Biochemical Pharmacology, Vol. 29, pp. 3215-3218.

0006-2952/80/1201-3215$02.00/0

PRELIMINARY COMMUNICATIONS

NUTRIENT IMBALANCE CAUSES THE LOSS OF CYTOCHROME P-450 IN LIVER CELL CULTURE: FORMULATION OF CULTURE MEDIA WHICH MAINTAIN CYTOCHROME P-450 AT IN VIVO CONCENTRATIONS

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The primary culture of hepatic parenchymal cells represents an ideal system to study mechanisms of hepatotoxicity and chemical carcinogenesis under defined conditions i.e. uninfluenced by blood flow, nutritional and hormonal status. However the toxicity and carcinogenicity of many natural and synthetic chemicals are frequently determined by the activity of the hepatic microsomal cytochrome P-450 system which spontaneously falls to low levels during the first twenty four hours of hepatocyte culture (1,2). In the course of other studies, we found that the culture of hepatocytes in a simple balanced salt solution prevents the loss of cytochrome P-450. This finding suggests that a component(s) of the standard culture medium is responsible for the loss of the cytochrome. The present work shows that these components are cystine and cysteine. Accordingly hepatocytes cultured in a complete medium without cystine and cysteine contain high concentrations of cytochrome P-450. Supplementing these media with 5-amino laevulinic acid, a precursor of the haem prosthetic group of cytochrome P-450, results in a concentration of cytochrome P-450 in hepatocytes cultured for 24 h that is not significantly different from that found in intact liver.

MATERIALS AND METHODS

Isolation and culture of hepatocytes

Hepatocytes were isolated and 20 x 10^6 cells were cultured in 150mm diameter petri dishes in 20ml of the respective medium as previously described (3). L-15, Waymouth's MB752/1 and William's medium E were purchased from Flow Labs. Irvine, Scotland, U.K. RPMI 1640 medium was purchased from Gibco-Biocult Ltd., Paisley, Scotland. The special media with different amino acid compositions are based on Earle's balanced salt solution (Gibco Biocult Ltd) containing 1ml "BME vitamin solution x 100 strength" (Flow Labs) per 100ml medium. Amino acids were added to this medium as concentrates supplied in an "RPMI Selectamine Kit" (Gibco Biocult Ltd). Their final concentrations are given in the legends to Table 1 and 2. All media were serum free and contained 10^{-6}M insulin, 10^{-4}M hydrocortisone-21-sodium succinate (both from Sigma Chemical Co., Poole, Dorset, U.K.) and 5mg gentamicin (Flow Labs.)/100ml medium. 5-Amino laevulinic acid was purchased from The Sigma Chemical Co., Poole, Dorset, U.K.

Cytochrome P-450 and protein were determined as previously described (4).

RESULTS AND DISCUSSION

Table 1 shows that hepatocytes cultured for 24 h in any one of a number of commercially available culture media lose 60-70% of their cytochrome P-450 concentration. In contrast hepatocytes cultured for 24 h in a simple balanced salt solution (BSS) contain a concentration of cytochrome P-450 that is not significantly different ($p > 0.05$) from that found in freshly isolated cells. Since the serum free, bicarbonate buffered, balanced salt solution contains glucose, vitamins, phenol

Table 1 Effect of different culture media on the loss of cytochrome P-450 in hepatocytes cultured for 24 h.

Medium	Cytochrome P-450 (% initial)
L-15	32 ± 10
Waymouth's MB752/1	26 ± 11
William's medium E	39 ± 4
RPMI 1640	36 ± 5
BSS	84 ± 7
BSS+ all RPMI amino acids	42 ± 2*
BSS+ neutral amino acids	76 ± 11
BSS+ aromatic amino acids	77 ± 12
BSS+ dicarboxylic acids and their amides	80 ± 11
BSS+ imino amino acids	72 ± 9
BSS+ basic amino acids	71 ± 7
BSS+ sulphur amino acids	38 ± 4*
BSS+ 0.2mM cystine	41 ± 8*
BSS+ 0.1mM methionine	84 ± 13
BSS+ 1.0mM cysteine	42 ± 4*
BSS+ all RPMI amino acids except cystine	72 ± 4

* denotes significantly different ($p < 0.05$) by Student's t-test from BSS (Earle's Balanced Salt solution + "BME vitamins": see Materials and Methods section) and not significantly different ($p > 0.05$) from complete RPMI 1640 medium. The results are means ± S.D. for individual values found in preparations derived from three separate rat livers (i.e. n=3). The initial cytochrome P-450 concentration of hepatocytes was 180 ± 20 (n=3) pmoles/mg of protein. All media were used serum free and contained 10^{-6}M insulin, 10^{-4}M hydrocortisone-21-sodium succinate, and 5mg gentamicin/100ml. RPMI medium comprises the following amino acids (mM concentrations in parenthesis):-

Neutral = Gly (0.13) Ile (0.38) Leu (0.38) Ser (0.28) Thr (0.17) Val (0.17)
Aromatic = Phe (0.09) Trp (0.025) Tyr (0.11)
Dicarboxylic acids and their amides = Asp (0.15) Asn (0.33) Glu (0.14) Gln (2.0)
Imino = Pro (0.17) Hyp (0.15)
Basic = Arg (1.15) His (0.10) Lys (0.27)
Sulphur = Cys Cys (0.20) Met (0.10)

red, gentamicin, insulin and hydrocortisone these components are not responsible for the loss of cytochrome P-450 in hepatocyte culture. A major difference between the balanced salt solution and the complete media is the prescence of amino acids. Addition of the twenty amino acids present in "RPMI 1640 medium" to the balanced salt solution caused the loss of cytochrome P-450 (Table 1). When these amino acids, subdivided into groups based on their chemical structure, were added to the balanced salt solution only the addition of the sulphur containing amino acids, cystine and methionine, caused the loss of cytochrome P-450 (Table 1). Table 1 shows that this is due to cystine and not methionine. Thus the balanced salt solution containing

all the amino acids present in RPMI 1640 medium except cystine prevents the loss of cytochrome P-450. This suggests that cystine is responsible for the loss of cytochrome P-450 in cultured hepatocytes.

However L-15 medium does not contain cystine but contains 1mM cysteine. Hepatocytes cultured in L-15 medium lose their cytochrome P-450 suggesting that cysteine can also cause the loss of the cytochrome in cultured hepatocytes. Table 1 shows that the culture of hepatocytes in the balanced salt solution to which cysteine has been added also results in the loss of cytochrome P-450. We therefore conclude that the presence of cystine and/or cysteine in media results in the loss of cytochrome P-450 in hepatocytes cultured for 24 h.

The loss of cytochrome P-450 in hepatocyte culture is due to its impaired synthesis and enhanced degradation (5). We have found that the culture of hepatocytes in media which do not contain either cystine or cysteine prevents the loss of the cytochrome by inhibiting its enhanced degradation rather than by affecting its synthesis. Since the culture of hepatocytes for 24 h in a media containing all the RPMI amino acids except cystine results in the maintenance of only 70% of the initial concentration of cytochrome P-450 we attempted to increase its concentration to the same level as found in the initial cell preparation by stimulating its synthesis. Guzelian and Bissell (6) have shown that the incubation of cells with 5-amino laevulinic acid increases both the synthesis of haem and the concentration of cytochrome P-450. Accordingly we examined the effect of incorporating 5-amino laevulinic acid into media with and without cystine on the concentration of cytochrome P-450 in hepatocytes cultured for 24 h. Table 2 shows that cytochrome P-450 can be maintained in hepatocytes cultured for 24 h at a concentration that is not significantly different ($p > 0.05$) from the initial cell preparation if the cells are cultured in media comprising all the RPMI amino acids except cystine and which contains 100μM 5-amino laevulinic acid. Neither the absence of cystine or presence of 5-amino laevulinic acid alone maintains such high levels of cytochrome P-450 (Table 2).

In conclusion we have found that the loss of cytochrome P-450 observed in liver cell culture can be prevented by the culture of hepatocytes in either a simple balanced salt solution or a complete medium supplemented with 5-amino laevulinic acid but which does not contain cystine and/or cysteine. Hepatocytes cultured in these media should therefore be useful for the study of cytochrome P-450 mediated mechanisms of hepatotoxicity and hepatocarcinogenesis.

Table 2. Effect of 5-amino laevulinic acid on the concentration of cytochrome P-450 in hepatocytes cultured for 24 h in media ± cystine.

Medium supplemented with:		Cytochrome P-450 (% initial)
200μM Cystine	100μM aminolaevulinic acid	
+	-	48 ± 4
+	+	71 ± 13
-	-	72 ± 4
-	+	90 ± 6*

* denotes not significantly different ($p > 0.05$) by Student's t-test from initial cell preparation. The results are means ± S.D. for individual values found in preparations derived from three separate rat livers (i.e. n=3). The initial cytochrome P-450 concentration of hepatocytes was 155 ± 20 (n=3) pmoles/mg protein.

The media to which cystine and 5-aminolaevulinic acid were added comprised Earle's balanced salt solution containing 1ml "BME vitamins x 100 strength" (see Materials and Methods) per 100ml medium, 0.5 mg phenolred/100ml, 5 mg gentamicin/100ml, 10^{-6}M insulin, 10^{-4}M hydrocortisone-21-sodium succinate. The millimolar concentrations of amino acids in the media were: 1.15 Arg, 0.15 Asp, 0.33 Asn, 0.14 Glu, 2.0 Gln, 0.13 Gly, 0.1 His, 0.15 Hyp, 0.38 Ile, 0.38 Leu, 0.27 Lys, 0.1 Met, 0.09 Phe, 0.17 Pro, 0.28 Ser, 0.17 Thr, 0.025 Trp, 0.11 Tyr, 0.17 Val.

REFERENCES

1. Guzelian, P.S., Bissell, D.M. & Meyer, U.A. (1977) Gastroenterology 72, 1232-1239.
2. Paine, A.J. & Legg, R.F. (1978) Biochem.Biophys.Res.Commun. 81, 672-679.
3. Paine, A.J., Hockin, L.J. & Legg, R.F. (1979) Biochem.J. 184, 461-463.
4. Paine, A.J., Williams, L.J. & Legg, R.F. (1979) Life Sci.24, 2185-2192.
5. Paine, A.J. & Villa P. (1980) Biochem.J. submitted.
6. Guzelian, P.S. & Bissell, D.M. (1976) J.biol.Chem. 251, 4421-4427.