Vol. 150, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1988 Pages 436-443

LIGAND-DEPENDENT MAINTENANCE OF ETHANOL-INDUCIBLE CYTOCHROME P-450 IN PRIMARY RAT HEPATOCYTE CELL CULTURES

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Received October 26, 1987

Administration of ethanol, dimethylsulphoxide, 2-propanol or imidazole to rats caused 2-7-fold increases in the level of hepatic ethanol-inducible cytochrome P-450 (P-450j), without any concomitant enhancement of corresponding mRNA. All the compounds were able to stabilize P-450j in hepatocyte cultures for at least three days, whereas P-450j mRNA rapidly disappeared from the cultures. A correlation was reached between the concentration of Me_2SO, ethanol and 2-propanol necessary to maintain P-450j in the cell cultures and their binding affinities to the enzyme. It is suggested that the ligand-bound form of P-450j in the hepatocytes is protected from degradation.

The liver microsomal cytochrome P-450 system is in a sense similar to the immune system. The expression of various forms of hepatic cytochrome P-450 depends on the type and concentration of chemicals transported to the liver. Over 300 different compounds are known to increase their own metabolism (1). In principle, their rate of oxidation can be enhanced by the induction of various types of cytochrome P-450, belonging to over 9 different gene families (2), having different, but partially overlapping substrate specificities. Recently, increasing interest has been focused on the mechanisms by which these chemicals are able to affect the level of different P-450 isozymes. Apparently, mechanisms for P-450 induction, involving increased rate of gene transcription (3, 4), mRNA stabilization (5) and protein stabilization (6), have been described.

Abbreviations used: Me_2SO, dimethylsulphoxide; P-450j, ethanol-inducible cytochrome P-450 from rat liver microsomes.

0006-291X/88 $1.50

The ethanol-inducible type of cytochrome P-450 (7-11) is different from many other forms of P-450, since it is induced by numerous structurally different compounds (12-14), many of which are known to effectively bind to the enzyme. Thereby, they induce a change from a high spin to a low spin state of the enzyme (7, 10). Furthermore, compared to other cytochrome P-450-dependent reactions, the microsomal ethanol oxidation, which is catalyzed to a great extent by the ethanol-inducible isozyme of cytochrom P-450 (10, 15), has a rapid turnover (16).

In the present paper we describe that the ethanol-inducible form of P-450 from rat (P-450j) is maintained in hepatocyte cultures, provided that enzyme ligands are present in the medium. A correlation between the binding affinities of the compounds and their capabilities to stabilize the enzyme is apparent, which indicates that the ligand-bound enzyme is protected from degradation.

EXPERIMENTAL PROCEDURES

Materials.

Imidazole, 2-propanol, 1-butanol and dimethylsulphoxide (Me_2SO) were obtained from Merck. Isoniazid was a gift from Ferrosan, Malmö, Sweden. Collagenase (batch 69) was purchased from Boehringer Mannheim. Hepes was obtained from Gibco and cycloheximide was from Sigma. [^{3}H]leucine (specific activity 160 Ci/mmol) was purchased from Amersham.

Methods.

Animal treatment. Male Sprague-Dawley rats (170 g) were used. Animals used for preparation of cells were starved and treated with acetone (5 ml/kg, given as 33 % (v/v) acetone in 0.9 % (w/v) NaCl) intragastrically for two days, as previously described (17). Other rats were treated with three daily intraperitoneal injections of imidazole (250 mg/kg), or 2-propanol (2 ml/kg). Another group of rats was injected intraperitoneally with Me_2SO (2 ml/kg) twice daily for three days. Some rats were fed the liquid alcohol diet (Bioserv Inc.) as described by DeCarli and Lieber (18) for 20 days. Control rats received food and water ad libitum.

Isolation of hepatocytes. Cell isolation was performed under sterile conditions basically as described by Seglen (19). The rats were anestezised with ether and the livers were perfused at 37^{0}C with oxygen-saturated buffer containing 0.14 M NaCl, 6 mM KCl, 0.01 M Hepes, pH 7.4 at a rate of 50 ml/min, for 10 minutes. The perfusions were continued at 37^{0}C for 10 minutes at a rate of 20 ml/min with a oxygen-saturated buffer containing collagenase (0.45 mg/ml), 0.07 M NaCl, 6.7 mM KCl and 4.8 mM $CaCl_2$, pH 7.6. The cells were suspended in 30 ml Waymouth medium (Gibco MB 752/1 with L-glutamine) and filtered through nylon filter (250 µm, 3 times and 100 µm, 2 times), before cooling on ice. The cells were washed three times in the Waymouth

medium. Usually about 250 x 10^6 cells were recovered from one liver and 80-90 % of the cells excluded trypan blue.

Cell culture. The cells were diluted to a concentration of 1.33 x 10^6/ml with Waymouth medium containing 10 % (v/v) Newborn Calf Serum (GIBCO), penicillin (100 IU/ml) and streptomycin (100 µg/ml, GIBCO, Penicillin-Streptomycin solution) and 5 mM Hepes (GIBCO). Aliquots of 3 ml were seeded on 60 mm Nunclon-Delta culture dishes, coated with 20 µg rat-tail collagen (Sigma, type VII). Various substrates were added to the cultures and, in case of volatile substrates, the dishes were sealed with parafilm. The dishes were incubated in a humidified atmosphere containing 5 % CO_2 at 37 ^{0}C and medium was changed every 24 h.

Isolation of microsomes. After culturing for 72 h, the cells were washed three times with 2 ml cold solution containing 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl and 140 mM NaCl and subsequently suspended in 1.5 ml of this buffer. The cells were then centrifuged at 350 x g for 2 minutes at 4^{0}C and suspended in 1 ml 100 mM potassium phosphate buffer, pH 7.4, containing 10 mM EDTA. At this point the cells were stored at -70^{0}C until analysis. The cell suspension was thawed and sonicated for 20 x 1 sec using a MSE Soniprep 150. The cell lysate was centrifuged at 10 700 x g for 10 minutes and the resulting supernatant was centrifuged at 100 000 x g for 60 minutes, all centrifuations at 4^{0}C. The pellet was homogenized in 100 - 150 µl 50 mM sodium phosphate buffer, pH 7.4, containing 0.1 mM EDTA and 10 % (v/v) glycerol. The protein concentration was determined according to Lowry et al. (20) using bovine serum albumin as standard. Usually 100-400 µg of microsomal protein was recovered from each culture dish.

Assay for P-450j apoprotein and mRNA. The amount of P-450j in microsomes from livers and cell cultures was determined by Western-blot using a monospecific polyclonal antiserum (21). Gel electrophoresis (22) was performed using a Bio-Rad mini protean cell with 3 µg of protein in each well. Total RNA was isolated using the minimethod described in Davis et al. (23). An oligonucleotide complimentary to bp 771-820 of the P-450j cDNA sequence (24) was synthesized as described (21) and 5'-end labeled with [γ-^{32}P]ATP (specific activity 3000 Ci/mmol, NEN) and T_4 polynucleotide kinase (NEN), essentially as described by Richardson (25). Blot hybridization to the ^{32}P-labeled probe (specific activity 2-6 x 10^6 dpm/pmol) was performed at 60^{0}C with 2 x 10^6 dpm/ml of the oligomer, according to Davis et al. (23).

RESULTS

Various compounds known to be metabolized by or bound to the ethanol-inducible form of cytochrome P-450, were tested for their ability to induce the level of the protein *in vivo* as well as to prevent P-450j loss from primary hepatocyte cultures. As shown in Table I, the four compounds used did induce the amount of P-450j in rat liver microsomes 2-7 fold, compared to control, when administrated to the animals *in vivo*. RNA from the livers was isolated and hydridized to a ^{32}P-labeled oligonucleotide recognizing P-450j mRNA. As shown in table I, P-450j induction with ethanol, imidazole, 2-propanol or Me_2SO was not accompanied with an increase at the mRNA level.

Table I. Apparent levels of P-450j and P-450j mRNA in rat liver and cultured hepatocytes after treatment with various inducers. The amount of P-450j in microsomes was determined by Western-Blot analysis. P-450j mRNA was quantified by dot blot hybridization to total RNA using a ^{32}P-labeled oligonucleotide.

Inducer a/	Conc. used mM b/	Ks mM	Total P-450 liver nmol/mg c/	P-450j liver pmol/mg	P-450j cells % d/	P-450j mRNA liver %	P-450j mRNA cells % e/
None (5)	-	-	0.58±0.07	50±9	2±2	100±73	<0.5
Ethanol (5)	100	15	1.14±0.14	330±60	45±21	n.d.	n.d.
Imidazole (4)	0.5	0.02	0.96±0.15	116±19	41±14	96±48	<0.5
2-Propanol (4)	50	9	0.53±0.07	113±33	54±28	40±24	<0.5
Me_2SO (4)	50	7	0.89±0.07	206±53	49±29	99±38	<0.5

a/ The number of animals used in each group is given within parenthesis; b/ the concentrations of the chemicals used in the cell experiments are given; c/ determined spectrophotometrically; d/ the level of cytochrome P-450j in microsomes of primary hepatocyte cultures is given after three days in culture and is expressed in relation to the amount of P-450j present in the microsomal fraction of the cells when isolated; e/ these data are given after 3 days in culture and are expressed in relation to the level of P-450j mRNA in the cells when isolated; n.d., not determined.

Culturing of hepatocytes, isolated from livers of starved and acetone-treated rats, caused a rapid disappearence of P-450j from the cells. After 24 h, the amount of enzyme had decreased to about 30 % of the original level (Fig. 1) and after 3 days in culture, almost no P-450j was remaining. However, the presence of 14 mM 1-butanol in the medium prevented the enzyme loss (Fig. 1) and about 70 % of the original amount of P-450j was present after three days in culture (Fig. 1).

All compounds acting as inducers of P-450j *in vivo* were also capable to prevent loss of P-450j from hepatocyte cultures by about 50 % (Table I). This corresponds to a 20-fold higher amount of P-450j in microsomes from cells cultured in the presence of ligands, compared to control cells. The doses used for stabilization of P-450j in the cultures were well above the half-maximal concentration necessary for conversion of the high spin form of P-450j to the low spin form (Table I). The relationship between the affinities of the ligands for P-450j and their capabilities to prevent P-450j loss from the cultures was

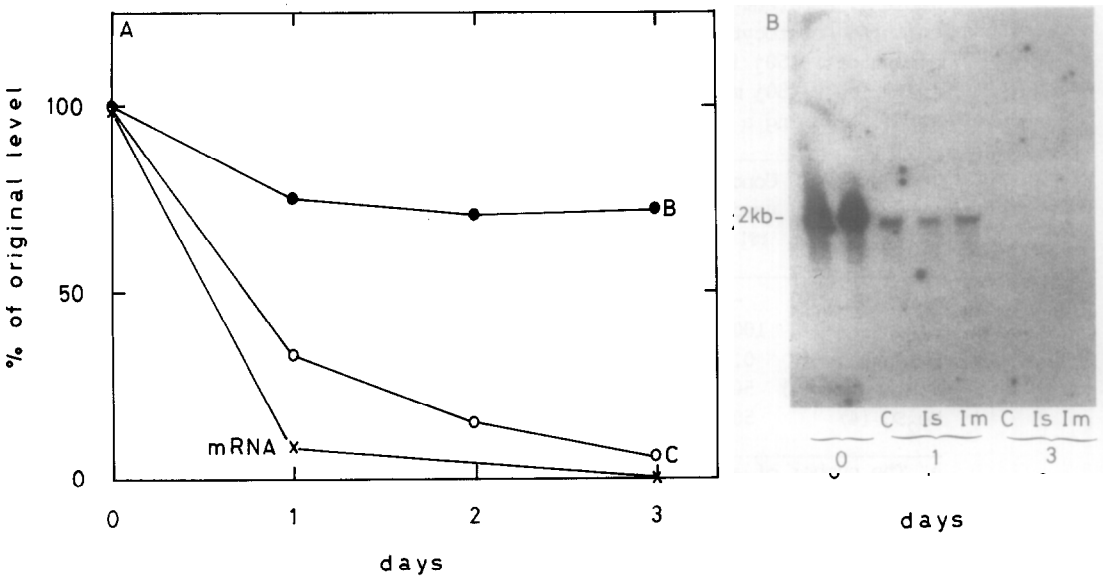

Fig. 1. A. Effect of time on the level of immunodetectable P-450j in liver microsomes from hepatocytes isolated from starved and acetone-treated rats and cultured in the absence (O—O) (C) or in the presence (●—●) (B) of 1-butanol and on the level of P-450j mRNA in cells cultured in the absence of inducer (x—x). The results are mean values of three different experiments. B. Northern blot analysis, using an oligonucleotide recognizing P-450j mRNA, of RNA isolated from hepatocytes cultured in the absence (C) or in the presence of isoniazid (Is) or imidazole (Im) for 0, 1 or 3 days.

analyzed in detail using Me_2SO, 2-propanol and ethanol. As shown in Fig. 2, the concentrations of the compounds necessary for maintaining the enzyme in culture agreed very well with the half-maximal doses required for the spin shift conversion. By contrast, the level of P-450j mRNA from control cells, or cells treated with inducers, decreased rapidly and the mRNA was apparently not detectable from cells cultured for three days (Fig. 1 & Table I).

In separate experiments, cells were treated with cycloheximide between 24 and 48 h of culture. After addition of 0, 0.4 and 1.0 µg/ml of cycloheximide, the presence of Me_2SO (50 mM) during this time period caused, compared to control, an enhanced amount of P-450j by 157, 162 and 147 %, respectively. Under similar conditions, the rate of ^{3}H-leucine incorporation into TCA-precipitable protein was inhibited by 85 % at 0.4 and by 93 % at 1.0 µg of cycloheximide/ml, whereas the viability of the cells was not significantly affected.

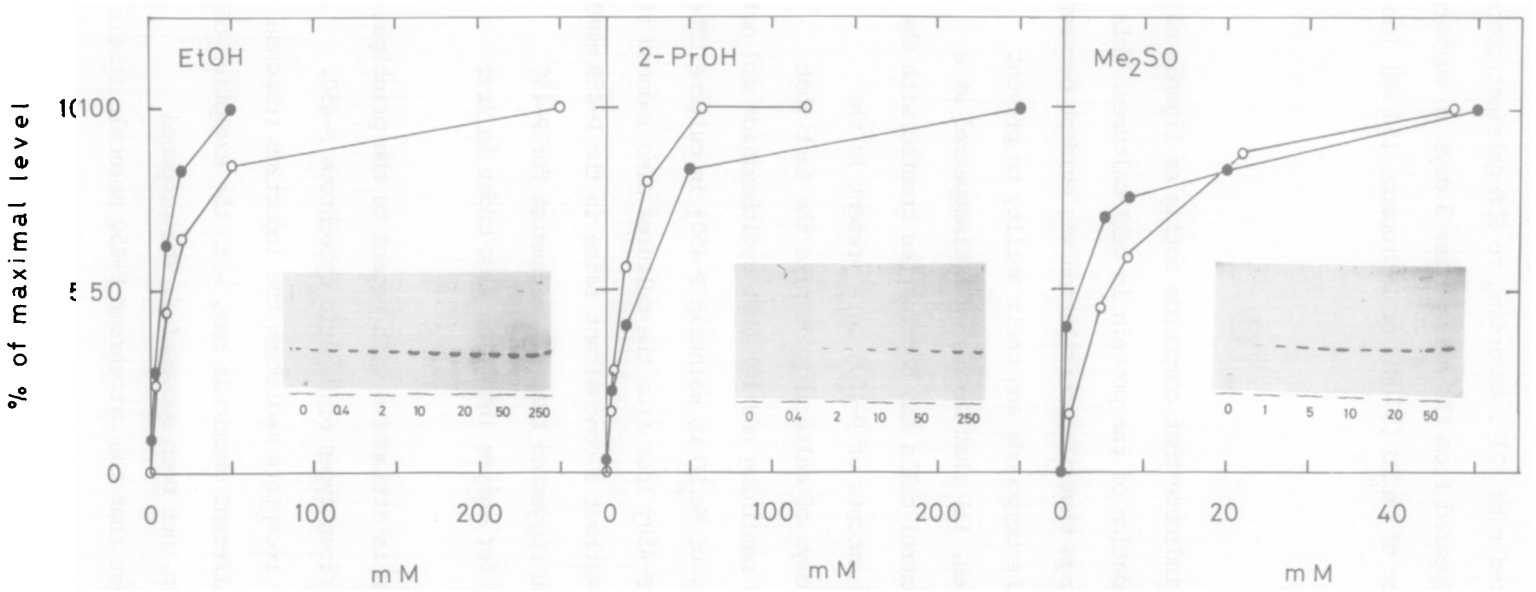

Fig. 2. Effect of the ligand concentration on the extent of low spin shift of purified P-450j (O---O) and the amount of immunodetectable P-450j (●---●) in liver microsomes from primary hepatocytes cultivated for three days. Low spin shift analysis was carried out at 20°C in 50 mM potassium phosphate buffer, pH 7.4, using a Lambda 5 spectrophotometer. The enzyme concentration was 0.3 µM. The results are mean values of two different experiments performed in duplicate with each compound and expressed in relation to the maximum level reached (cf. Table I). Regression analysis revealed correlation coefficients between binding and P-450j maintenance of 0.96 (ethanol), 0.98 (2-propanol) and 0.96 (Me_2SO). Inserts show Western-blot analysis of one of the experiments with each compound.

Cytochrome P-450b is present in large amounts in liver cells from starved and acetone-treated rats (21). According to SDS-gelelectrophoresis, this protein disappeared from the cells after 3 days in culture, regardless of the presence of Me_2SO (50 mM) or 2-propanol (50 mM) (not shown in figure).

DISCUSSION

The results presented indicate that compounds acting as ligands of P-450j also prevent degradation of the protein in cell cultures. This is supported by the findings that i) a correlation was evident between the binding affinity of the compounds and their ability to prevent P-450j loss in the cultures, ii) that P-450j mRNA disappeared at a similar rate from both control cells and hepatocytes treated with the ligands iii) that similar amounts of P-450j were present in the cells both after 1 and 3 days of culturing, despite the fact that almost no P-450j mRNA was remaining and iv) that cycloheximide did not interfere with the ability of Me_2SO to maintain P-450j in culture. The compounds that prevented P-450j loss from the cultures also induced the level of P-450j *in vivo*, without a concomitant raise in the P-450 mRNA level. It can therefore be suggested that the mechanism for P-450 stabilization is relevant for enzyme induction also under *in vivo* conditions.

The suggested mechanism is attractive with regard to the principal action of inducers for various types of hepatic cytochrome P-450. Generally, the search for receptors mediating the induction response of cytochrome P-450 to different chemicals has, with the exception of the Ah-receptor for P-450c, not been successful. The proposed mechanism includes the fact that the cytochrome P-450 molecule itself constitutes a receptor for the chemical. It remains to be established to what extent this principle of induction is common among hepatic cytochromes P-450.

ACKNOWLEDGEMENTS

We are indebted to Asa Johansson for skilful assistance and to Drs Ralf Pettersson and Sune Kvist for synthesis of the oligonucleotide probe. This work was supported by grants from Magn. Bergvalls Stiftelse and from the Swedish Medical Research Council.

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