EFFECTS OF VARIOUS INDUCERS ON THE ACTIVITIES OF CYTOCHROME P-450-MIXED FUNCTION OXIDASES AND AFLATOXIN B₁ ACTIVATION IN MICROSOMES OF HAMSTER LIVERS

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Abstract.....The effects of various inducers on the activities of drug-metabolizing enzymes including aflatoxin B₁ activation were studied in Syrian golden hamsters. Activity for aflatoxin B₁ was determined by aflatoxin B₁-DNA adducts formation. The treatments of hamsters with 3-methylcholanthrene, α -naphthoflavone and benzo(a)pyrene elevated markedly the activity for aflatoxin B₁ by 2460, 1380 and 450%, respectively. Phenobarbital induced slightly and isosafrole and ethanol did not induce the activity for aflatoxin B₁. Pregnenolone-16 α -carbonitrile decreased aflatoxin B₁ activation to 51% of that of the non-treated animals. These results were in good accordance with the induction rate of a form of cytochrome P-450 (P-450_{AFB}) which has potent activity to aflatoxin B₁. Characteristics in the induction of mixed function oxidases of hamsters by these inducers, especially in respect to benzo(a)pyrene metabolizing enzyme, seemed to differ from those of rats. These results suggest that the activity for aflatoxin B₁ in hamster is inducible by 3-methylcholanthrene-type inducers and that hamster is a suitable animals model to study the mechanism of aflatoxin B₁-hepatocarcinogenesis.

Key words: Aflatoxin B₁, methylcholanthrene, α -naphthoflavone, benzo(a)pyrene, pregnenolone-16 α -carbonitrile, isosafrole, cytochrome P-450_{AFB}, hamster.

INTRODUCTION

Aflatoxin B₁(AFB₁), a potent hepatocarcinogen and mutagen in experimental animals (Newberne and Bulter, 1969 ; Wong and Hsieh, 1976) is known to contaminate various foods and feeds. The contamination of AFB₁ has been shown to correlate well with the occurrence of human liver cancer by epidemiological studies (Alpert et al., 1971).

AFB₁ is transformed to various metabolites, such as AFB₁ 8,9-oxide, aflatoxicol, AFM₁, AFQ₁ and AFP₁ by liver microsomal mixed function oxidases (Garner, 1973 ; Miller, 1978 ; Masri et al., 1974 ; Dalezios et al., 1971). It is known that the mutagenicity of AFB₁ is obtained with the formation of AFB₁ 8,9-oxide (Wong and Hsieh, (1976) and that binding of AFB₁ to cellular macromolecules such as DNA occurs via formation of AFB₁ 8,9-oxide, which is thought to initiate carcinogenesis (Gurtoo and Bejba, 1972).

Species differences in the sensitivity in carcinogenesis and in the activity to induce mutagenicity of AFB₁ have been reported by Bulter (1969), Herrold (1969) and Wong and Hsieh (1976). They indicated that the rat was more susceptible than mouse and hamster to AFB₁-hepatocarcinogenesis. In the ability to induce AFB₁ mutagenicity, the hamster is more potent than the rat and mouse. The discrepancy of species differences between the susceptibility in hepatocarcinogenesis and the activity to induce mutagenicity of AFB₁ could be explained by the existence of different forms of cytochrome P-450 in non-treated animals and in polychlorinated biphenyls(PCB) or 3-methylcholanthrene(MC)-treated animals.

We demonstrated in a previous paper that a form of cytochrome P-450 purified from MC-treated hamsters was highly active in the activation of AFB₁ and that its potency was about 50-to 250-fold greater than the other forms of cytochrome P-450 purified from the livers of MC- and phenobarbital(PB)-treated rats(Mizokami et al., 1986). Further, we found that this form of cytochrome P-450 is specific to hamster and not to rat, rabbit, guinea pig, mouse and *Suncus murinus*(Sunouchi et al., 1988).

Since this form of cytochrome P-450 (cytochrome P-450_{AFB} or P-450-I) is specific to hamster and to AFB₁ activation, it is important to study the inducing effects of various inducers on mixed function oxidase, especially on the AFB₁ activation in hamsters. This communication deals with the effects of various type of inducers on the activity of AFB₁ - DNA adduct formation, the activities of mixed-function oxidases and the induction of cytochrome P-450_{AFB} in the liver microsomes.

MATERIALS AND METHODS

Chemicals : 3-Methylcholanthrene, benzo(a)pyrene(BP), α -naphthoflavone(α NF), isosafrole(ISF), ethanol(EtOH), phenobarbital and 3,3'-diaminobenzidine hydrochloride were purchased from Wako Pure Chemical Industry Ltd. (Osaka, Japan). Pregnenolone-16 α -carbonitrile(PCN) was donated by Shionogi & Company Ltd.(O-

saka, Japan). Benzphetamine(BPT) was a generous gift from Dr. Yuji Nakahara of National Institute of Hygienic Sciences. [^3H]-Aflatoxin B₁ (AFB₁, 20 Ci/mmol) was purchased from Moravek Biochemicals Inc. (Brea, MO). [7,10- ^{14}C]-Benzo(a)pyrene was purchased from Amersham Co.(Amersham, U.K.). [Dimethyl- ^{14}C]-aminopyrine (AM, 70 mCi/mmol) and [2- ^{14}C]-hexobarbital (HB, 8.58 mCi/mmol) were obtained from New England Nuclear Co.(Boston, Mass.). Calf thymus DNA and N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid (HEPES) were purchased from Sigma Chem. Co.(St. Louis, MO.). NADP and glucose-6-phosphate(G6P) were purchased from Oriental Yeast Co. Ltd. (Tokyo, Japan). G6P dehydrogenase(G6PDH) was obtained from Boehringer Mannheim (Mannheim, F.R.G.). Anti mouse Ig G(γ -chain, rabbit) and mouse peroxidase anti-peroxidase were purchased from Medical & Biological Laboratories Co. Ltd. (Nagoya, Japan) and from Jackson Immunoresearch (West Grove, PA.), respectively. The other chemicals used were of reagent grade.

Treatments of animals : Male Syrian golden hamsters weighing from 90 to 100 g bred in Shizuoka Laboratory Animals Co. (Shizuoka, Japan), were housed in plastic cages, fed F2 standard chow diet (Funabashi Farm Co. Ltd., Funabashi, Japan) and given water *ad libitum* except for the experiment with EtOH-treatment. The animals were maintained on a constant 12-hr light : dark cycle with temperature and humidity kept at $25 \pm 1^\circ\text{C}$ and $55 \pm 5\%$, respectively. MC and α NF (25 mg/kg of body weight, daily) were administered intraperitoneally for 3 and 2 consecutive days, respectively. BP and PB (50 mg/kg, daily) were injected intraperitoneally for 3 consecutive days. PCN (40 mg/kg, daily) and ISF (150 mg/kg, daily) were administered intraperitoneally for 4 consecutive days, EtOH was given as 10% solution in drinking water for 10 days until sacrifice. Livers were removed 48 hours after the last injection of MC, α NF, and 24 hours after the last injection of the other chemicals except for EtOH. MC, BP, α NF, ISF and PCN were dissolved or suspended in olive oil and PB was dissolved in saline.

Preparation of microsomes : Hamsters were killed by decapitation and the livers were dissected and homogenized in 3 vol. of ice cold 1.15% KCl solution by a Teflon-glass homogenizer and were centrifuged at 10,000g for 15 min. The supernatant fraction was recentrifuged at 105,000g for 60 min. The precipitated microsomal pellets were rinsed and resuspended with ice cold 1.15% KCl solution.

Monoclonal antibody and Western blots : Monoclonal antibody (Mab. MIT604), which was prepared as described previously (Sunouchi et al., 1988) was used for the detection of the induction of cytochrome P-450_{AFB} by Western blots. Electrophoresis on a SDS-polyacrylamide gel (10% acrylamide, Laemmli, 1970) was done and Western blots were carried out by the methods of Guengerich et al. (1982).

AFB₁-DNA adduct formation : Assay of AFB₁-DNA adduct formation was measured by the modified method of Yoshizawa et al. (1982). The reaction mixture contained G6P (10 mM), NADP (81 mM), MgCl₂ (6.25mM), HEPES buffer (pH7.4, 52mM), G6P-DH (0.7U/ml), calf thymus DNA (0.6 mg/ml), [^3H]-AFB₁ (7.5

nmol/0.57 μ Ci) and microsomes (non-treated, ISF, PCN and EtOH-treated ; 30 μ g, PB, α NF and BP-treated ; 10 μ g, MC-treated ; 8 μ g of protein) in a total volume of 0.25 ml. The reaction mixture was incubated at 37°C for 60 min and [3 H]-AFB₁ bound to DNA was measured.

Assays : The contents of cytochrome P-450 and cytochrome b₅ of hepatic microsomes were measured by the methods of Omura and Sato (1964). NADPH-cytochrome c reductase was assayed by the method of Philips and Langdon (1962). The activities of other microsomal enzymes assayed were [14 C] BP-metabolizing activity determined by the method of Nesnow et al. (1977), 7-ethoxycoumarin (7ET) O-deethylation activity by the method of Greenlee and Poland (1978), [14 C]AM-metabolizing activity by the method of Poland and Nebert (1973), BPT N-demethylation activity by the method of Nash et al. (1953), [14 C] HB-metabolizing activity by a modification (Inoue et al., 1981) of Cooper and Brodie (1955) and aniline hydroxylation activity by the method of Mieyal et al. (1976). The reaction mixture of these assays consisted of 0.1 to 0.2 ml of the microsomal fraction, 1 mM NADP, 30mM G6P, 5 mM MgCl₂, 0.24 ml of 0.2 M phosphate buffer (pH 7.4) and a substrate (BP; 80 mM, 7ET; 50 mM, AM, BPT, HB, AN; 1 mM) in a final volume of 0.5 ml and was incubated at 37°C for 10 min. Microsomal protein was determined according to the method of Lowry et al. (1951).

Statistics : Statistical significances of differences between the non-treated group and the treated-groups were assayed by Student's *t* test.

RESULTS

Effects of inducers on AFB₁-DNA adduct formation : The results of the activity to form AFB₁-DNA adducts by liver microsomes from hamsters treated with various inducers are shown in Table 1. The level of AFB₁-DNA adduct formation by microsomes from the non-treated animals was 4.07 pmol-AFB₁/min/mg protein. MC and α NF-treatments to hamsters markedly elevated the formation of AFB₁-DNA adducts to 2460 and 1380% of that of the non-treated hamsters. BP-treatment increased the activity to 450% of that of the non-treated animals. A slight increase in the formation of AFB₁-DNA binding was observed with PB-treatment. On the contrary, PCN-treatment decreased the activity to 51% of that of the non-treated animals. Either of ISF and EtOH-treatments did not evoke any significant changes in the formation of AFB₁-DNA adducts compared with that of the controls.

Effects of inducers on the induction of cytochrome P-450_{AFB} in hamster liver microsomes : To compare the AFB₁-DNA adducts formation with cytochrome P-450_{AFB}, which has high potency in AFB₁ activation, identification of cytochrome P-450_{AFB} was done by Western blots in liver microsomes obtained from hamsters treated with various inducers. The profile of immunochemical staining of cytochrome P-450_{AFB} of liver microsomes is shown in Figure 1. The cytochrome P-450_{AFB} was highly induced following the treatment with MC, α NF and BP. The

Inducers on hamster drug-metabolizing enzymes

Table 1. Effects of various inducers on the formation of AFB₁-DNA adduct in liver microsomes in hamsters^a.

None	Inducers						
	MC	BP	α NF	PB	ISF	EtOH	PCN
4.07	100.07 ^b	18.34 ^c	56.36 ^b	5.49 ^c	4.33	3.44	2.09 ^b
$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$
0.20	8.67	2.57	5.97	0.45	0.36	0.33	0.34

a Values(AFB₁-DNA adduct pmol/min/mg) are expressed as mean $\pm$ SE(n= 4).

b Significantly different from the value of non-treated animals, P<0.01

c Significantly different from the value of non-treated animals, P<0.05

Fig. 1. Western blots of Mab. MIT604 with liver microsomes from hamsters treated with various inducers. Microsomes (1,2,4-6 ; 10 μ g of protein : 3,8,9 ; 1 μ g of protein) or cytochrome P-450_{AFB}(0.2 μ g of protein) were applied to each well. (1 ; Control, 2 ; PB, 3 ; MC, 4 ; EtOH, 5 ; PCN, 6 ; ISF, 7 ; P-450_{AFB}, 8 ; BP, 9 ; α NF)

slight induction was observed with PB-treatment. On the contrary, ISF, EtOH and PCN-treatments did not induce cytochrome P-450_{AFB}.

Effects of inducers on mixed function oxidases : Changes in the activity of mixed function oxidases by treatments of various inducers are shown in Table 2 and 3. MC, α NF and PB-treatments significantly increased the level of microsomal cytochrome P-450 to 179, 125 and 220% of that of the non-treated animals, respectively. In contrast, PCN-treatment decreased the level of microsomal cytochrome P-450 to 65% of that of the non-treated animals. The level of microsomal cytochrome P-450 in BP, ISF and EtOH-treated animals was unchanged. In the non-treated hamsters, the maximum absorption in CO-difference spectra of reduced cytochrome P-450 in the hepatic microsomes was observed at 450.1 nm. In the treatment with MC and α NF, the maximum absorption was observed at 448.8 nm and 449.5 nm, respectively. In PB, EtOH and PCN-treatments, the maximum absorption was observed in the range of 450.5 and 450.9 nm. The cytochrome b₅ level was increased by the treatments with BP, ISF and PCN but not with other inducers. NADPH-cytochrome c reductase activity was decreased by the treatments with α NF, ISF, EtOH and PCN.

BP-metabolizing activity was not induced by MC-treatment, but the activity was significantly decreased with α NF, ISF and PCN-treatments to about 50% of that of the non-treated animals. 7ET O-deethylation activity was significantly increased following MC, α NF and PB-treatments to 269, 195 and 175% of that of the non-treated animals, while the activity was decreased significantly following PCN-treatment. AM-metabolizing activity was significantly increased following PB and

Table 2. Effects of various inducers on cytochrome P-450 and b₅ contents and NADPH-cytochrome c reductase activity of liver microsomes in hamsters.^a

Inducers	P-450 content (nmol/mg)	b ₅ content (nmol/mg)	NADPH-cyt. c reductase activity (nmol/min/mg)
Control	1.023 ± 0.061	0.320 ± 0.036	201.11 ± 9.11
MC	1.830 ± 0.178 ^b	0.364 ± 0.052	184.82 ± 11.89
BP	0.998 ± 0.081	0.660 ± 0.019 ^b	210.27 ± 17.91
α NF	1.282 ± 0.087 ^c	0.396 ± 0.029	123.21 ± 11.71 ^b
PB	2.248 ± 0.118 ^b	0.388 ± 0.037	260.90 ± 33.70
ISF	0.920 ± 0.088	0.695 ± 0.038 ^b	162.00 ± 5.40 ^b
EtOH	1.003 ± 0.066	0.311 ± 0.065	133.79 ± 10.61 ^b
PCN	0.665 ± 0.056 ^b	0.512 ± 0.055 ^c	116.49 ± 9.83 ^b

^a Values are expressed as mean ± SE(n = 4).

^b Significantly different from the value of non-treated animals, P < 0.01

^c Significantly different from the value of non-treated animals, P < 0.05

Table 3. Effects of various inducers on mixed function oxidases in liver microsomes of hamsters^a.

Inducers	Benzo(a)pyrene metabolizing activity	Ethoxycoumarin O-deethylation activity	Aminopyrine metabolizing activity	Benzphetamine N-demethylation activity	Hexobarbital metabolizing activity	Aniline p-hydroxylation activity
NONE	1.648±0.162	3.301±0.100	4.329±0.150	11.871±0.809	2.132±0.069	0.710±0.028
MC	1.248±0.153	8.873±0.339 ^b	4.327±0.399	9.659±0.826	1.692±0.116 ^b	1.081±0.066 ^b
aNF	0.810±0.054 ^b	6.450±0.213 ^b	3.677±0.232	6.833±0.347 ^b	1.314±0.105 ^b	0.719±0.034
PB	1.833±0.317	5.780±0.665 ^c	19.190±2.767 ^b	9.669±0.438	4.575±0.732 ^c	1.376±0.065 ^b
ISF	0.864±0.065 ^b	3.911±0.121 ^b	7.955±0.438 ^b	9.003±0.339 ^c	1.795±0.044 ^b	3.239±0.081 ^b
EtOH	1.349±0.112	3.228±0.054	3.662±0.108 ^b	14.151±0.247 ^c	2.089±0.067	1.354±0.127 ^b
PCN	0.912±0.070 ^b	2.675±0.073 ^b	3.522±0.190 ^c	11.608±0.368	1.897±0.058 ^c	0.519±0.035 ^b

^a Values(nmol/min/mg protein) are expressed as mean ± SE(n=4).^b Significantly different from the value of non-treated animals, P<0.01^c Significantly different from the value of non-treated animals, P<0.05

ISF-treatments to 443 and 184% of that of the non-treated animals. In contrast, AM-metabolizing activity was decreased significantly following EtOH and PCN-treatments to about 85 and 81% of that of the non-treated animals. BPT N-demethylation activity was decreased by α NF and ISF-treatments. HB-metabolizing activity was decreased significantly following MC, α NF, ISF and PCN-treatments to 79, 62, 84 and 89% of that of the non-treated hamsters, while PB-treatment increased the activity to 215% of that of the non-treated hamsters. ISF, PB, EtOH and MC-treatments increased AN hydroxylation activity to 456, 194, 190 and 152% of that of the non-treated animals, respectively. On the other hand, PCN-treatments decreased AN hydroxylation activity to 73% of that of the non-treated animals.

DISCUSSION

The present results indicated that MC-type inducers such as α NF and BP have potency for inducing the capability of AFB₁-activation in hamsters. The level of AFB₁-DNA binding in liver microsomes of the non-treated hamsters is 4.0 pmol-AFB₁/min/nmol of microsomal cytochrome P-450, while the level induced by the treatment with MC is 55.5 pmol-AFB₁/min/nmol of microsomal cytochrome P-450. In the study of Yoshizawa et al. (1982), the level using reconstituted system of cytochrome P-448 purified from MC-treated rat livers is 11 pmol-AFB₁/min/nmol of purified cytochrome P-448. Thus, the activity for AFB₁ of hepatic microsomes of hamsters treated with MC is much higher than that of cytochrome P-448 reconstituted system of rats. Booth et al. (1981) have described that the amounts of AFB₁-DNA adducts formed by cytochrome P-450-oxidase systems in human livers were similar to those by hamster livers. If this is the case, the present results suggest that MC-type of inducers might be active inducers on AFB₁-activation in human, which might increase the human risk to AFB₁-hepatocarcinogenesis.

High activity for AFB₁ in hamster livers might be mainly due to cytochrome P-450_{AFB} induced by MC, α NF and BP. This is supported by the experiment of Western blots that demonstrated the induction of large amounts of cytochrome P-450_{AFB} in hamster livers by treatments with MC, α NF and BP. The results of the induction of cytochrome P-450_{AFB} by inducers are well correlated with those of the potency of AFB₁-DNA adduct formation.

All the inducers used in the present study may give both qualitative and quantitative alteration to components of cytochrome P-450. For example, the qualitative alteration was indicated by the induction pattern of different enzymes and also by the different absorption maximum in the spectrum of carbon monoxide-complex of reduced cytochrome P-450 observed in liver microsomes of hamsters treated with various inducers. Cytochrome P-450 of liver microsomes induced by MC and α NF might contain not only a large amount of cytochrome P-450_{AFB} but another forms of cytochrome P-450. Cytochrome P-450_{AFB} seems not to correlate

with the forms of hepatic cytochrome P-450 purified from MC-treated rats as demonstrated by the results on the potency of AFB₁-activation and the immuno-chemical properties (Mizokami et al., 1986 ; Sunouchi et al., 1988), although a high spin type of the forms from rats partly had similar enzyme activities to cytochrome P-450_{AFB} (Mizokami et al., 1986).

The comparison of the present results with those of rats (Ryan et al., 1979 ; Guengerich et al., 1982 ; Sunouchi et al., 1984), in respect to inducing effects of various types of inducers on mixed function oxidases, disclosed that the effects of MC-type inducers such as MC and α NF on BP-metabolizing activity differ between hamsters and rats. Contrary to rats, the MC-type of inducers decreased BP-metabolizing activity in hamsters. In respect to effects of MC on BP-metabolism, our data are in good agreement with the results of Wroblewski et al. (1988). They described that the metabolite profile of BP by hepatic microsomes from tetrachloro-dibenzo-*p*-dioxin (TCDD, a MC-type inducer)-pretreated hamsters was unchanged compared with that of the controls, and that the profile in TCDD-pretreated rats differ from that of the controls. These could be explained by the differences in the forms of cytochrome P-450 induced by MC-type of inducers between hamsters and rats.

Effects of the inducers on the other enzyme activities in hamsters are similar to those in rats. 7ET O-deethylation and AN p-hydroxylation were increased by both MC-type and PB-type of inducers, and HB- and AM-metabolism and BPT N-demethylation were increased by PB-type of inducers. PCN did not increase the activities of these enzymes, which might depend on the decrease in specific forms of cytochrome P-450, cytochrome P-450 content and NADPH-cytochrome c reductase activity in microsomes. AN p-hydroxylation in hamster was induced highly by ISF-treatment. The present results on the effects of these inducers on 7ET-, AM-, BPT- and AN-metabolizing enzymes are mostly similar to the results of Chiang and Steggle (1983) and Ardies et al. (1987).

In conclusion, in hamsters, MC-type of inducers including BP and α NF induced potentially the activity for AFB₁. The differences were observed between the hamster and rat in the effects of MC-type of inducers on BP-metabolizing enzymes which may be due to the different forms of cytochrome P-450. In respect to the other inducers, the effects on other mixed function oxidase enzymes in hamsters are mostly similar to those of the rats.

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