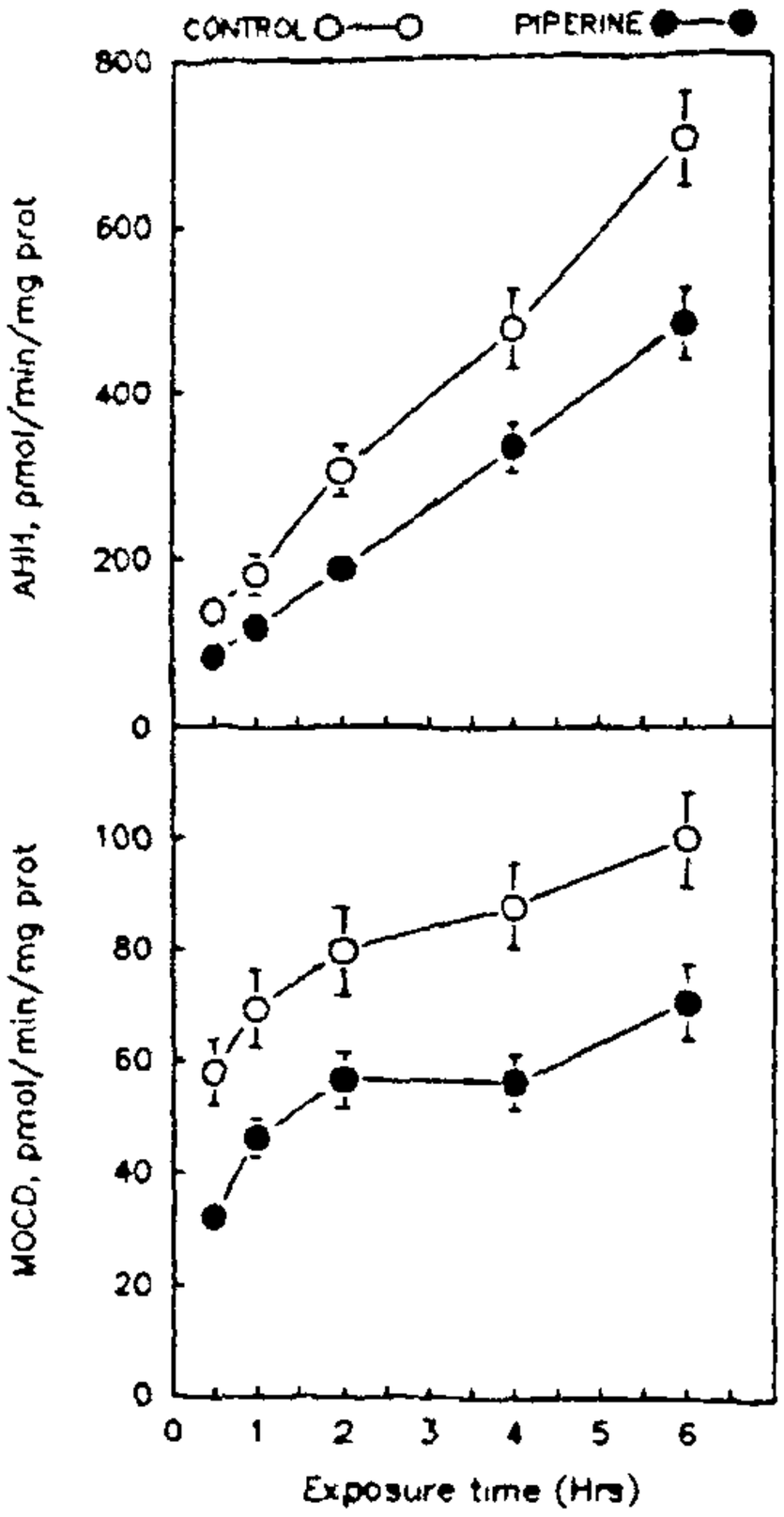

Figure 3. Influence of short-term exposure of piperine on the monooxygenase activities of H4/G⁻ cells in culture. Cells were seeded in 100 mm plates and after 24 h were treated with fresh medium containing 2 µM DEX for 72 h. Cultures were drained of old medium and fresh DEX medium was given. One hour later piperine at 50 µM in DMSO (10 µl/3 ml medium) was added to culture plates. Cells were collected at indicated time intervals for assay of CYP450 activities. Other conditions are given in Materials and Methods. Four plates were used for each value (Mean ± SD)

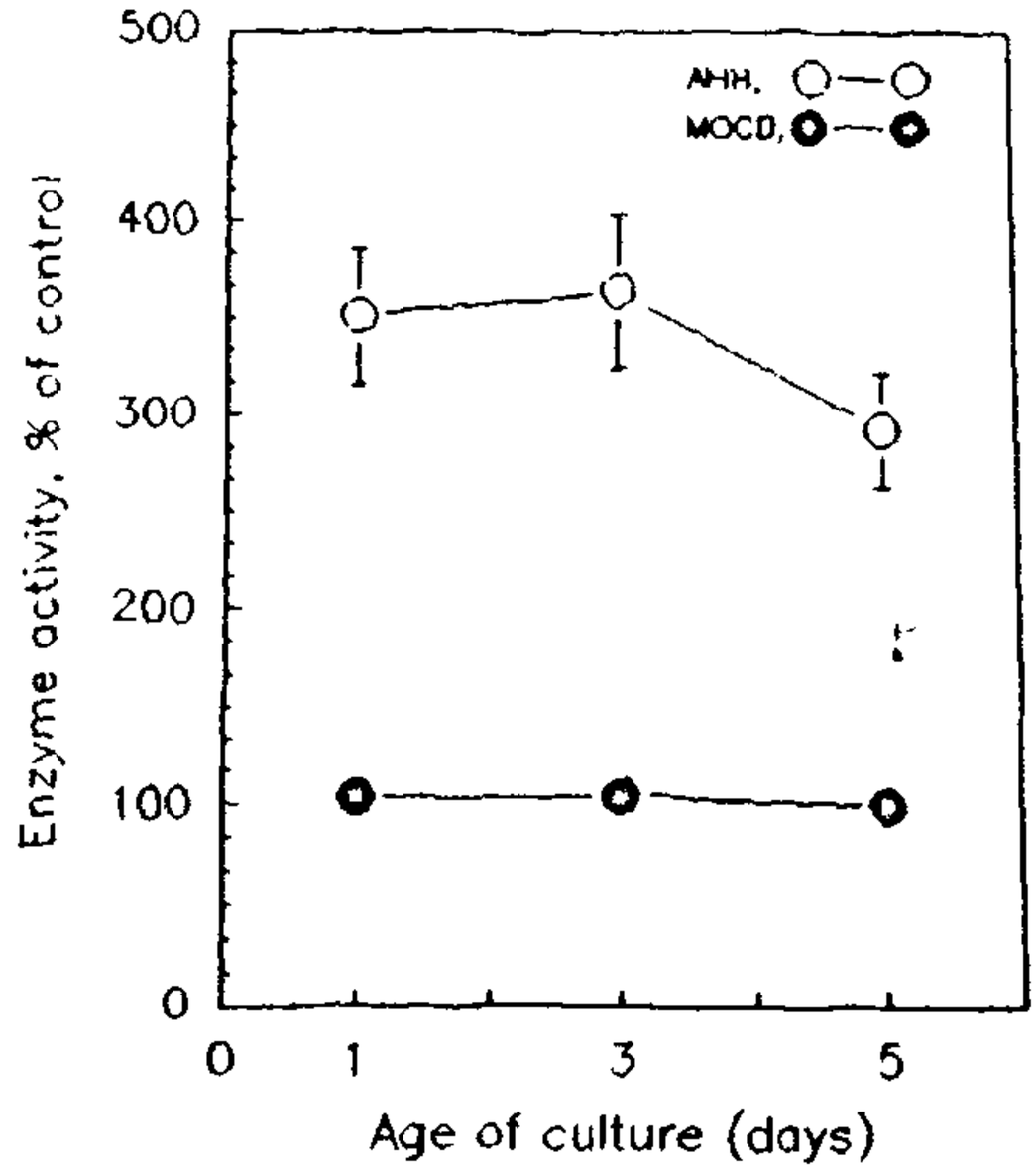

Figure 4. Influence of long-term exposure of piperine on the monooxygenase activities of H4/G⁻ cells in culture. Cells were seeded in 100 mm dishes and allowed to grow for 24 h in complete growth medium when it was replaced by fresh DEX-medium. After 48 h cell cultures were treated with conditioned medium containing 50 µM piperine. Medium in plates was renewed by fresh conditioned medium 24 h before the collection of cells when the cultures appeared nearly confluent. Treatment with piperine was staggered so as to collect the cells on the same day. Other conditions were the same as given in Figure 3. Control values, pmol/min/mg protein, AHH = 56 ± 7, MOCD = 17 ± 3.

factors or nutrients responsible for potentiation of CYP450IA1 catalytic activity.

The activities of CYP450s in cell cultures are known to be influenced by factors such as age of culture, density of cells, nature of growth medium and presence of chemicals[5,22], etc. All of these may have a profound influence on the xenobiotics activation and inactivation potentials of cells when the same cells are used for pharmacokinetic studies or as indicator of biological end-points of xenobiotic action. For instance, in this study renewal of cultures with fresh medium resulted in exponential increase in AHH activity during the first few hours of feeding cells. This initial upsurge in activity appeared as a consequence of the activation of CYP450IA1 gene resulting in higher transcriptional rate followed by stabilization of the activity. This is evidenced from the fact that cycloheximide completely blocked the synthesis of AHH activity while puromycin exerted its influence only after 5 h of the change of medium. The delayed effect of puromycin compared to cycloheximide appears to reflect either a different mechanism of action or a differential sensitivity of the cells to the protein translational inhibitors. Thus, the results suggested that drugs or chemicals should be added directly to cultures, at least 6 h after the change of medium to avoid variation in the interpretation of results.

To understand the influence of inhibitors of drug-metabolizing enzymes, our studies with H4/G⁻ cells showed that the magnitude of inhibition offered by piperine *in vitro* was similar to the one observed earlier with hepatic oxidases[13]. It caused concentration-dependant inhibition of both the constitutive and DEX-inducible AHH and MOCD activities, while the PB-inducible forms showed poor sensitivity than the

BA-inducible forms. All these inducers are known to induce different CYP450s in animal tissues[20] or several CYP450 mRNAs in hepatoma cells[23]; and induction of both *AHH* and *MOCD* by BA and PB, though unusual in hepatoma cells, is known earlier[7]. It is possible that piperine might be exhibiting differential specificity towards various inducible CYP450s. In cultures piperine exhibited biphasic effects on the activity of AHH, an early inhibitory followed by stimulatory one. Similar observations were made on the activity of hepatic AHH during a long treatment of piperine (15 mg/kg b.wt., p.o., daily) for 8 weeks in rats (our unpublished results). The hepatoma cells thus appear to mimic hepatic tissue *in vivo* in modulation of AHH activity by piperine. The cells may thus offer a system of choice for rapidly predicting the influence of inhibitor on modulation of toxicity and pharmacokinetics of drugs and chemicals.

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