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INDUCTION OF CYTOCHROME P-450 ASSOCIATED MONOOXYGENASE ACTIVITIES BY PHENOBARBITAL AND 3-METHYLCHOLANTHRENE IN PRIMARY CULTURES OF ADULT RAT HEPATOCYTES

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ABSTRACT: *In vitro induction of cytochrome P-450 associated monooxygenase activities by phenobarbital (PB) and 3-methylcholanthrene (MC) was investigated in primary cultures of adult rat hepatocytes. PB and MC were added to the culture 24 hr after the initial plating of hepatocytes. A signiftcant increase of the activities of 7-ethoxycoumarin 0-deethylase and aryl hydrocarbon hydroxylase were observed in MC and PB treated culture. MC caused about 500% induction of the initial oxidation rates of both enzymes in 48 hr. However the PB maintained both enzyme activities close to the level of freshly isolated hepatocytes. Biphenyl 4-hydroxylase and aminopyrine N-demethylase activities were also induced by MC and PB. But the level of induction was less than that occuring with 7-ethoxycoumarin 0-deethylase and aryl hydrocarbon hydroxylase. When aflatoxin B_1 was added to the hepatocyte cultures which have been treated with MC or PB, it caused a significant increase of the unscheduled DNA synthesis at higher dose of aflatoxin B_1 as compared to those of untreated control hepatocyte cultures. The results suggest that microsomal enzyme activities can be selectively controlled preferably in hepatocyte cultures by the in vitro induction method. This principle may be useful for studying the metabolism and other toxicological studies.*

Keywords: *Microsomal monooxygenase activities, in vitro induction, hepatocyte culture, unscheduled DNA synthesis, Aflatoxin B_1.*

INTRODUCTION

For the past several years, we have used primary cultures of nonproliferating hepatocytes prepared from adult rats as an alternative metabolic system to the microsomal fractions in the *in vitro* toxicity studies (Kim *et al.*, 1986.; Yang *et al.*, 1986; Kim *et al.*, 1987). However, due to the rapid loss of cytochrome P-450, the terminal oxidase responsible for metabolism of many drugs, carcinogens and endogenous substrates, there still exist serious limitations for the use of hepatocyte culture system in those studied. To overcome this particular limitation, we have shown that cytochrome P-450 can be maintained in the primary rat hepatocyte culture by adding hexobarbital to the culture medium (Kim *et al.*, 1987). In present study, we tried an *in*

vitro induction method to maintain the level of cytochrome P-450 associated monooxygenase activities close to the *in vivo* level. Phenobarbital (PB) and 3-methylcholanthrene (MC), traditional representatives of two classes of agents that induce seperate, distinct forms of cytochrome P-450 *in vivo* were used (Orrenius *et al.*, 1965; Mannering, 1971). We also studied unscheduled DNA synthesis caused by aflatoxin B_1 (AFB_1) in the PB and MC induced primary hepatocyte cultures.

MATERIALS AND METHODS

Primary Rat Hepatocyte Culture and Treatment

Rat hepatocytes were isolated by a collagenase perfusion technique as described previously (Yang *et al.*, 1983) and suspended in the modified Waymouth's medium supplemented with testosterone, thyroxine, hydrocortisone, estradiol, glucagon, insulin, linoleic acid, and oleic acid as described by Decad *et al.* (1977). The hepatocyte suspension was diluted to 1×10^6 cells/ml of the medium and 9 ml of the suspension was transfered into 100 x 20 mm plastic petri dishes (Falcon) which had been precoated with Vitrogen 100 (Collagen Corp.). The cultures were incubated at 37°C in a humidified 5% CO_2/95% air incubator. To remove the unattached cells and cellular debris, the medium was changed 4 hr after the initial plating and at every 24 hr thereafter. Phenobarbital (2 mM), dissolved in saline and 3-methylcholanthrene (2 μM), dissolved in dimethylsulfoxide (DMSO) were added to the culture 24 hr after the initial plating of the hepatocytes. The final concentration of DMSO in culture was 0.5% (v/v).

Preparation of Whole Cell Homogenates

At 24, 48, 72 and 96 hr after the treatment with PB or MC, the medium was removed by aspiration and 6 ml of a 66 mM Tris-HCl buffer (pH 7.4) was added to each culture dish. The attached hepatocytes were scraped off with a rubber policeman and transfered to a centrifuge tube and were pelleted by centrifugation at 1,000 g for 10 min. The pelleted cells were resuspended in 6 ml of fresh Tris-HCl buffer and pelleted a second time by centrifugation. After removing the buffer, the pellet was resuspended in 1.5 ml of 0.25 M sucrose and homogenized using a Potter-Elvehjem homogenizer. Homogenates of freshly isolated hepatocytes, prior to their being inoculated into culture dishes, were prepared similarly and used as the initial (0 hr) sample. These homogenates were stored at −70°C until use. Assays for the various mixed function oxidase activities were carried out within 24 hr after the preparation of whole cell homogenates.

Enzyme Activity Assays

Whole cell homogenates were used for enzyme assays. Activities of 7-ethoxycoumarin 0-deethylase (Greenlee and Poland, 1978), aryl hydrocarbon hydroxylase (Nebert and Gelboin, 1968), biphenyl 4-hydroxylase (Haugen, 1981), and aminopyrine N-demethylase (Holder *et al.*, 1976) were determined by published methods. Protein was determined by the method of Lowry *et al.* (1951).

Unscheduled DNA Synthesis Assay

Unscheduled DNA synthesis (UDS) caused by aflatoxin B_1 (AFB_1) was determined in control, MC-, and PB-induced hepatocyte cultures. MC and PB were added to the culture 24 hr after the initial plating and incubated for 48 hr. After the induction, the fresh medium containing hydroxyurea (10 mM), ^{3}H-thymidine (1 μCi/ml), and AFB_1 were added. After incubation for 4 hr, the medium was aspirated off and the plate was washed twice with phosphate-

buffered saline (PBS) containing thymidine (0.5 mg/ml) and the hepatocytes were harvested.

The filter elution method used for the detection of DNA single-strand breaks was used to measure the unscheduled DNA synthesis according to the modified method of Hsia *et al.* (1983). The plate which was rinsed with PBS containing 0.5 mg/ml cold thymidine was treated with EDTA solution for 10 min at 37°C and the cells were detached by gentle scrapping with a rubber policeman and dispersed in cold PBS solution. About 6×10^5 cells were loaded onto a 2.0 μm pore size polycarbonate filter in a 25 mm diameter filter holder. The cells were lysed in the dark with 10 ml of lysis solution (2% sodium dodecylsulfate, 0.025 M EDTA, 0.1 M glycine, pH 10.0) supplemented with thymidine (0.5 mg/ml) and proteinase K (0.2 mg/ml). The lysis solution allowed to flow by gravity. When 1 ml of the lysis solution remained on the filter, the outflow tube from the filter holder was clamped off and incubation was continued for 30 min at room temperature. After incubation, the samples were allowed to drip to dryness. The lysed samples were washed with 5 ml of 0.02 M EDTA solution (pH 10.0). After the final washing, each filter was carefully removed and placed in a scintillation vial to which 0.4 ml of HCl was added. The vial was heated for an hour at 70°C. After cooling, 0.5 ml of 2N NaOH was added. The vials were placed in a shaker bath at 25°C and were shaken vigorously for 1 hr. Finally, 0.4 ml of aliquots were taken and 7 ml of Aquasol containing 0.7% acetic acid were then added to each vial for radioactivity counting.

Statistical Analysis

Mean values obtained for control and MC or PB treated culture were compared by the Student's t-test. Significance was set at $p < 0.05$.

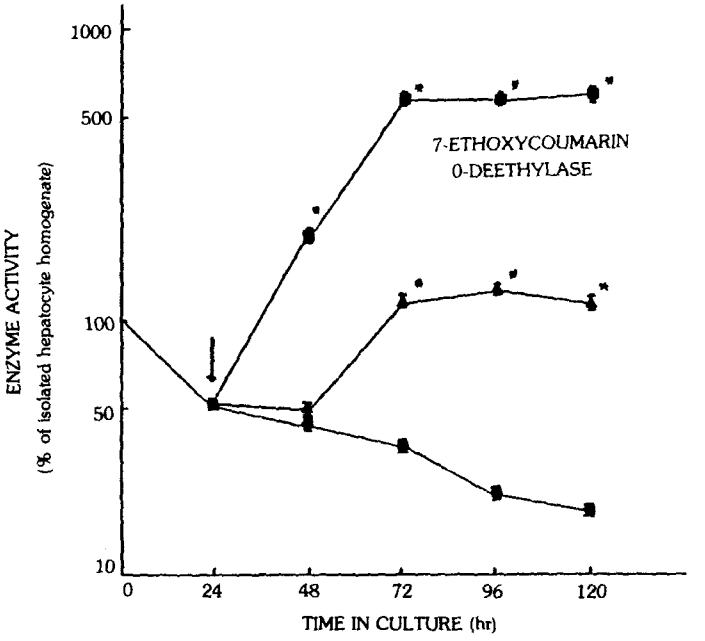

Fig. 1. Percent of initial 7-ethoxycoumarin O-deethylase activity of hepatocyte cultures treated with MC (-■-), PB (-▲-) and control (-●-)after various times in culture. MC and PB were added to the culture 24 hr after the initial plating (arrow). All values represent the mean ± SE of triplicate determinations. An asterisk indicates values significantly different from the control ($p < 0.05$).

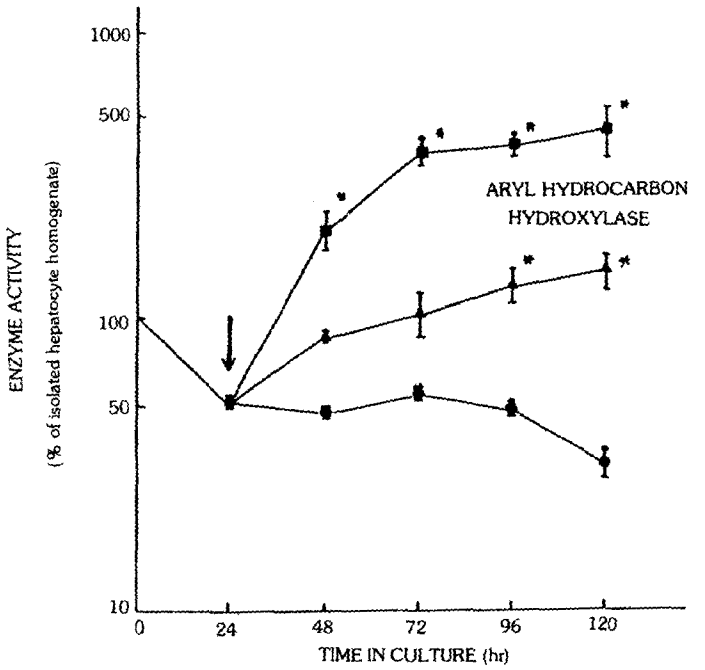

Fig. 2. Percent of initial aryl hydrocarbon hydroxylase activity of hepatocyte cultures treated with MC (-■-), PB (-▲-), and control (-●-) after various times in culture. MC and PB were added to the culture 24 hr after the initial plating (arrow). All values represent the mean ± SE triplicate determinations. An asterisk indicates values significantly different from the control ($p<0.05$).

RESULTS

Time dependent change in the activity of 7-ethoxycoumarin 0-deethylase in primary cultures of rat hepatocytes is shown in Fig. 1. As reported previously (Dickins and Peterson, 1980), the enzyme activity in the cells grown in control medium decreased rapidly with culture times. After 24 and 120 hr in culture, the enzyme activities were 60 and 22% of the initial rate (0 hr), respectively. Meanwhile, a significant increase of the enzyme activities was observed in MC treated cultures within 24 hr. Thus at 72, 96 and 120 hr, activities of the enzyme were more than 500% of the initial rate. A significant induction of the enzyme activity was also noticed after 48 hr in PB treated cell. However, the amounts of induction was much smaller when compared to that of MC. At 72, 96 and 120 hr, enzyme activities in the PB treated cell were close to those of the initial rate (0 hr).

The induction of aryl hydrocarbon hydroxylase activity in hepatocyte cultures treated with MC and PB are shown in Fig. 2. The induction patterns were quite similar to those of 7-ethoxycoumarin 0-deethylase activity as shown previously in Fig. 1. In the MC treated cultures, a significant increase was noticed within 24 hr and about 500% induction was observed at 120 hr (96 hr after the addition of MC). In the PB treated culture, significant induction was observed 72 hr after the addition of PB. At 120 hr, the enzyme activity was 140% of the initial rate (0 hr).

The activities of biphenyl 4-hydroxylase and aminopyrine N-demethylase are shown in Fig.

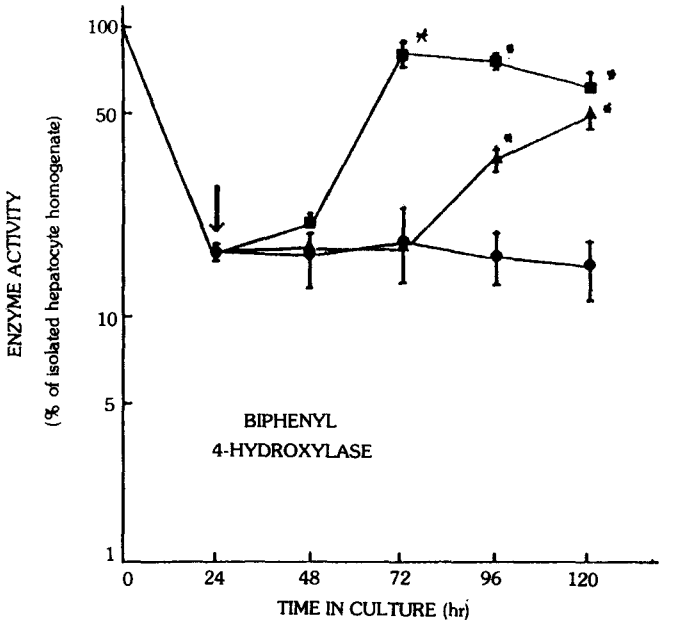

Fig. 3. Percent of initial biphenyl 4-hydroxylase activity of hepatocyte cultures treated with MC (-■-), PB (-▲-), and control (-●-)after various times in culture. MC and PB were added to the culture 24 hr after the initial plating (arrow). All values represent the mean ± SE triplicate determinations. An asterisk indicates values significantly different from the control ($p < 0.05$).

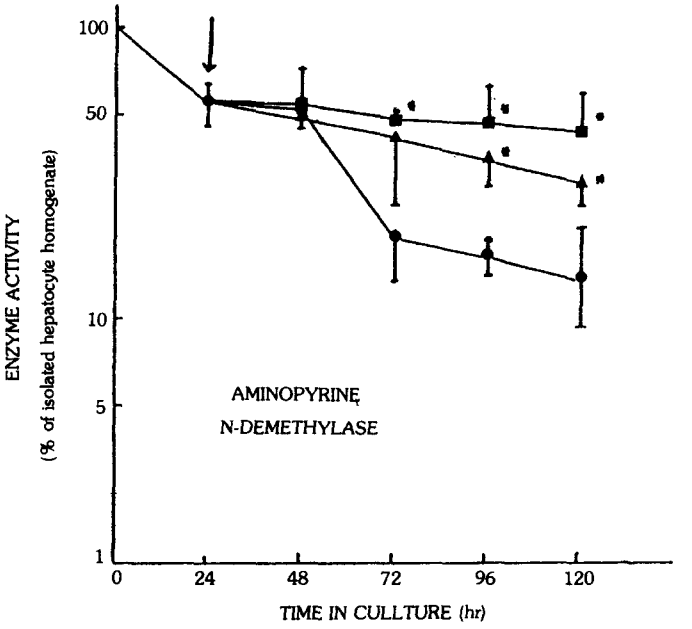

Fig. 4. Percent of aminopyrine N-demethylase activity of hepatocyte cultures treated with MC (-■-), PB (-▲-), and control (-●-) after various times in culture. MC and PB were added to the culture 24 hr after the initial plating (arrow). All values represent the mean ± SE triplicate determinations. An asterisk indicates values significantly different from the control ($p < 0.05$).

3 and 4. Similar to the activities of 7- ethoxycoumarin 0-deethylase and arylhydrocarbon hydroxylase, MC caused more rapid and higher induction of these enzymes than that observed with PB. However, the amounts of induction of biphenyl 4-hydroxylase and aminopyrine N-demethylase obtained by MC and PB treatments were much smaller than the previous two enzymes. Neither enzyme activities were increased to the level of freshly isolated hepatocytes after 96 hr of MC or PB treatments. The activities of biphenyl 4-hydroxylase at 120 hr in the MC and PB treated cultures were 60 and 48% of the initial rate, respectively. The activities of aminopyrine N-demethylase at 120 hr were 44 and 29% of the initial rate for MC and PB treated cultures, respectively.

Unscheduled DNA synthesis (UDS) induced by aflatoxin B_1 $(AFB)_1$ was measured in untreated and PB or MC treated hepatocyte cultures. As shown in Fig. 5, AFB_1 caused a dose dependent increase of UDS in the control culture. At lower concentrations of AFB_1 (10^{-9}, 10^{-8} and 10^{-7} M,) the extent of UDS in both the MC and PB treated cultures were similar to that of the control cells. At a higher concentration of AFB_1 (10^{-6} M), however, a significant increase in UDS was observed in both the MC and PB treated cultures when compared to that of controls.

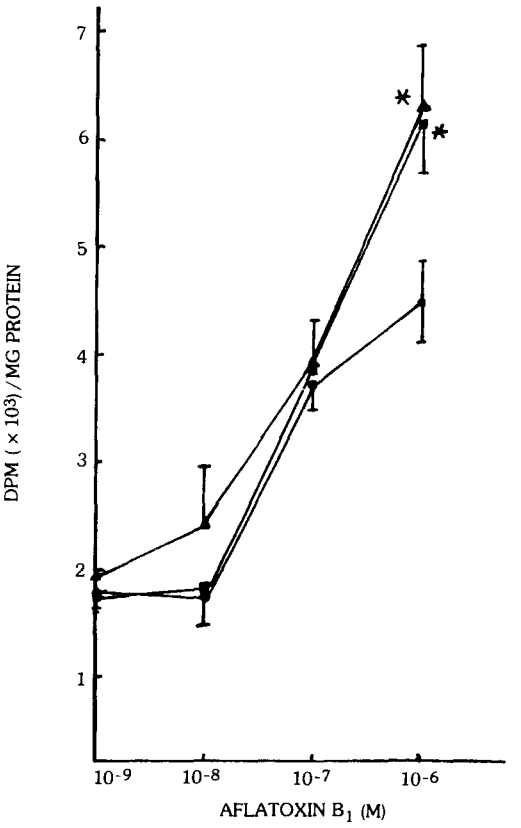

Fig. 5. Unscheduled DNA synthesis induced by aflatoxin B_1 in hepatocyte cultured treated with MC (-■-), PB (-▲-), and control (-●-). Hepatocytes were treated with MC or PB for 48 hr after the initial 24 hr attachment period and then aflatoxin B_1 was treated for 4 hr. All values represent the mean ± SE of triplicate determinations. An asterisk indicates values significantly different from the control ($p<0.05$).

DISCUSSION

It has been well documented that the level of cytochrome P-450 and the associated monooxygenase activities decrease rapidly during cultivation of isolated rat hepatocytes. This decrease appears to be an adaptive response of the cells grown under culture conditions, rather than a reflection of declining viability of cells (Bissel and Guzelian, 1975). To overcome this particular limitation, several investigators have shown that the levels of cytochrome P-450 can be maintained in primary hepatocytes culture by adding certain hormones and other substances to the medium (Decad *et al.*, 1977; Nelson and Acosta, 1982; Pain and Legg; 1978). At present, however, the culture conditions vary widely and the ideal standardized conditions have not yet been obtained. In the present study, we have demonstrated that additions of either MC or PB to the hepatocyte culture medium resulted in an increase of the cytochrome P-450 associated monooxygenase activities. The activities of 7-ethoxycoumarin 0-deethylase and aryl hydrocarbon hydroxylase were increased to a higher or closely equal to the level of freshly isolated hepatocytes by MC and PB treatment. Biphenyl 4-hydroxylase and aminopyrine N-demethylase activities were also induced by MC and PB pretreatments but the levels of induction were less than that occuring in 7-ethoxycoumarin 0-deethylase and aryl hydrocarbon hydroxylase. These results tentatively suggest that microsomal enzyme activities can be selectively controlled preferably in hepatocyte cultures by an *in vitro* induction method applicable for studying the metabolism of drugs and also in other toxicological assays.

In previous reports, we have demonstrated that aflatoxin B_1 was successfully metabolized by the primary hepatocyte culture and caused a dose-dependent response in the unscheduled DNA synthesis and also in immunosuppression (Kim *et al.*, 1986; Yang *et al.*, 1986). The result obtained in the present study shows that aflatoxin induced a significant increase of UDS in hepatocyte cultures treated with MC and PB at higher doses. Since aflatoxin B_1 was incubated in hepatocyte culture for 4 hr in this experiment, control culture may not have sufficient enzyme activities to metabolize the higher dose of aflatoxin in that period while MC and PB induced cultures did. Decad *et al.* (1977) also reported that hepatocyte cultures with *in vivo* level of cytochrome P-450 completely metabolized aflatoxin B_1 in 10 hr.

The molecular forms of cytochrome P-450 induced by MC and PB are known to differ with respect to substrate specificity and the absorption maxima of their reduced CO and ethylisocyanide, etc. And hence the terms cytochrome P-448 and cytochrome P-450 were used for the cytochromes induced by treatment MC and PB, respectively (Alvares *et al.*, 1967; Saldek and Mannering, 1966). The results obtained in this study also show the differences in the kinetics of induction by MC and PB in hepatocytes cultures and suggest that different mechanisms of induction must be involved.

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