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HORMONAL REQUIREMENTS FOR THE INDUCTION OF CYTOCHROME P450 IN HEPATOCYTES CULTURED IN A SERUM-FREE MEDIUM

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Summary: Drug mediated induction of cytochrome P450 was studied in cultures of hepatocytes that had never been cultured in the presence of serum. Propylisopropylacetamide induced a five-fold increase in cytochrome P450, approximating *in ovo* induced levels, when triiodothyronine and/or dexamethasone were included in the culture medium. Insulin was apparently not required for this induction. Cytochrome P450, free of cytochrome oxidase, could be fully recovered from cell homogenates in a 8700g supernatant, by use of a buffer containing 0.2% Emulgen.

Cytochrome P450 (P450) is the terminal oxidase of the mixed function oxidases of the liver, a system that metabolizes many drugs and activates several carcinogens. Many investigators have been trying to retain both *in vivo* levels and inducibility of P450 in cultured hepatocytes (1,2,3). We recently presented evidence for the induced synthesis of at least two different species of P450 in primary cultures of chick embryo hepatocytes (4,5), the only primary culture in which δ-aminolevulinate synthase (δ-ALAS) is inducible (6, 7,8). δ-ALAS is the rate-limiting enzyme in the hepatic synthesis of heme, the prosthetic group of P450. In the induction studies with P450, serum was present during the first 24h of culture and absent during the induction period (h 24 through 44). Here we show that P450 can be induced even when serum is eliminated altogether from the culture. Furthermore, by including triiodothyronine and dexamethasone in the medium, the levels of microsomal P450 induced by propylisopropylacetamide approximate *in ovo* induced levels.

Abbreviations: WE: Williams E medium; P450: Cytochrome P450; Dex, Dexamethasone; T3: 3,3',5-Triiodothyronine; δ-ALAS: δ-aminolevulinate synthase; β-NF: β-naphthoflavone; and PIA: Propylisopropylacetamide.

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Insulin was not necessary for induction of cytochrome P450 although it is required for optimal induction of δ-ALAS (6,7,9,10).

MATERIALS AND METHODS

Reagents: Williams E (WE) (Flow Labs) contained 2mM glutamine, 100 U penicillin/ml, 100 µg streptomycin/ml. Trypsin (TC59) was from Wellcome; 2-propyl-2-isopropylacetamide (PIA), a gift from Hoffmann La Roche; β-naphthoflavone (β-NF), Aldrich; Insulin (Sigma bovine pancreas) was dissolved in 0.02N HCl; Dexamethasone (Dex) (Maurry Biological Co., sodium phosphate salt, 4mg/ml) was diluted with sterile H_2O; 3,3',5-triiodothyronine (T3) (Sigma, sodium salt) was dissolved in 0.05N NaOH and diluted ten-fold with H_2O. Both Dex and T3 were made fresh before use. Phosphate-glycerol buffer was 0.1M sodium phosphate pH 7.4-20% glycerol (v/v). Phosphate-glycerol-Emulgen buffer contained, in addition, 1mM EDTA, 1mM Dithiothreitol, and 0.2% (v/v) Emulgen (12). Tris-sucrose was 0.02M Tris Cl pH 7.4 and 0.25M sucrose.

Culture of chick embryo hepatocytes: Cultures of 16 to 18-day embryonic livers were prepared by a modification of several procedures (4,7,11). The livers were minced with a razor blade and digested in 0.25% trypsin in Hanks Balanced Salt solution without Ca^{++}. Contaminating red blood cells were removed (7) and the hepatocytes were resuspended in WE containing, unless indicated, (per ml): 1 µg insulin, 0.3 µg Dex, and 1.0 µg T3. After 20h, the medium was changed to WEH (WE containing Dex and T3 but no insulin). A second change to WEH was made at the 22h of culture. At the 24h of culture, chemicals were added (in 2 µl ethanol per ml medium) at the following final concentrations: Control, ethanol as indicated; PIA, 20 µg/ml; β-NF, 4 µg/ml. The exposure varied from 18 to 21h.

Cytochrome P450 was determined by the method of Omura and Sato (13) for all preparations except the whole homogenates of embryonic livers where, because of the contamination of hemoglobin in the samples, P450 was measured from dithionite-reduced CO vs. CO difference spectra (16).

Preparation of subcellular fractions from cultured hepatocytes and intact liver: Cultured cells: 8700g supernatant: Each 6cm petri dish of cells was rinsed with 5ml phosphate-glycerol buffer (room temperature). The cells were harvested in 0.9ml phosphate-glycerol-Emulgen buffer (4°C). The cell suspension was homogenized in a Potter-Elvehjem homogenizer (600 RPM, 10 strokes), and centrifuged at 8700g in a Microfuge B (Beckman Instruments) for 8 min. Microsomes: Dishes were rinsed in Tris-sucrose at room temperature and harvested in the same buffer at 4°C. The cell suspensions from three 10cm dishes were combined and homogenized in a 5ml Potter-Elvehjem homogenizer (600 RPM, 11 strokes). The homogenate was then precooled for 20 sec in an ice-salt bath (-8°C) and sonicated for 13 sec at a setting of 2.5, using a Model W140D Sonifier Cell Disrupter (Heat Systems Ultrasonics, Inc.). This sonicate was centrifuged at 10,000g for 12 min and the supernatant recentrifuged at 105,000g for 1h. The microsomal pellet was resuspended in phosphate-glycerol buffer.
Intact liver: All chemicals were injected in 0.2ml DMSO in the fluid surrounding the 17-day old embryo. Chemicals injected and time of treatment: Control (DMSO), 44h; PIA, 2 mg/egg, 20h; β-NF, 1.5 mg/egg, 44h. Chick embryos were perfused through the heart with 20ml ice cold saline. The livers were excised and a 20% homogenate (w/v) of 3 livers made in Tris-sucrose buffer. Microsomes were prepared as for the cultured cells. They were washed once in 0.15M KCl.

Table 1. Comparison of cytochrome P450 in homogenates and microsomes of cultured hepatocytes and embryonic livers.

Treatment	Cytochrome P450 (p moles/mg protein)			
	Microsomes		Whole Homogenate	
	Cultured Cells	Embryo	Cultured Cells	Embryo
Control	105 (120, 90)	215 (210, 220)	14 (16, 12)	139 ± 9
PIA	545 (530, 560)	645 (650, 640)	157 (149, 165)	315 ± 16
β-NF	230 (210, 250)	510 (550, 470)	100 (106, 095)	245 ± 15

All P450 values given are the means of duplicate samples, except for whole homogenates of chick embryo livers, in which the values are the means ± SEM for six individual embryos.

Other assays: Porphyrins were measured spectrofluorometrically (6). Protein concentrations were determined (14), using crystalline BSA as standard.

RESULTS AND DISCUSSION

The data shown in Table 1 were obtained from cells cultured in serum-free WE, containing insulin, Dex, and T3 for the first 24h; insulin was deleted during the induction period (24 through 44h). The specific activity of microsomal P450 induced by PIA was three times the value we reported previously (4,5), and approximates the level in hepatic microsomes from embryos treated with PIA. The specific activities of P450 in microsomes, from both control cells and cells treated with β-NF, were about 50% those obtained for microsomes of livers from similarly treated embryos, in agreement with previous findings (4,5).

The levels of P450 in homogenates of treated cells were about 50% of the values for the corresponding liver homogenates from intact embryos. Table 2 shows that the recovery of total P450 in the microsomal fractions varied with the inducers used. These results suggest that in hepatocytes of chicken embryo, as well as rats (15), the measurement of P450 in microsomes may not accurately reflect homogenate levels.

Table 2. Recovery of homogenate cytochrome P450 in subcellular fractions.

	Microsomes		8700g Supernatant
	Cultured Cells %	Embryo %	Cultured Cells %
Control	66.3 (66, 66.7)	13.9 (13.9, 14.0)	157 (137, 183)
PIA	64.2 (63.1, 65.3)	20.8 (19.9, 21.7)	96 (84, 108)
β-NF	38.5 (31.8, 45.2)	14.7 (13.6, 15.9)	117 (108, 125)

8700g supernatant was prepared in phosphate-glycerol-Emulgen buffer as in Methods. The values are the percent of total P450 determined in homogenates. The numbers in parentheses are values from duplicate preparations. Homogenate levels were identical whether measured in Tris-sucrose or phosphate-glycerol-Emulgen.

In the case of untreated cells and untreated embryonic liver the homogenate P450 of cells was only 1/10 the value of liver whereas the microsomal level of cells was 1/2 the microsomal level of liver. This apparent discrepancy results from interference by cytochrome oxidase in the spectral measurement of homogenate P450 (16,17), when the concentration of P450 is less than 60 pmoles per mg protein (3,16). The CO-reduced vs. reduced spectra of control homogenates from cultured hepatocytes has a major peak at 430 nm and the 450 nm peak was shifted to 454 nm, indicating interference from cytochrome oxidase (results not shown). This interference probably accounts for the apparent low measurement of P450 in homogenates of control cells.

Total recovery of cytochrome P450 in 8700g supernatant

Cytochrome oxidase can be removed from the cell homogenate, with 100% recovery of P450 in the supernatant, by using a phosphate-glycerol-Emulgen buffer (Table 2, Figure 1). The spectra of the 8700g supernatants have only a small shoulder at 430 nm and have peaks similar to those obtained for microsomes, with β-NF causing a shift to the blue. The apparent high recovery (157%) in 8700g supernatant prepared from control cells reflects the underestimation

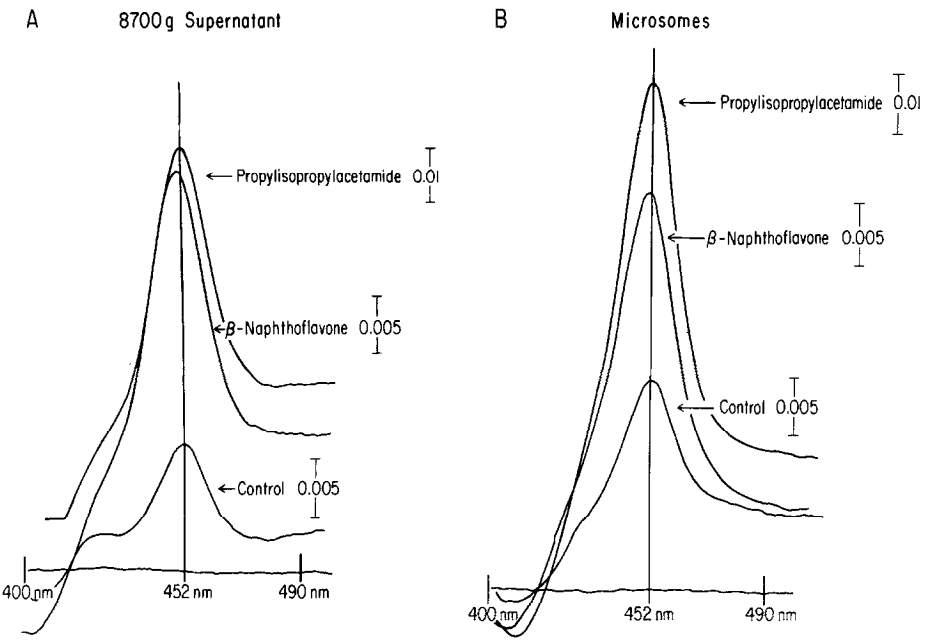

Figure 1. A. Cells were scraped and homogenized in Phosphate-glycerol-Emulgen buffer and the 8700g supernatant prepared according to the procedure given in the Methods section. Protein concentration: Control, 1.8 mg/ml; PIA, 2.9 mg/ml; β-NF, 2.7 mg/ml Note each spectrum was measured at a different sensitivity. B. Cells were scraped and homogenized in Tris-sucrose buffer. The homogenate was sonicated and centrifuged at 10,000g for 12 min. The resultant supernatant was centrifuged at 105,000g for 1h, and the microsomal pellet was resuspended in Phosphate-glycerol buffer. Protein concentration: Control, 1.8 mg/ml; PIA, 1.4 mg/ml; β-NF, 1.3 mg/ml. Cytochrome P450 was determined according to the method of Omura and Sato (13).

of P450 in the homogenate due to interference from cytochrome oxidase. Cytochrome P450 is stable for at least 24h at -20°C in phosphate-glycerol-Emulgen buffer. This new method permits measurement of P450 on only 2mg of cellular protein.

Hormonal supplements increase induction of cytochrome P450 and porphyrin accumulation

As a rapid screening procedure, P450 was measured in 8700g supernatants of homogenates prepared in phosphate-glycerol-Emulgen buffer. We investigated the conditions for induction of P450 and δ-ALAS by PIA. Total porphyrin accumulation in cells plus media was used as an indication of the induced level of δ-ALAS (6).

Table 3. Effect of hormones and serum on the induction of cytochrome P450 and porphyrins in cultured chick embryo hepatocytes.

Media Supplements		Cytochrome P450		Porphyrin
0 - 24h	24 - 44h	Control	PIA	PIA
5% Fbs	a) Insulin	56.7 ± 4.0	104.7 ± 9.1	2370 (2430, 2310)
	b) Dex T3, Insulin	37.2 ± 4.7	125 ± 7.6	1930 (2080, 1780)
	c) 5% Fbs	52.5 ± 7.7	121 ± 6.9	1670 (1820, 1520)
Insulin	d) Insulin	49.5 ± 5.6	94 ± 4.2	611 (587, 643)
	e) Insulin	32.5 ± 4.5	114.5 ± 5.5	1118 (1133, 1104)
Insulin Dex T3	f) Insulin Dex, T3	33 ± 1.6	119 ± 8.6	2095 (1970, 2220)
	g) Dex, T3	32.7 ± 4.2	178.7 ± 6.7	737 (634, 839)
Insulin Dex	h) Dex	55.7 ± 3.0	195.5 ± 18.9	215 (196, 235)
Insulin T3	i) T3	42.7 ± 5.9	169 ± 5.2	202 (196, 209)
Insulin	j) --	49.2 ± 1.5	93.2 ± 2	75 (72, 78)
------	k) --	43.5 (40, 47)	79.5 (73, 86)	104 (100, 108)

Cultures of chick embryo hepatocytes were prepared according to the procedure outlined in the Methods section. Cells were inoculated in WE containing the supplements indicated. Cytochrome P450 is in p moles of P450 per mg protein in 8700g supernatant. All values given are the mean ± SEM for 4-6 separate experiments except for the condition where there are no additional supplements to WE (k). In this case, the values given represent the means of duplicate samples from one experiment. Porphyrins are in p moles of total porphyrins (media plus cell homogenate) per mg homogenate protein. The values given are the means of duplicate samples from one representative experiment. Individual values are shown in parentheses. The range of the control values was 34 to 138.

Table 3 shows that P450 can be increased two-fold even if no hormonal supplements are added to WE (Table 3, row k), whereas induction of δ-ALAS, as others have shown (6,7,18,19), requires hormonal supplementation (compare rows j through k with a through i). Even though induction of P450 occurs in the

continual presence of serum (Table 3-c), the induced amount is greatest when serum is omitted altogether (Table 3-f through i). Induction of δ-ALAS (Table 3-a, -b, -d, -f) was greatest in the presence of insulin in agreement with previous findings (6,7,9,10). On the other hand, only Dex and T3, alone or in combination, are required for greatest induction of P450 (Table 3-g, -h, -i). The combination of Dex and T3 provided a synergistic effect on induction of porphyrins compared to the presence of either hormone alone (Table 3-g vs. -h and -i). The induction of P450, however, was the same in all three conditions. We added insulin during the first 24h of culture because in its absence only 50% of the cell inoculum adhered to the dish.

In summary, chick embryo hepatocytes cultured in a chemically defined medium devoid of serum retain the full physiological ability to respond to drugs with induction of cytochrome P450. The conditions for inducing δ-ALAS differ from those for induction of cytochrome P450.

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REFERENCES

1. Michalopoulos, G., Sattler, C.A., Sattler, G.L., and Pitot, H.C. (1976) Science 193, 907-909.
2. Decad, G.M., Hsieh, D.P.H., and Byard, J.L. (1977) Biochem. Biophys. Res. Commun. 78, 279-287.
3. Paine, A.J., and Legg, R.F. (1978) Biochem. Biophys. Res. Commun. 81, 672-679.
4. Althaus, F.R., Sinclair, J.F., Sinclair, P., and Meyer, U.A. (1979) J. Biol. Chem., in press.
5. Althaus, F.R., Sinclair, J.F., Sinclair, P., and Meyer, U.A. (1978) Fed. Proc. 37, 1759.
6. Granick, S., Sinclair, P., Sassa, S., and Grieninger, G. (1975) J. Biol. Chem. 250, 9215-9225.
7. Sassa, S., and Kappas, A. (1977) J. Biol. Chem. 252, 2428-2436.
8. Rifkind, A.B., Gillette, P.N., Song, C.S., and Kappas, A. (1973) J. Pharmacol. Exp. Ther. 185, 214-225.
9. Fischer, P., Morgan, R., Krupa, V., and Marks, G. (1976) Biochem. Pharmacol. 25, 687-693.
10. Bonkowsky, H.L., Collins, A., Doherty, J.M., and Tschudy, D.P. (1973) Biochim. Biophys. Acta. 326, 561-576.
11. Grieninger, G., Hertzberg, K.A., and Pindyck, J. (1978) Proc. Natl. Acad. Sci. USA 75, 5506-5510.
12. Imai, Y., and Sato, R. (1974) Biochem. Biophys. Res. Commun. 60, 8-14.
13. Omura, T., and Sato, R.(1964) J. Biol. Chem. 239, 2370-2385.
14. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.

15. McLean, A.E.M., and Day, P.A. (1974) Biochem. Pharmacol. 23, 1173-1180.
16. Kowal, J., Simpson, E.R., and Estabrook, R.W. (1970) J. Biol. Chem. 245, 2438-2443.
17. Pohl, R.J., Philpot, R.M., and Fouts, J.R. (1976) Drug Metab. Dispos. 4, 442-450.
18. Morgan, R., Stephens, J., Fischer, P., and Marks, G. (1977) Biochem. Pharmacol. 26, 1389-1394.
19. Lane, S., Gidari, A., Levere, R. (1977) Biochem. Biophys. Res. Commun. 74, 57-63.