*Biochem. J.* (1983) **210**, 855–857
*Printed in Great Britain*



# The role of 5-aminolaevulinate synthase, haem oxygenase and ligand formation in the mechanism of maintenance of cytochrome *P*-450 concentration in hepatocyte culture

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(*Received 17 September 1982/Accepted 2 December 1982*)

The present work shows that the ability of pyridines, e.g. metyrapone, to maintain the cytochrome *P*-450 concentration in cultured hepatocytes is not due to their ability to alter the 5-aminolaevulinate synthase and haem oxygenase activities of the hepatocytes. Since ligands such as metyrapone will prevent the cobalt-mediated loss of hepatic cytochrome *P*-450 in rats, the hypothesis that ligand formation is the mechanism of maintenance of the cytochrome in hepatocyte culture was tested. The observation that non-pyridine ligands will maintain the cytochrome *P*-450 concentration supports this hypothesis.

Rat hepatocytes lose their cytochrome *P*-450 when cultured for 24h (Paine & Legg, 1978). This is due to an enhanced degradation and the failure of cultured rat hepatocytes to synthesize cytochrome *P*-450. Metyrapone [2-methyl-1,2-di-(3-pyridyl)-propan-1-one], and other pyridines, prevent this loss by permitting the synthesis and reducing the degradation of the cytochrome (Paine & Villa, 1980). A clue to the mechanism underlying the synthetic effects of metyrapone is the observation that 5-aminolaevulinic acid, a precursor of the haem prosthetic group of cytochrome *P*-450, is able to produce a partial maintenance of the cytochrome (Paine & Hockin, 1980). This finding suggests that cultured rat hepatocytes may have a defect in haem synthesis before the formation of 5-aminolaevulinic acid. In addition to this, since haem oxygenase is a pathway of cytochrome *P*-450 degradation (De Matteis, 1978) the effects of pyridines on degradation could reflect an ability to inhibit the induction of haem oxygenase (EC 1.14.99.3), which is known to occur when rat hepatocytes are placed in culture (Paine & Legg, 1978). Accordingly, we have determined the effect of different culture conditions on 5-aminolaevulinate synthase (EC 2.3.1.37) and haem oxygenase activities of rat hepatocytes.

## Materials and methods

### *Isolation and culture of hepatocytes*

Hepatocytes were isolated as previously described (Paine *et al.*, 1982) and cultured at a density of $20 \times 10^6$ cells/20ml of medium in 150mm-diameter Petri dishes (Lux Scientific; supplied by Flow Laboratories, Irvine, Scotland, U.K.). The medium used was Williams medium E supplemented with 5% (v/v) foetal-calf serum, 5mg of gentamycin/100ml (all from Flow Laboratories), 1$\mu$M-insulin and 0.1mM-cortisol-21-sodium succinate (both from Sigma Chemical Co., Poole, Dorset, U.K.). The compounds studied were dissolved directly in the culture medium.

### *Whole-animal experiments*

Adult (180–200g) male rats of the Porton-derived Wistar strain, fed *ad libitum* on MRC 41B diet, were injected intraperitoneally with metyrapone, dissolved in 0.15M-NaCl at a dose of 100mg/kg body wt. $CoCl_2$ in 0.15M-NaCl was injected subcutaneously at a dose of 60mg/kg body wt. Control rats received appropriate volumes of 0.15M-NaCl intraperitoneally and subcutaneously. [Rats treated only with metyrapone or $CoCl_2$ received 0.15M-NaCl either subcutaneously or intraperitoneally.] At 24h after injection the rats were anaesthetized, by intraperitoneal injection with 60mg of pentobarbital/kg body wt. and their livers were perfused with 250ml of ice-cold 0.15M-NaCl, excised and homogenates (20%, w/v) were prepared in 0.15M-KCl containing 50mM-Tris/HCl buffer, pH7.4, using an Ultra-Turrax TP18/10 blender for 10s.

### *Chemicals*

Imidazole, isonicotinamide, 3-acetylpyridine, 3-aminopyridine and 3-hydroxypyridine were pur-



0306-3283/83/030855-03$2.00

chased from Aldrich Chemical Co., Gillingham, Dorset, U.K. Pyridine, sodium succinate and $CoCl_2$ were from BDH, Poole, Dorset, U.K. [2,3-$^{14}$C]-Succinic acid was purchased from Amersham International, Amersham, Bucks., U.K. Metyrapone was a gift from Ciba Laboratories, Horsham, Sussex, U.K. 1-(2-Cyanophenyl)imidazole was synthesized as described by Johnson *et al.* (1969).

*Cytochrome P-450 and protein*

These were determined as previously described (Paine *et al.*, 1979). The addition of ligands to cell homogenates was not found to interfere with the determination of cytochrome *P*-450 or protein. Furthermore carbon monoxide is known to displace ligands, such as metyrapone, from the cytochrome (Hildebrandt *et al.*, 1969).

*5-Aminolaevulinate synthase activity*

This was determined by using [2,3-$^{14}$C]succinic acid (sp. radioactivity 2.73 Ci/mol) as described by De Matteis *et al.* (1981). The incubation mixture (volume 2 ml) contained 2 mg of cell protein. Under these conditions conversion of [2,3-$^{14}$C]succinate into 5-aminolaevulinate was proportional to time for up to 2 h.

*Haem oxygenase activity*

This was assayed in homogenates (5–8 mg of protein/ml of 0.15 M-KCl in 50 mM-Tris/HCl buffer, pH 7.4) on a Perkin–Elmer 356 spectrophotometer in the dual-beam mode as described by De Matteis & Gibbs (1976).

**Results and discussion**

The results in Table 1 show that freshly isolated hepatocytes and hepatocytes cultured for 24 h without treatment have the same 5-aminolaevulinate synthase activity as reported by De Matteis *et al.* (1981) for intact liver ($0.9 \pm 0.1$ pmol/min per mg of protein). Therefore the loss of cytochrome *P*-450 in cultured hepatocytes is not due to a decrease in activity of the rate-limiting enzyme of haem biosynthesis. However, Table 1 shows that there is a marked induction of haem oxygenase activity in hepatocytes cultured for 24 h. Thus it may be necessary to induce the 5-aminolaevulinate synthase activity in order to maintain the haem pool in the hepatocyte. Indeed, Table 1 shows that some of the pyridines that are able to prevent the loss of cytochrome *P*-450 induce 5-aminolaevulinate synthase activity. However, since they do not all induce 5-aminolaevulinate synthase activity it is unlikely that an effect solely on haem biosynthesis is their mechanism of action. Another mechanism of maintenance could be that pyridines are able to prevent the induction of haem oxygenase. Table 1 shows that some of the pyridines decrease the induction of haem oxygenase but none of them inhibit it completely. Therefore an effect solely on haem oxygenase is not their mechanism of action. Since these pyridines produce every possible combination of effect on 5-aminolaevulinate synthase and haem oxygenase activities is is unlikely that these variable effects on haem metabolism are the mechanism underlying their common ability to maintain the cytochrome *P*-450 concentration in hepatocyte culture.

However, these pyridines have one known, common, effect, which may relate to the maintenance of the cytochrome *P*-450 concentration, and this is their ability to bind to the cytochrome (Paine *et al.*, 1980). When metyrapone, the most potent of these ligands, is administered to rats, it is able to protect hepatic cytochrome *P*-450 against the degradation

Table 1. *Effect of pyridines on the concentration of cytochrome P-450 and activities of 5-aminolaevulinate synthase and haem oxygenase in hepatocytes cultured for 24 h*

Isolated hepatocytes and rat liver cells cultured for 24 h in Williams medium E with or without pyridines at the concentrations shown were assayed for cytochrome *P*-450, 5-aminolaevulinate synthase and haem oxygenase activities as described in the Materials and methods section. The initial cytochrome *P*-450 concentration of isolated hepatocytes was $196 \pm 10$ ($n = 3$) pmol/mg of protein. The results are means $\pm$ S.D. for individual values found in preparations derived from three separate rat livers (i.e. $n = 3$). * denotes significantly different values ($P < 0.05$) from hepatocytes cultured without treatment.

| Treatment | Cytochrome *P*-450 concn. (% of initial value) | 5-Aminolaevulinate synthase activity (pmol of aminolaevulinate formed/min per mg of protein) | Haem oxygenase activity (pmol of bilirubin formed/min per mg of protein) |
|---|---|---|---|
| Isolated cells | 100 | $1.0 \pm 0.2$ | $8 \pm 3$ |
| Cells cultured for 24 h | | | |
| (*a*) Without treatment | $49 \pm 10$ | $0.8 \pm 0.1$ | $62 \pm 6$ |
| (*b*) +0.5 mM-Metyrapone | $99 \pm 4$ | $1.9 \pm 0.3$* | $27 \pm 4$* |
| (*c*) +5 mM-3-Hydroxypyridine | $90 \pm 11$ | $0.7 \pm 0.1$ | $67 \pm 7$ |
| (*d*) +10 mM-Acetylpyridine | $77 \pm 10$ | $1.8 \pm 0.3$* | $33 \pm 8$* |
| (*e*) +10 mM-Aminopyridine | $77 \pm 10$ | $1.4 \pm 0.1$* | $61 \pm 15$ |
| (*f*) +10 mM-Isonicotinamide | $79 \pm 10$ | $0.5 \pm 0.2$ | $25 \pm 4$* |
| (*g*) +25 mM-Pyridine | $106 \pm 9$ | $1.8 \pm 0.3$* | $40 \pm 9$ |

Table 2. *Effect of metyrapone on the cobalt-mediated loss of cytochrome P-450 in rat liver*

Rats were treated and cytochrome *P*-450 concentration and haem oxygenase activity were determined as described in the Materials and methods section. The results are means ± S.D. for individual values found in rat livers (i.e. $n = 3$). * denotes significantly different values ($P < 0.05$) from control rats.

| Treatment | | Cytochrome *P*-450 (pmol/mg of protein) | Haem oxygenase activity (pmol of bilirubin formed/min per mg of protein) |
|---|---|---|---|
| 100mg of metyrapone/kg body wt. | 60mg of $CoCl_2$/kg body wt. | | |
| – | – | 174 ± 20 | 8 ± 1 |
| – | + | 100 ± 9* | 112 ± 6* |
| + | – | 235 ± 18 | 13 ± 2 |
| + | + | 210 ± 16 | 200 ± 45* |

Table 3. *Effect of 1-(2-cyanophenyl)imidazole and imidazole on the cytochrome P-450 concentration of hepatocytes cultured for 24 h*

Hepatocytes were cultured in Williams medium E with or without 250 μM-1-(2-cyanophenyl)imidazole or 2.5 mM-imidazole for 24 h when their cytochrome *P*-450 content was determined. The results are means ± S.D. for individual values found in preparations derived from three separate rat livers ($n = 3$). * denotes values not significantly different ($P < 0.05$) from the cytochrome *P*-450 content of isolated hepatocytes before culture, which was 196 ± 10 pmol/mg of protein.

| Addition to medium | Cytochrome *P*-450 concn. (% of initial value) |
|---|---|
| None | 51 ± 2 |
| 250 μM-1-(2-Cyanophenyl)imidazole | 101 ± 14* |
| 2500 μM-Imidazole | 97 ± 13* |

associated with the administration of $CoCl_2$ (Table 2). These observations are in agreement with those of Drummond *et al.* (1982), who found that SKF 525A (2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride), which is also known to bind to cytochrome *P*-450, could protect against the loss of hepatic cytochrome *P*-450 in rats without inhibiting the cobalt-mediated increase in haem oxygenase activity. Thus ligands may protect cytochrome *P*-450 from degradation by binding to it and making it inaccessible to degradation, via haem oxygenase, rather than by preventing the induction of haem oxygenase activity. If this is the case then ligands other than pyridines should protect cytochrome *P*-450 in hepatocyte cultures. To examine this hypothesis we have determined the effects of 1-(2-cyanophenyl)imidazole and imidazole, two non-pyridine ligands, on the cytochrome *P*-450 concentration of hepatocytes cultured for 24 h. The results in Table 3 show that both 1-(2-cyanophenyl)imidazole and imidazole will prevent the loss of cytochrome *P*-450 in hepatocyte culture.

In conclusion the only common denominator between the pyridines that maintain the cytochrome *P*-450 concentration in hepatocyte culture appears to be their ability to form ligands with the cytochrome. The finding that ligands can protect cytochrome *P*-450 in hepatocyte culture from degradation and the observation that they can protect hepatic cytochrome *P*-450 in rats treated with $CoCl_2$ suggest that cytochrome *P*-450–ligand complexes are inaccessible to degradation via haem oxygenase. Although it is possible to explain the effect of ligands on the degradation of cytochrome *P*-450, the mechanism by which they permit its synthesis is unclear.

## References


De Matteis, F. (1978) in *Heme and Hemoproteins* (De Matteis, F. & Aldridge, W. N., eds.), pp. 95–127, Springer-Verlag, Berlin

De Matteis, F. & Gibbs, A. H. (1976) *Ann. Clin. Res.* **8**, 193–197

De Matteis, F., Zetterlund, P. & Wetterberg, L. (1981) *Biochem. J.* **196**, 811–817

Drummond, G. S., Rosenberg, D. W. & Kappas, A. H. (1982) *Biochem. J.* **202**, 59–66

Hildebrandt, A. G., Leibman, K. C. & Estabrook, R. W. (1969) *Biochem. Biophys. Res. Commun.* **37**, 477–485

Johnson, A. L., Kauer, J. C., Sharma, D. C. & Dorfman, R. I. (1969) *J. Med. Chem.* **12**, 1024–1028

Paine, A. J. & Hockin, L. J. (1980) *Biochem. Pharmacol.* **29**, 3215–3218

Paine, A. J. & Legg, R. F. (1978) *Biochem. Biophys. Res. Commun.* **81**, 672–679

Paine, A. J. & Villa, P. (1980) *Biochem. Biophys. Res. Commun.* **97**, 744–750

Paine, A. J., Hockin, L. J. & Legg, R. F. (1979) *Biochem. J.* **184**, 461–463

Paine, A. J., Villa, P. & Hockin, L. J. (1980) *Biochem. J.* **188**, 937–939

Paine, A. J., Allen, C. M., Durkacz, B. W. & Shall, S. (1982) *Biochem. J.* **202**, 551–553