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THE RELATIONSHIP BETWEEN THE ABILITY OF PYRIDINE AND SUBSTITUTED PYRIDINES TO MAINTAIN CYTOCHROME P-450 AND INHIBIT PROTEIN SYNTHESIS IN RAT HEPATOCYTE CULTURES

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Abstract—In order to gain an insight into the mechanism of maintenance of cytochrome P-450 by nicotinamide (pyridine-3-carboxylic acid amide) in liver cell culture, the ability of pyridine and substituted pyridines to maintain cytochrome P-450 were compared. The structure–activity relationship suggests that the feature of nicotinamide important for the maintenance of cytochrome P-450 is a primary amide group attached to a pyridine ring. Pyridine itself, but not secondary or tertiary amides of pyridine, is also able to maintain cytochrome P-450. To examine the hypothesis that the ability of compounds to maintain cytochrome P-450 in liver cell culture may be related to their ability to inhibit protein synthesis, the effect of pyridine analogues and cycloheximide were compared. The results suggest that the inhibition of protein synthesis is not a mechanism underlying the maintenance of cytochrome P-450 in hepatocyte culture.

Rat liver parenchymal cells lose 70–80 per cent of their cytochrome P-450 concentration during the first 24 hr of culture [1, 2]. This spontaneous loss of cytochrome P-450 makes it difficult to use hepatocyte culture to understand precisely cytochrome P-450-mediated mechanisms of hepatotoxicity and hepatocarcinogenesis. However, it has been shown that the incorporation of high, unphysiological concentrations of nicotinamide (pyridine-3-carboxylic acid amide) in the cell isolation and culture medium maintains the cytochrome P-450 concentration of hepatocytes cultured for 24 hr at the same level as that found in intact liver [3].

An obvious consequence of nicotinamide administration to experimental animals is an increase in the hepatic concentration of NAD [4]. However, the ability of nicotinamide to increase the NAD content of cultured hepatocytes has been shown to be unrelated to the maintenance of cytochrome P-450 [5].

Nicotinamide and its analogues have many diverse pharmacological effects (e.g. refs. 4, 6–10), but the way in which these could be related to the maintenance of cytochrome P-450 is unclear. In order to gain an insight into the mechanism of maintenance of cytochrome P-450 by nicotinamide, the present study examines the ability of nicotinamide analogues to maintain cytochrome P-450 in cultured hepatocytes. Two main conclusions result from this work. The first, purely practical point is that isonicotinamide is the most efficient analogue of nicotinamide examined at maintaining cytochrome P-450 in cultured hepatocytes. Secondly, the proposal that the degradation of cytochrome P-450 in hepatocyte culture involves a process dependent on protein synthesis and hence can be prevented by inhibitors of protein synthesis [11] is shown not to be the mechanism regulating the level of cytochrome P-450 in cultured hepatocytes.

METHODS

Isolation of hepatocytes. Hepatocytes were isolated from adult (180–250 g) male rats of the Porton derived Wistar strain, fed *ad lib.* on MRC 41B diet, by perfusion of the liver with 0.05% (w/v) collagenase as previously described [12]. This process yielded 5×10^8 parenchymal cells per liver with a viability of 88 ± 6 per cent (mean ± S.D. of 18 perfusions) as assessed by trypan blue exclusion.

Culture of hepatocytes. Isolated hepatocytes were added to the required volume of Williams medium E containing the respective nicotinamide analogue and 5% (v/v) foetal calf serum plus 50 μg gentamicin/ml (all media from Flow Labs, Irvine, Scotland, U.K.). Hepatocytes were cultured at a density of 10×10^6 cells/10 ml medium in 100 mm diameter petri dishes (Lux Scientific, supplied by Gateway International, Cleveland, U.K.) for the determination of cytochrome P-450 or a density of 3×10^6 cells/3 ml medium in 60 mm diameter dishes (Lux Scientific) for the measurement of incorporation of ^{14}C-nicotinamide or ^{14}C-leucine.

Assays

Cytochrome P-450 and cell protein. These were measured as previously described [2].

Uptake of [carbonyl-^{14}C]-nicotinamide. This was obtained from the Radiochemical Centre, Amersham, Bucks, U.K., and was measured after incubating hepatocyte monolayers for the time stated in

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Williams medium E containing 25 mM nicotinamide at a specific activity of 54 μCi/mmole. The cells were harvested after aspirating the culture medium and washing the monolayer with 6 × 5 ml changes of unlabelled 25 mM nicotinamide in 0.15 M NaCl. The washes were discarded and the cells scraped into 1 ml 'emulgen buffer' [13] and homogenized. A 0.75 ml aliquot was then added to 10 ml Instagel (Packard Instrument Co., Downers Grove, Illinois, U.S.A.) and the radioactivity determined by liquid scintillation counting; the d.p.m. values were computed by using automatic external standardization.

L-[U-^{14}C]-*leucine incorporation into cellular protein.* This was determined by incubating cells in a 60 mm diameter dish in 3 ml Williams medium E containing 1 μCi L-[U-^{14}C]-leucine (Radiochemical Centre, Amersham, Bucks, U.K.) at a final specific activity of 0.6 μCi/μmole for 1 hr. The medium was then aspirated and the monolayer washed with 6 × 5 ml of 0.57 mM leucine in 0.15 M NaCl. The washes were discarded and the cells scraped into 0.6 ml 'emulgen buffer' [13] and homogenized. A 0.4 ml aliquot of the cell homogenate was added to 0.1 ml of a 'cold carrier' solution composed of 5 mg L-leucine/ml 0.15 M NaCl containing 1% bovine serum albumin. This was then diluted to 2 ml with 0.15 M NaCl and the protein precipitated with 2 ml 10% (w/v) trichloroacetic acid. One hour later the precipitate was collected by centrifugation, washed once with 4 ml 5% trichloroacetic acid, dissolved in 1 M NaOH, and the radioactivity determined by liquid scintillation counting in Instagel. The c.p.m. values were corrected for quenching by using automatic external standardization.

Conversion of pyridine-3-carboxaldehyde to nicotinic acid. This was determined by thin-layer chromatography of 100 μl samples of medium on aluminium oxide plates of 0.25 mm thickness (Merck F-254 Type E supplied by Anderman & Co., East Molesey, Surrey, U.K.) The plates were developed in 0.33 M ammonium acetate in 70% (v/v) aqueous ethanol. Pyridine-3-carboxaldehyde and nicotinic acid had R_f values of 0.83 and 0.43, respectively, when viewed under u.v. light. Nicotinic acid was not observed in medium containing pyridine-3-carboxaldehyde incubated without hepatocytes.

Chemicals. Nicotinamide, pyridine and leucine were purchased from British Drug House, Poole, Dorset, U.K. Isonicotinamide, nipecotamide, 3-acetylpyridine, 3-aminopyridine and pyridine-3-carboxaldehyde were purchased from the Aldrich Chemical Co., Gillingham, Dorset, U.K. Nicotinic acid, nicotinic acid hydrazide, isonicotinic acid hydrazide, *N,N*-diethylnicotinamide, thionicotinamide and cycloheximide were purchased from The Sigma Chemical Co., Poole, Dorset, U.K.

The pyridines were directly dissolved in the cell culture medium, neutralized with NaOH or HCl as required, and sterilized by passage through a 0.22 μm Millipore filter except for aminopyridine which was used without filter sterilization.

RESULTS

Structure–activity relationship. Table 1 shows the dose-dependent effect of nicotinamide added only to the culture medium on the concentration of cytochrome P-450 found in hepatocytes cultured for 24 hr. Nipecotamide (hexahydronicotinamide), a non-aromatic analogue of nicotinamide, is unable to maintain cytochrome P-450, suggesting that the ability of nicotinamide to maintain this cytochrome is dependent on its pyridine nucleus.

Indeed, pyridine itself is able to maintain cytochrome P-450 at high levels, as are substituted pyridines such as 3-acetyl- or 3-aminopyridine (Table 1). However, with reference to nicotinamide, the amide group also seems to be important, as thionicotinamide, which has a pyridine nucleus, but has the oxygen of the carbonyl group of nicotinamide

Table 1. Dose–response relationship of the effect of analogues of nicotinamide on the loss of cytochrome P-450 in rat hepatocytes cultured for 24 hr*

Analogue	Cytochrome P-450 (as % of freshly isolated cell concn) after 24 hr culture with varying analogue concn (mM)					
	0	1	5	10	15	25
Nicotinamide	26 ± 11	21 ± 8	32 ± 5	49 ± 14	60 ± 2	74 ± 3†
Nipecotamide	33 ± 4	29 ± 1	36 ± 4	Cytotoxic	—	—
Pyridine	31 ± 4	40 ± 10	59 ± 5	78 ± 18	78 ± 7	72 ± 7†
3-Aminopyridine	33 ± 5	55 ± 6	71 ± 15	70 ± 13†	Cytotoxic	—
3-Acetylpyridine	33 ± 5	68 ± 16	65 ± 1	86 ± 20†	Cytotoxic	—
Thionicotinamide	34 ± 4	40 ± 3	Cytotoxic	—	—	—
Nicotinic acid hydrazide	30 ± 3	34 ± 12	32 ± 10	36 ± 9	Cytotoxic	—
N,N-diethylnicotinamide	25 ± 8	17 ± 3	39 ± 8	Cytotoxic	—	—
Nicotinic acid	37 ± 5	21 ± 8	27 ± 12	29 ± 6	Cytotoxic	—
Pyridine-3-carboxyaldehyde	28 ± 1	23 ± 6	37 ± 1	45 ± 12	Cytotoxic	—
Isonicotinamide	32 ± 9	35 ± 8	59 ± 4	88 ± 9†	65 ± 2	Cytotoxic
Isonicotinic acid hydrazide	28 ± 1	43 ± 10	43 ± 10	Cytotoxic	—	—

* Rat liver parenchymal cells were isolated as described in Methods and cultured in Williams medium E containing the respective analogue at the concn shown above for 24 hr. Cells were then harvested for assay of cytochrome P-450 as described in Methods. The results above are the means ± S.D. of the per cent of cytochrome P-450 remaining in cells prepared from 3 different rat livers (viz. N = 3) after 24 hr culture under the respective condition.

† Indicates significantly different from untreated cells ($P < 0.05$). The initial cytochrome P-450 concn of hepatocytes used in these experiments was 188 ± 27 (N = 36) pmoles/mg protein.

Table 2. Effect of nicotinamide, isonicotinamide and cycloheximide on cytochrome P-450 concentration and protein synthesis in hepatocytes cultured for 24 hr*

Addition to culture medium	Cytochrome P-450 in hepatocytes cultured for 24 hr (as % freshly isolated cell concn)	Inhibition of protein synthesis (%)
None	37 ± 2 (8)	—
Nicotinamide (25 mM)	63 ± 4 (4)	48 ± 11 (4)
Isonicotinamide (10 mM)	81 ± 4 (8)	60 ± 10 (8)
Cycloheximide (0.1 μM)	44	0
Cycloheximide (0.25 μM)	46	38
Cycloheximide (0.5 μM)	48 ± 5 (5)	46
Cycloheximide (1.0 μM)	52 ± 5 (6)	76
Cycloheximide (2.5 μM)	47 ± 9 (6)	85
Cycloheximide (5.0 μM)	51 ± 6 (5)	93
Cycloheximide (10.0 μM)	51 ± 7 (5)	95

* Rat liver parenchymal cells were isolated and cultured for 23 hr as described in Methods in Williams medium E + the additions referred to above. After 23 hr of culture the respective medium was changed to this medium containing 0.6 μCi/μmole ^{14}C-leucine and the hepatocytes allowed to incorporate ^{14}C-leucine over the next hour before harvesting the cells and assay of the radioactivity incorporated into trichloroacetic acid insoluble material and determination of cytochrome P-450 as described in Methods. The results are expressed as the average percentage value of cytochrome P-450 and the inhibition of protein synthesis compared to untreated cells produced by the treatment found in cultures prepared from two different rat livers except when given as the mean ± S.D. The number in parentheses indicates the number of different cell preparations. The P-450 concentrations of freshly isolated hepatocytes used for these experiments was 180 ± 37 pmoles/mg protein (N = 8) and the incorporation of ^{14}C-leucine into untreated cells prepared from different rat livers was 7000 ± 1500 d.p.m/hr/mg protein (N = 8).

replaced by sulphur, is unable to maintain cytochrome P-450 when incorporated into the medium at either millimolar (Table 1) or micromolar concentrations (data not shown). Substitution of the amide group of nicotinamide, as in the secondary amide (nicotinic acid hydrazide) or the tertiary amide (*N,N*-diethylnicotinamide), leads to an inability to maintain cytochrome P-450 in cultured hepatocytes. Nicotinic acid is also ineffective and the failure of pyridine-3-carboxaldehyde to maintain cytochrome P-450 is apparently due to its metabolism, by hepatocytes, to nicotinic acid. Thus, after 4 hr of culture in medium initially containing 10 mM pyridine-3-carboxaldehyde, the hepatocytes were found to convert all the pyridine-3-carboxaldehyde to nicotinic acid (shown by thin layer chromatography: see Methods.

In summary, the results presented above suggest that a primary amide group attached to a pyridine ring is the feature of nicotinamide which is important for the maintenance of cytochrome P-450 in hepatocyte culture. Whether the amide group is attached to position 3 of the pyridine ring, as in nicotinamide, or to position 4, as in isonicotinamide, seems unimportant as isonicotinamide will also maintain cytochrome P-450 in cultured hepatocytes (Table 1). As in the example of nicotinamide, a secondary amide analogue of isonicotinamide (such as isonicotinic acid hydrazide) is also unable to maintain cytochrome P-450 (cf. effects of nicotinic acid hydrazide with isonicotinic acid hydrazide in Table 1).

Effect on protein synthesis. Guzelian and Barwick [11] have proposed that the conditions of hepatocyte culture stimulate the degradation of cytochrome P-450 by a process that is inhibited by cycloheximide and hence may require protein synthesis. A possible common denominator between the ability of pyridine and substituted pyridines to maintain cytochrome P-450 in hepatocyte culture could therefore be related to their ability to inhibit protein synthesis. Accordingly, the effects of nicotinamide analogues, used in the present study, and cycloheximide on cytochrome P-450 and protein synthesis were compared.

Table 2 shows that the concentrations of nicotinamide (25 mM) or isonicotinamide (10 mM) that are effective in maintaining cytochrome P-450 in cultured hepatocytes do indeed produce a marked, 48 ± 11 per cent and 60 ± 10 per cent, inhibition of protein synthesis, respectively.

The inhibition of protein synthesis found 24 hr after culture in media containing either 25 mM nicotinamide or 10 mM isonicontinamide is similar to the inhibition produced by culture in medium containing between 0.5 and 1 μM cycloheximide (Table 2). Although the culture of hepatocytes in media containing different concentrations of cycloheximide produced a dose-dependent inhibition of protein synthesis, measured after 23 hr of culture, which ranged between 38 and 95 per cent; cycloheximide was considerably less effective than isonicotinamide at maintaining cytochrome P-450 (Table 2). However, since cycloheximide is generally considered to inhibit protein synthesis very rapidly after its addition to the culture medium, the question was posed as to whether the more efficient maintenance of cytochrome P-450 after 24 hr of culture by nicotinamide was due to a different time course of inhibition of protein synthesis.

Table 3 shows the time course of the accumulation, in cultured hepatocytes, of the radiolabel derived from [carbonyl-^{14}C]-nicotinamide and the subsequent inhibition of protein synthesis. The data in Table 3 shows that protein synthesis is maximally inhibited 6 hr after the addition of nicotinamide and that this inhibition coincides with the time course of

Table 3. Time course of ^{14}C-nicotinamide uptake and the inhibition of protein synthesis in hepatocytes cultured with 25 mM nicotinamide*

Time of culture (hr)	Incorporation of ^{14}C-nicotinamide d.p.m/mg protein	Inhibition of protein synthesis of untreated cells (%)
2	1920	19
4	3960	28
6	4440	53
18	2760	59
24	2280	53

* Rat liver parenchymal cells were isolated as described in Methods and cultured in Williams medium E containing 25 mM nicotinamide labelled with ^{14}C-nicotinamide (specific activity 54 μCi/mmole) for the time shown. Replicate culture dishes containing unlabelled 25 mM nicotinamide were used for the determination of the rate of protein synthesis. One hour prior to the time shown, each 60 mm diameter dish had 1 μCi ^{14}C-leucine added to give a specific activity of 0.6 μCi ^{14}C-leucine/μmole and the cells allowed to incorporate ^{14}C-leucine for 1 hr. Cells were harvested for assay of radioactivity associated with ^{14}C-nicotinamide or ^{14}C-leucine as described in Methods. The results above are the average of values obtained from duplicate culture dishes prepared from the same rat liver. Variation between replicate dishes did not exceed 10 per cent.

accumulation of ^{14}C derived from nicotinamide. The time course of the inhibition of protein synthesis produced by culturing cells in medium containing 10 mM isonicotinamide is similar to that shown in Table 3 for nicotinamide. After 2, 4, 6, 18 and 24 hr of incubation, medium containing 10 mM isonicotinamide produced 19, 28, 53, 59 and 60 per cent inhibition of protein synthesis, respectively. Since nicotinamide and isonicotinamide efficiently maintain the concentration of cytochrome P-450 and produce maximal inhibition of protein synthesis after 6 hr incubation, the possibility that cycloheximide could produce levels of cytochrome P-450 higher than those shown in Table 2, if it was added after 2, 4 and 6 hr of hepatocyte culture, was investigated. However, the addition of 0.25, 0.5, 1, 2.5 or 5 μM cycloheximide after 2, 4 or 6 hr of culture did not increase the concentration of cytochrome P-450 found at 24 hr above the level found in untreated cells. Although these results suggest that an inhibition of protein synthesis is not a mechanism underlying the maintenance of P-450 in hepatocyte culture, Table 4 shows that there is an apparent correlation between the ability of substituted pyridines to maintain cytochrome P-450 after 24 hr of culture and their effect on protein synthesis. Thus, nipecotamide, nicotinic acid hydrazide, isonicotinic acid hydrazide and nicotinic acid, which do not maintain cytochrome P-450 in hepatocyte culture, do not inhibit protein synthesis to the same extent as those compounds which maintain cytochrome P-450, e.g. nicotinamide, isonicotinamide, 3-acetylpyridine and 3-aminopyridine. Pyridine, however, maintains cytochrome P-450 at high levels, yet does not significantly inhibit protein synthesis (Table 4).

DISCUSSION

The present work shows that although cycloheximide produces a more efficient inhibition of protein synthesis than nicotinamide or isonicotinamide, it does not result in the maintenance of cytochrome P-450 in hepatocyte culture. This conclusion is in contrast to that of Guzelian and Barwick [11]. When our results of cycloheximide treatment are expressed in the same manner as Guzelian and Barwick [11],

Table 4. Effect of nicotinamide analogues on protein synthesis and cytochrome P-450*

Analogue	Inhibition of protein synthesis due to analogue (%)	Cytochrome P-450 (as per cent freshly isolated cell concn) after 24 hr culture with analogue
None	—	26 ± 11
Nipecotamide (5 mM)	18 ± 7	36 ± 4
Nicotinic acid hydrazide (10 mM)	21 ± 4	36 ± 9
Isonicotinic acid hydrazide (5 mM)	0	43 ± 10
Nicotinic acid (10 mM)	24 ± 1	29 ± 6
Nicotinamide (25 mM)	48 ± 11	63 ± 4†
Isonicotinamide (10 mM)	60 ± 10	88 ± 9†
3-Acetylpyridine (10 mM)	48 ± 4	86 ± 20†
3-Aminopyridine (10 mM)	41 ± 6	70 ± 13†
Pyridine (25 mM)	12 ± 6	72 ± 7†

* Rat liver parenchymal cells were cultured in medium containing the respective analogue for 23 hr when protein synthesis and cytochrome P-450 were measured as described in Table 2. The results are given as the means ± S.D. of the per cent inhibition of protein synthesis and cytochrome P-450 concn found in cells prepared from three different rat livers.

† Indicates significantly different from untreated cells ($P < 0.05$).

that is as a percentage of the low P-450 value of untreated hepatocytes cultured for 24 hr, they fall in the range reported by Guzelian and Barwick [11] i.e. 136–175 per cent. However, the specific activity of cytochrome P-450 in cycloheximide-treated hepatocytes is only 50 per cent of that found in intact liver or freshly isolated cells and accordingly this does not represent a maintenance of cytochrome P-450 in our system. Furthermore, pyridine efficiently maintains cytochrome P-450 at high levels but does not significantly inhibit protein synthesis. These findings that pyridine can maintain cytochrome P-450 without producing a significant inhibition of protein synthesis, while cycloheximide inhibits protein synthesis without maintaining cytochrome P-450, clearly demonstrate that the inhibition of protein synthesis is not a mechanism underlying the maintenance of cytochrome P-450 in hepatocyte culture.

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