CYTOCHROME *P*-450 SYNTHESIS IN VIVO AND IN A CELL-FREE SYSTEM FROM RAT LIVER

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1. Introduction

Cytochrome *P*-450, the key liver endoplasmic reticulum protein playing a vital role in drug metabolism, undergoes marked changes in level under a wide variety of conditions [1,2]. While information is available on the effect of drugs on the synthesis and turnover of cytochrome *P*-450–heme [3,4], very little is known about the synthesis of the apo-protein species under these conditions. Recent attempts to evaluate the rate of apo-cytochrome *P*-450 synthesis have essentially involved the measurement of the rate of labeled amino acid incorporation into a protein band on SDS–acrylamide gels, which includes the species of cytochrome *P*-450 [5,6]. In the present study, antibody has been raised to cytochrome *P*-450, purified from the livers of phenobarbital-treated rats and this antibody has been used to follow the rate of synthesis of this protein in vivo and in a cell-free system from the livers of rats subjected to treatments which are known to alter the content of this protein.

2. Experimental

Cytochrome *P*-450 was purified from the livers of phenobarbital-treated rats as in [7]. For this purpose male rats (60–70 g) which had received daily injections of phenobarbital (80 mg/kg) for 4 days were used. The final cytochrome *P*-450 preparation had a specific heme content of 17 nmol heme/mg protein.

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Antibody was raised in rabbits against the purified cytochrome *P*-450 preparation and the schedule of antigen injection was as in [8]. 1 mg immunoglobulin G fraction was found to quantitatively precipitate 45 μg purified cytochrome *P*-450 preparation.

To measure cytochrome *P*-450 synthesis in vivo, 30 μCi ^{14}C-labeled *Chlorella* protein hydrolysate was injected intraperitoneally into rats at different time intervals after suitable drug treatments. The schedule of drug injections is described in the appropriate figure and tables. The animals were killed 1 h after the tracer administration. The microsomal pellet (about 20 mg protein) was solubilized in 4 ml 0.1 M sodium phosphate buffer (pH 7.4) containing 20% glycerol, 1 mM EDTA and 1% cholate and the clear solution was centrifuged at 2500 rev./min for 15 min. To 0.3 ml (2 mg protein) of the soluble preparation 0.4 ml immunoglobulin G fraction representing 2-fold excess of antibody was added. The incubation mixture also contained 0.5 mM phenylmethyl sulfonyl fluoride. The mixture was incubated at room temperature for 1 h and then at 4°C overnight. The immunoprecipitate was collected by centrifugation and suspended in 0.4 ml saline. The suspension was layered over 1 ml 0.5 M sucrose containing 1% deoxycholate in 20 mM Tris–HCl (pH 7.5) buffer and centrifuged. The pellet was dissolved in formic acid and plancheted on filter paper discs for radioactivity measurement.

Protein synthesis in vitro was studied using the post-mitochondrial supernatant. The preparation and assays conditions were as in [9].

At the end of the incubation, the mixture was

solubilized and aliquots were taken for precipitation with trichloroacetic acid and for treatment with the antibody after the addition of carrier cytochrome *P*-450.

Antibody to bovine serum albumin was used to measure non-specific precipitation in the in vivo and in vitro experiments. Non-specific precipitation accounted for about 10% radioactivity in the immunoprecipitate. Some of the immunoprecipitation experiments were cross-checked using monospecific antibody to cytochrome *P*-450. The preparation was based on the general procedure in [10]. Protein was estimated as in [11] using bovine serum albumin as the standard. Radioactivity measurements were made in a Beckman LS-100 liquid scintillation counter using 0.5% PPO in toluene as the scintillant.

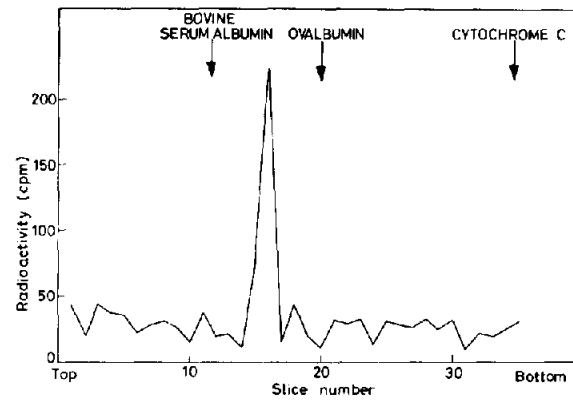

Fig. 1. Radioactivity profile of the cytochrome *P*-450 immunoprecipitate on SDS–gels. 30 μCi ^{14}C-labeled *Chlorella* protein hydrolysate was injected to rats 12 h after a single injection of phenobarbital. The immunoprecipitation was carried out using solubilized microsomal preparations and anti-PB–*P*-450 as described in the text. Electrophoresis was carried out as in [16].

3. Results and discussion

The immunoglobulin G fraction (anti-PB–*P*-450) isolated from rabbits immunized against purified cytochrome *P*-450 from phenobarbital-treated rats was found to be quite specific and reacted weakly in Ouchterlony plates with purified cytochrome *P*-448 preparations from 3-methyl cholanthrene-treated rats, as already demonstrated in [12]. The use of anti-PB–*P*-450 for immunoprecipitating cytochrome *P*-450 from microsomal preparations labeled in vivo and in vitro was examined by subjecting the immunoprecipitate to SDS–acrylamide gel electrophoresis.

The results in fig. 1 indicate a single prominent radioactive peak in the cytochrome *P*-450 region. This pattern was quite similar and reproducible for the preparations obtained from in vivo and in vitro experiments, when the monospecific antibody was used. The use of immunoglobulin G fraction as such for precipitation sometimes resulted in the detection of low molecular weight proteins on the gel. However, this could be suppressed by the inclusion of the protease inhibitor phenyl methylsulphonyl fluoride and the immunoprecipitable radioactivity recovered with both antibody preparations was similar.

In the in vivo experiments, a labeling period of 1 h was employed and the results have been taken to essentially represent the rate of cytochrome *P*-450 synthesis. However, a striking change in the rate of degradation of the protein under conditions of drug treatments contributing to the net incorporation into cytochrome *P*-450 is not ruled out. Therefore additional confirmation has been obtained by measuring the rate of cytochrome *P*-450 synthesis in vitro using the post-mitochondrial supernatant obtained from the livers of animals given the different drug treatments. In these in vitro experiments, essentially the already initiated protein chains are completed and would thus reflect the effect of the drug on the rate of protein synthesis. Differential degradation of cytochrome *P*-450 in the in vitro experiments is unlikely in view of the similar recoveries in the immunoprecipitates obtained from labeled solubilized microsomes added to different preparations of the post-mitochondrial supernatant incubated under conditions of protein synthesis.

The rate of cytochrome *P*-450 synthesis was examined at different time intervals after the administration of phenobarbital to rats. The rate of synthesis was determined both in vivo and in vitro using anti-PB–*P*-450 for immunoprecipitation. Representative experiments were cross-checked using the monospecific antibody. The results presented in table 1 indicate that 6 h after phenobarbital administration there is nearly a 2-fold increase in the rate of cytochrome *P*-450 synthesis. Maximum rate of synthesis is observed at 12 h after phenobarbital administration. A similar pattern of results is obtained in vivo and in vitro. The

Table 1
Rate of cytochrome *P*-450 synthesis in vivo and in vitro after phenobarbital administration

Time (h) after phenobarbital administration	Cytochrome *P*-450 synthesis			
	In vivo			In vitro
	Total microsomal protein (cpm/mg protein)	Cytochrome *P*-450 (% total microsomal protein)	Total protein (cpm/mg protein)	Cytochrome *P*-450 (% total protein)
0	19 671	3.8	14 745	0.98
6	21 735	6.4	15 625	1.52
12	23 523	15.0	17 326	3.61
18	22 625	7.8	17 459	1.54

The in vitro synthesis was carried out with the S-17 liver fraction as in [10]. The incubation mixture in 1 ml contained: 20 mM Tris–HCl, pH 7.5, 60 mM KCl, 5 mM $MgCl_2$, 75 mM sucrose, 1 mM DTT, 1.6 mM ATP, 0.6 mM GTP, 10 mM phosphoenolpyruvate, 40 μg pyruvate kinase, 25 μM each of 19 amino acids, 10 μCi [^{3}H]leucine and 0.2 ml S-17 fraction. The mixture was incubated for 20 min at 28°C. The other experimental details are given in text

Table 2
Effect of actinomycin D and 3-amino-1,2,4-triazole on the rate of cytochrome *P*-450 synthesis

Treatment	Cytochrome *P*-450 synthesis (% total protein)	
	In vivo	In vitro
Saline	3.50	0.96
Phenobarbital (12 h)	15.20	3.61
Actinomycin D (12 h)	3.10	0.99
Phenobarbital (12 h) + actinomycin D (12 h)	6.40	0.89
Phenobarbital (16 h)	11.20	1.96
3-Amino-1,2,4-triazole (16 h)	2.90	0.62
Phenobarbital (16 h) + 3-amino-1,2,4-triazole (16 h)	6.40	1.26
Phenobarbital (16 h) + 3-amino-1,2,4-triazole (16 h) + hemin	9.80	Not done

Actinomycin D (1.5 mg/kg) or 3-amino-1,2,4-triazole (3 g/kg) was injected intraperitoneally 15 min before phenobarbital (80 mg/kg) administration. Actinomycin D injection was repeated 6 h after the first. Hemin (20 mg/kg) was injected before the administration of the label. The animals were killed at the time intervals indicated. The other experimental details are given in text

effect of phenobarbital has also been confirmed in in vivo experiments, where the labeling period was reduced to 15 min (data not presented).

Actinomycin D and 3-amino-1,2,4-triazole, an inhibitor of heme synthesis, have been reported to block the increase in the content of cytochrome *P*-450 induced by phenobarbital [13,14]. In the present study the effects of these two inhibitors on the rate of cytochrome *P*-450 synthesis were studied after phenobarbital administration. The results presented in table 2 indicate that at the time intervals examined, both actinomycin D and 3-amino-1,2,4-triazole bring about a significant decrease in the rate of cytochrome *P*-450 synthesis. The effect of 3-amino-1,2,4-triazole is counteracted partially by the administration of exogenous hemin. It it likely that the effect of phenobarbital on cytochrome *P*-450 synthesis is mediated through enhanced RNA synthesis and that prolonged inhibition of heme synthesis would decrease the rate of apo-protein synthesis. It has recently been suggested that heme is possibly not directly involved in the binding of this protein to the endoplasmic reticulum [15].

Studies are in progress to examine the site of heme action and quantitation of cytochrome *P*-450 messenger RNA.

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References

[1] Conney, A. H. (1967) Pharmacol. Rev. 19, 317–366.
[2] Kuntzman, R. (1969) Annu. Rev. Pharmacol. 9, 21–36.
[3] Levin, W. and Kuntzman, R. (1969) J. Biol. Chem. 244, 3671–3676.
[4] Greim, H., Schenkman, J. B., Klotzbucher, M. and Remmer, H. (1970) Biochem. Biophys. Acta 201, 20–25.
[5] Rajamanickam, C., Satyanarayana Rao, M. R. and Padmanaban, G. (1975) J. Biol. Chem. 250, 2305–2310.
[6] Haugen, D. A., Coon, M. J. and Nebert, D. W. (1976) J. Biol. Chem. 251, 1817–1827.
[7] Ryan, D., Lu, A. Y. H., Kawalek, J., West, S. B. and Levin, W. (1975) Biochem. Biophys. Res. Commun. 64, 1134–1141.
[8] Thomas, P. E., Lu, A. Y. H., Ryan, D., West, S. B., Kawalek, J. and Levin, W. (1976) J. Biol. Chem. 251, 1385–1391.
[9] Iynedjian, P. B. and Hanson, R. W. (1977) J. Biol. Chem. 252, 655–662.
[10] McCans, J. L., Lane, L. K., Lindenmeyer, G. E., Butler, V. P., jr and Schwartz, A. (1974) Proc. Natl. Acad. Sci. USA 71, 2449–2452.
[11] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[12] Thomas, P. E., Lu, A. Y. H., Ryan, D., West, S. B., Kawalek, J. and Levin, W. (1976) Mol. Pharmacol. 12, 746–758.
[13] Marver, H. S. (1969) in: Microsomes and Drug Oxidation (Gillette, J. R. et al. eds) pp. 495–515, Academic Press, New York.
[14] Baron, J. and Tephly, T. R. (1969) Mol. Pharmacol. 5, 10–20.
[15] Bhat, K. S., Sardana, M. K. and Padmanaban, G. (1977) Biochem. J. 164, 295–303.
[16] Laemmli, U. K. (1970) Nature (London) 227, 680–685.