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7-ETHOXYCOUMARIN *O*-DEETHYLASE INDUCTION BY PHENOBARBITONE AND 1,2-BENZANTHRACENE IN PRIMARY MAINTENANCE CULTURES OF ADULT RAT HEPATOCYTES

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Abstract—The microsomal monooxygenase system of adult rat hepatocytes in short-term non-proliferating culture could be induced by phenobarbitone and benzanthracene. Differences in the kinetics of induction and the additive nature of the inductions indicated that induction by the two agents occurred by different mechanisms and the use of haemoprotein-selective inhibitors demonstrated the induction of different haemoproteins by the two agents. The type of haemoprotein present in the cells altered during a four day culture period.

The microsomal monooxygenase (MMO)§ system of mammalian liver, centred around the haemoprotein cytochrome P_{450}, has a major responsibility for the metabolism of a wide range of endogenous and foreign chemicals [1–3]. An important property of this MMO system is its ready inducibility by a large number of foreign chemicals, including drugs, pesticides and environmental contaminants [1, 2]. These chemical inducing agents can broadly be categorized into two main classes, one class being exemplified by the barbiturates [e.g. phenobarbitone (PB)] and the other by the polycyclic aromatic hydrocarbons [e.g. 1,2-benzanthracene (BA)] [1, 2, 4]. The classes of inducer may be distinguished by differences in the types of reaction induced and their sensitivity to inhibitors, and by spectrophotometrically- or electrophoretically-determined differences in the haemoprotein induced [1, 2, 4–7]. Phenobarbitone-type induction commonly occurs with drugs and is by far the more important form of induction in the clinical situation [8], and therefore knowledge of the mechanism(s) of induction by PB-like compounds is of both fundamental and clinical significance.

The induction of one MMO enzyme, aryl hydrocarbon hydroxylase (AHH), has been extensively studied in a variety of mammalian cell lines [9–15], including some liver-derived ones [9, 16–18], and in primary foetal rat hepatocyte cultures [19–21], and such studies have proved valuable in elucidating the mechanisms by which the polycyclic aromatic hydrocarbons exert their inducing effect [22, 23]. However, with the exception of the foetal hepatocyte cultures and a few liver-derived cell lines, PB is generally ineffective as an inducer of AHH activity in these various cell systems. Even in those cultures in which PB has been shown to be an effective inducer, there is strong evidence that the haemoprotein induced is not the normal cytochrome P_{450} induced *in vivo* but behaves much more like the BA-inducible cytochrome $P_{1_{450}}$ variant. Cell division actively occurs in both the foetal hepatocyte cultures and the PB-responsive liver-derived cell-lines and this is unlike the normal *in vivo* situation in adult liver in which cell division occurs only at a very low level. There is now good evidence that the MMO system in foetal liver is also qualitatively different from that of adult liver [25–27]. Finally, there is evidence that AHH is not a typical example of an enzyme of the MMO system [23]. These points taken together strongly indicate that the cell systems already mentioned are not appropriate for the study of the mechanism of PB-mediated induction of the MMO system.

It is suggested from these previous studies that the best prospect of producing an appropriate model to study PB-mediated induction of the MMO system is likely to be primary maintenance cultures of functional adult rat hepatocytes. The techniques required to produce and maintain such cells in primary culture have recently been established (for review see [28]). It has been reported that PB does not induce MMO activity in such a culture system [29] although MMO activity can be induced by polycyclic aromatic hydrocarbons [29]. Others workers, using a somewhat modified culture system, have, however, reported PB-mediated induction of the haemoprotein [30].

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§ Abbreviations used: AHH, aryl hydrocarbon hydroxylase; ANF, α-naphthoflavone; BA, benzanthracene; 7-ECOD, 7-ethoxycoumarin *O*-deethylase; 7-HC, 7-hydroxycoumarin; MMO, microsomal monooxygenase; PB, phenobarbitone; PPO, diphenyloxazole; P_{450}, the microsomal haemoprotein induced by PB; $P_{1_{450}}$ the microsomal haemoprotein induced by BA.

We have studied the induction of MMO activity by PB and BA in primary maintenance cultures of adult rat hepatocytes using 7-ethoxycoumarin *O*-deethylation as a more typical index of the function of the MMO system than AHH.

MATERIALS AND METHODS

Animals. Male Wistar/albino rats (60–80 g) were used in this study. The animals were bred in the University of Surrey Animal Unit and, once delivered to the laboratory, were maintained on synthetic 'Sterolit' bedding (Engelhard, NJ) for a minimum of three days prior to use in order to minimize any induction caused by the wood shavings on which the animals were reared.

Materials. The suppliers of the majority of chemicals and media used in this study have been listed in a previous paper. Additional chemicals were obtained from the following sources: cycloheximide (Sigma Chemical Co., Poole, Dorset, U.K.), phenobarbitone sodium and α-naphthoflavone (ANF) (BDH, Poole, Dorset, U.K.), and 2,5-diphenyloxazole (PPO) (Packard Instrument Co.). SKF 525-A was a gift of Smith, Kline and French (Welwyn Garden City, Herts, U.K.), and metyrapone was a gift of Ciba Geigy (Horsham, Sussex, U.K.).

Preparation of inducer- and inhibitor-containing media. Phenobarbitone sodium and cycloheximide were made up as ×100 stock solutions in isotonic saline solution, filter sterilized and diluted in culture medium prior to use. BA, SKF 525-A, metyrapone, ANF and PPO were made up as ×2000 stock solutions in dimethylformamide and diluted in culture medium prior to use.

Cell isolation and culture details together with details of the measurement of 7-ethoxycoumarin *O*-deethylase (7-ECOD) activity and nuclei counts have been described in detail previously [31, 32].

RESULTS

The morphological characteristics of the cultures used in this study have been described in detail previously [31–33]. Briefly, hepatocytes are the predominant cell type between the first and fourth days in culture whereas fibroblasts predominate beyond the fourth day in culture, and there is extensive loss of loosely attached, "rounded-up" early necrotic cells between the first and second days in culture.

The level of 7-ECOD activity in control uninduced cells doubled between the first and second days in culture (due to loss of non-viable cells as described previously [31, 32]), it remained static for two days and then fell again between the fourth and seventh days in culture due to the overgrowing fibroblasts effectively "diluting out" the enzyme activity in the hepatocytes (Fig. 1). The level of 7-ECOD activity between the first and fourth day in culture was approx. 25–40 per cent of that found in freshly isolated cells. The overgrowing fibroblasts appeared to possess a low level of 7-ECOD activity.

Exposure of the cultured liver cells to 2 mM PB, commencing 24 hr after initiating the culture led to a significant increase in 7-ECOD activity (Fig. 1). Furthermore, this enhancement in enzyme activity

Fig. 1. Induction of 7-ECOD activity in adult rat liver cells in culture. Adult rat liver cells were isolated and cultured as described in the text and 24 hr after initiating the culture, the cell monolayers received fresh medium containing either no added inducer (●), 13 μM BA (■), 2 mM PB (○), or 13 μM BA + 2 mM PB (□). Flasks were assayed at various time points for 7-ECOD activity and nuclei counts. Appropriate medium (with or without inducer) was changed on the fourth day in culture. Each point is the mean of 2 experiments (three flasks at each treatment group point).

reached a plateau value 3 days after commencing PB exposure (i.e. day 4 in culture), thus demonstrating that the increase is mediated through the hepatocytes and not through the fibroblasts. This has been confirmed by cytochemical studies into the induction of NADPH oxidase by PB and polycyclic hydrocarbons which demonstrated that this enzyme, believed to be closely involved in the MMO system, is localized in the hepatocytes in both the control and induced situations [34–36]. Although the data presented in Fig. 1 regarding induction by PB refer to only 2 experiments (for reasons of comparison between the two inducers), it is important to note that 4 other separate experiments confirmed the inductive effect of PB and proved that the level of 7-ECOD activity after 3 days exposure to PB was statistically ($P < 0.01$) greater than that in the uninduced cells.

Incubation of cultured rat hepatocytes with PB and cycloheximide, at a concentration of cycloheximide which inhibited general protein synthesis by 90–95 per cent, greatly diminished the response to PB (Fig. 2b), indicating that the PB-mediated increase in enzyme activity is a true induction, i.e. requires *de novo* protein synthesis. This must be regarded with some caution, however, as the level of cycloheximide used was cytotoxic (Fig. 2a) and also decreased 7-ECOD activity in cells not exposed to PB (Fig. 2b).

Benzanthracene, an example of an inducer of the polycyclic aromatic hydrocarbon type, also induced 7-ECOD activity (Fig. 1), the maximum induction occurring in the hepatocytes after 24 hr exposure.

A mixture of PB and BA as inducers produced a pattern of induction indicative of additive induction (Fig. 1). There was an initial early increase in enzyme activity, presumably due to the BA component, and this was followed by a further rise which tended to plateau between days 3 and 4; this latter phase was probably due to the PB component.

The differences between PB and BA in the kinetics

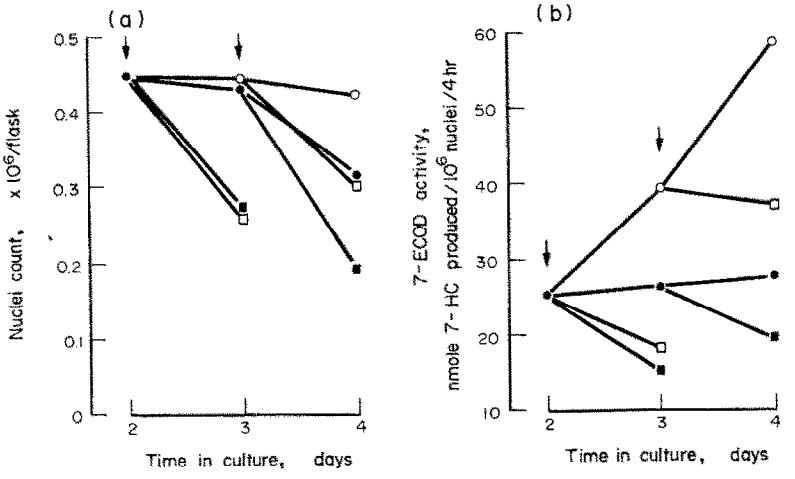

Fig. 2. Effect of cycloheximide on (a) nuclei count and (b) 7-ECOD activity in control and PB-treated rat hepatocytes in primary culture. Adult rat hepatocytes were isolated and cultured as described in the text and 48 hr after initiating the culture the cells received fresh medium containing either no added inducer (closed symbols) or 2 mM PB (open symbols). Cycloheximide (final conc. 1 μg/ml) was added to some of the flasks for 24 hr prior to assay of nuclei counts and 7-ECOD activity at the times indicated by the arrows. One typical experiment of three. Round symbols represent cultures without cycloheximide and the square symbols represent cultures incubated with cycloheximide.

of induction and the additive nature of these inductive processes (Fig. 1) strongly suggested that different forms of haemoprotein were being induced, as occurs *in vivo*. This possibility was investigated by using inhibitors selective for either cytochrome P_{450} (i.e. SKF 525-A and metyrapone) or cytochrome P_{1450} (i.e. ANF and PPO). The results of this study are presented in Figs. 3 and 4.

Freshly-isolated hepatocytes appeared to contain both P_{450} and P_{1450} (Fig. 3). However, when the cells were cultured for up to 4 days in the absence of any known exogenous inducer (although some may be present in the foetal calf serum), the P_{450} component decreased, whereas the contribution by the P_{1450} component increased, so that by day 4 almost all the haemoprotein was present as P_{1450} (Fig. 3). As expected, the haemoprotein induced by BA had the characteristics of P_{1450} and the inhibitor profile was similar to that in the control medium (Fig. 4). On the other hand, PB induced the normal P_{450} (i.e. that inhibited by SKF 525-A and metyrapone) even against a high background of P_{1450} (Fig. 4).

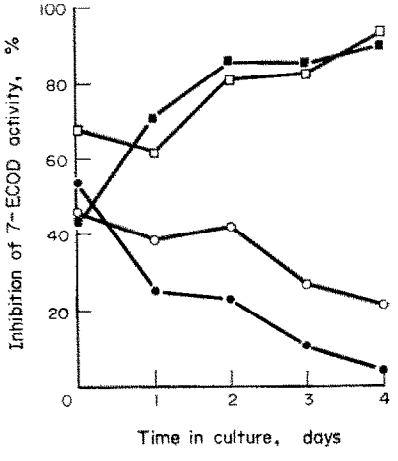

Fig. 3. Effect of inhibitors on 7-ECOD activity in control rat hepatocytes in primary culture. Adult rat hepatocytes were isolated and cultured as outlined in the text. At various times the cells were assayed for 7-ECOD activity in the absence or presence of the various inhibitors (final conc. of inhibitor, 100 μM). The cells were preincubated for 30 min with the inhibitor prior to the addition of 7-EC. Each point is the mean of 2–3 different experiments, there being approximately 10 per cent variation between each experiment. Inhibitors used: ●, SKF 525-A; O, metyrapone; ■, ANF; □, PPO.

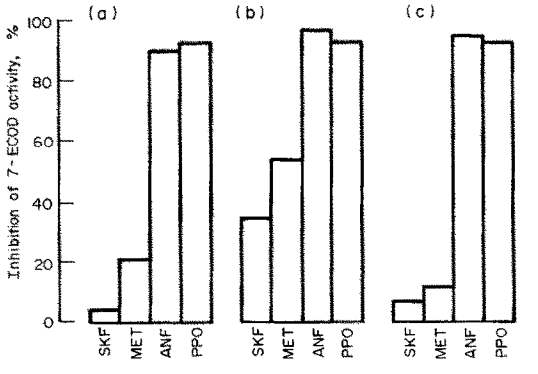

Fig. 4. Effect of various inhibitors on 7-ECOD activity in control and induced cultured rat hepatocytes. Adult rat hepatocytes were isolated and cultured as described previously. Twenty-four hours after commencing cultures, the cells were refed with either control medium (a), medium containing 2 mM PB (b) or medium containing 13 μM BA (c) and cultured for a further 3 days. After this time the effect of various inhibitors on 7-ECOD activity was assessed as outlined in the legend to Fig. 3. Each value is the mean of 2 different experiments (4 different flasks each point per experiment) with means differing by less than 10 per cent.

DISCUSSION

The results presented in this paper demonstrate that activity of the MMO system of adult rat hepatocytes in primary maintenance culture can be induced by both PB and BA. Furthermore, a number of differences can be discerned between the forms of induction produced by these two agents, e.g. differences in the kinetics of induction, additive induction and the differential effects of selective inhibitors, and this suggests that different mechanisms of induction must be involved. These findings are in accord with previous *in vivo* studies (see Introduction) and they confirm that this primary cell culture system can be of value in the study of liver-specific functions, particularly that of induction of the MMO system. The results confirm the findings of Michalopoulos *et al.* [30] regarding induction of haemoprotein by PB and BA, but they are in apparent disagreement with the findings of Guzelian *et al.* [29] who were unable to detect PB-mediated induction of the MMO system. This discrepancy may be readily explained by the short (24 hr) time allowed for exposure of the cells to PB in the studies of Guzelian *et al.* [29], for we have found that cultured rat hepatocytes are largely refractory to the inductive effects of PB in the first 24 hr of exposure.

It has previously been shown that although induction of AHH activity in cultured foetal rat hepatocytes and some liver-derived cell lines by PB and BA is additive, for both inducers the induced haemoprotein possesses predominantly P_{1450} character [22, 24]. This is not the case in the present study, for inhibitor studies clearly indicate that different haemoproteins are induced by the two inducers (Fig. 4) and that these haemoproteins are similar to those induced by the two inducer types *in vivo* [4, 24]. It is important to note that induction of the normal P_{450} by PB occurred against a very high background of P_{1450} and this is reminiscent of similar *in vivo* work carried out by Tsyrlov *et al.* [37] who studied PB induction in animals preinduced with polycyclic aromatic hydrocarbons. These workers showed that PB induced further increases in the haemoprotein subsequent to hydrocarbon induction, but that the PB-induced haemoprotein had the spectral characteristics of P_{1450} even though the reactions induced by PB were typical for that inducer. From these findings, these authors suggested that caution must be exercised in interpreting changes in carbon monoxide-binding spectra of cytochrome P_{450}; such caution may also have to be exercised when using spectral techniques to assess the "normality" of P_{450} in cultured hepatocytes.

Previous studies from this and other laboratories have indicated that adult rat hepatocytes isolated by a collagenase dissociation technique and maintained in short term non-proliferating culture retain a number of structural and functional characteristics specific or selective to such cells *in vivo* [28]. These characteristics that are retained included: typical ultrastructural morphology [38, 39], the sex differences in steroid metabolism [40], plasma protein synthesis and secretion [41–43], arginine synthesis [43], bromosulphophthalein uptake [J. Fry, unpublished], the pattern of glucose metabolism [44] and insulin stimulation of glycogen synthesis [45–47]. However, one variable that is drastically and seemingly selectively altered very early on in culture of rat hepatocytes is the MMO system. A number of studies have now shown that within the first 24 hr in culture, the level of cytochrome P_{450} and related enzyme activity in the cells falls to about 20–40 per cent of the freshly-isolated cells [29, 30, 42, 48–50]. There is no loss of cytochrome P_{450} during the isolation of liver cells [23]. This pattern of rapid loss in activity of the MMO system on culturing rat hepatocytes has been confirmed in the present study and it has been established that the level of MMO activity is stable during the 1–4 day culture period subsequent to the initial 60–75 per cent loss of activity within the first 24 hr in culture. However, more detailed analysis involving the use of P_{450}- or P_{1450}-selective inhibitors revealed that throughout the first 4 days in culture, there is a gradual shift in the types of haemoprotein present. In freshly-isolated hepatocytes, P_{450} and P_{1450} are present in approximately equal catalytic amounts but during the culture period there is a decrease in the P_{450} form with a concomitant increase in the P_{1450} form. This change in the haemoprotein composition does not appear to be due to conversion of P_{450} to P_{1450}, but rather it involves *de novo* protein synthesis of the P_{1450} as judged by the inhibitory effects of cycloheximide on control 7-ECOD activity (Fig. 2b). The reasons for the loss of phenobarbitone type haemoprotein composition are unknown but it is possible that deficiencies in the culture medium may lead to increased destruction of the endoplasmic reticulum membrane, or that there is a need for the continual presence of MMO substrates (endogenous or exogenous) to keep the normal P_{450} cycling and hence minimize its destruction. Conversely, the induction of P_{1450} may be ascribed to the presence of inducing agents (e.g. hydrocortisone) in foetal calf serum.

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