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INDUCTION, MODIFICATION AND INHIBITION OF RAT LIVER MICROSOMAL BENZO(a)PYRENE HYDROXYLASE ;CORRELATION WITH THE S-9-MEDIATED MUTAGENICITY OF BENZO(a)PYRENE

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SUMMARY : The effects of various pretreatments in vivo (3MC, PB, 2 and 4FAA) and of various inhibitors in vitro (7,8 BF, SKF525A and MN R) on the activity of rat liver microsomal BP hydroxylase were analyzed and correlated with the S-9 mediated mutagenicity of BP. 3MC is the only treatment which both induces and modifies the hydroxylase activity; it also specifically increases the enzyme mediated mutagenicity. Miconazole R which inhibits all the tested microsomal preparations, also reduces the mutagenicity mediated by all the S-9 preparations whereas the inhibitory effects of 7,8 BF and SKF525A are limited respectively to enzyme preparations from 3MC induced and control or PB treated rats.

As reported by many authors including ourselves, pretreatment of the rat by 3-methylcholanthrene not only induces the benzo(a)pyrene hydroxylase activity (increased V_{max}) but it also reduces its K_M value [1-4]. The expression"enzyme modification"was proposed to characterize this last effect [3] which could be an intrinsic property of some chemical carcinogens. In the case of 3 methylcholanthrene, this in vivo enzyme induction and modification is related to the appearance of cytochrome P_{448} and it could be due to a change in the cytochrome-phospholipid interaction within the endoplasmic reticulum membranes [5].

As demonstrated many times, liver microsomal aryl hydrocarbon hydroxylase from 3 methylcholanthrene induced rat is catalytically different from the same enzyme isolated from control animal. Not only does it produce different metabolic patterns [6] but it is also inhibited by specific molecules such as 7-8 benzoflavone which do not act on the activity of control preparations [7-11].

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Abbreviations: 3MC, 3 methylcholanthrene ; BP, benzo(a)pyrene ; PB, Phenobarbital ; 2FAA, 2 fluorenylacetamide ; 4FAA, 4-fluorenylacetamide ; BF, 7,8 benzoflavone ; MN, miconazole R (1-(2-(2,4-dichlorophenyl)-2-((2,4-dichlorophenyl-methoxy)-1-H-imidazole.

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The present study was initiated to determine whetherthe biochemical induction and modification or the inhibition of benzo(a)pyrene hydroxylase could correlate with respectively an increased or a decreased capacity of the enzyme preparations (S-9) to activate benzo(a)pyrene to reactive intermediates that cause mutations in strain TA1538 of Salmonella typhimurium. 3-methylcholanthrene induced rats were compared not only with control but also with phenobarbital and 2-acetylaminofluorene pretreated animals.
This last compound was choosen because it is a well known inducer and modifier of an other microsomal activating enzymatic activity, the arylamine and arylamide N-hydroxylase [12].

MATERIALS AND METHODS

Chemicals : Benzo(a)pyrene (BP) and 3-methylcholanthrene (3MC) from Fluka, 7,8 benzoflavone (BF) from Eastman Organic Chemicals, 2-fluorenylacetamide (2FAA) from Aldrich and 4-fluorenylacetamide (4FAA) from Suchardt were of the purest grade available.SKF525A was a gift from Smith, Kline and French Laboratories and miconazole R (MN) 1-(2-(2,4-dichlorophenyl)-2-((2,4-dichlorophenyl)-methoxy-ethyl)-1-H-imidazole, was kindly supplied by Janssen Pharmaceutica, Beerse, Belgium.
Animals and treatments : Adult male Wistar rats weighing 200 to 250 g were used for all the experiments. They received the following treatments : PB, 75 mg/kg in water ip., 48 and 24h before sacrifice ; 3MC, 40 mg/kg ip. and 2FAA or 4FAA, 10 mg/kg in corn oil ip. 24h before the sacrifice by decapitation.
The liver microsomes were prepared according to the method of de Duve as described by Amar Costesec et al. [13]. The post mitochondrial S-9 fractions obtained from 3 pooled rat livers as well as the S-9 mix were prepared according to Ames et al [14].
Enzymatic assay : The microsomal benzo(a)pyrene hydroxylase activity was measured by applying the spectrofluorimetric assay of Dehnen [15] with slight modifications as previously reported [3]. The various inhibitors were added to the incubation medium after the addition of the microsomal proteins (final concentration : 3-6 µg/ml) and before the addition of BP (final concentration from 0.5 up to 5.10^{-6} with microsomes from control, PB, FAA treated rats and from 0.05 up to 2 µM with microsomes from 3MC induced rats) in 0.2 ml acetone to reach a final incubation volume of 3.55 ml. SKF525A and MN were added as a water solution to a final concentration of respectively 0.05 to 1. 10^{-6}M and 0.1 to 3. 10^{-6}M. BF was solubilized in acetone, its final concentration was 4 to 8.10^{-6}M. All the inhibitors were added to the incubation medium after a 30 min preincubation period (generation of NADPH) and 2 min after the addition of the microsomal proteins.
BP was added immediately after the inhibitor except for SKF525A which was previously preincubated for 15 min. The cytochrome P_{450} content and the concentration of the proteins in the microsomes were determined by applying respectively the method of Raj and Estabrook [16] and the assay of Lowry [17].
Mutagenicity assays : Salmonella typhimurium strain TA1538 was kindly provided by Professor B.N.Ames. The plate tests were per-

formed by mixing successively in histidine-biotin (0.05 mM) supplemented top agar (2 ml/plate) : 2-8.10^7 viable bacteria from an overnight culture in nutrient broth (Difco)/plate, inhibitors dilutions final concentrations (5.10^{-6}M), S-9 mix (0.5 ml/plate), substrate dilutions (0.1 ml/plate). The mixture was layered on minimal glucose agar and the plates incubated for 48h at 37°C in the dark. The numbers of his + revertant macroscopic colonies were calculated. In the experimental conditions, the optimal amounts of the different S-9 in the mix were found to be ranging from 100 µl/ml to 150 µl/ml mix. S-9 mix were utilized in the composition of 100 µl/ml mix.
The toxicity of BP was evaluated by determining the bacterial survival : a lower (10^4 fold dilution) bacterial inoculum incorporated in the top agar was layered on nutrient agar plates ; the other experimental conditions remained unchanged.
Statistical analysis : The program of Cleland[14] was applied for the quantitative estimation of the enzymic kinetic parameters. To study the influence of the inhibitors on the hydroxylation kinetics, this program has been extended [18] for the analysis of all types of inhibition of the enzymatic catalysis allowing :
- on a F test basis, the choice of the most probable kinetic model i.e. competitive, uncompetitive, mixed type or non-competitive inhibition.
- the quantitative estimation of the parameters V_{max}, K_M and K_i with their standard deviation. An iterative process of non linear regression "steepest descent" [19], further corrected by using a taylor series linearization was applied for all these analyses.

RESULTS

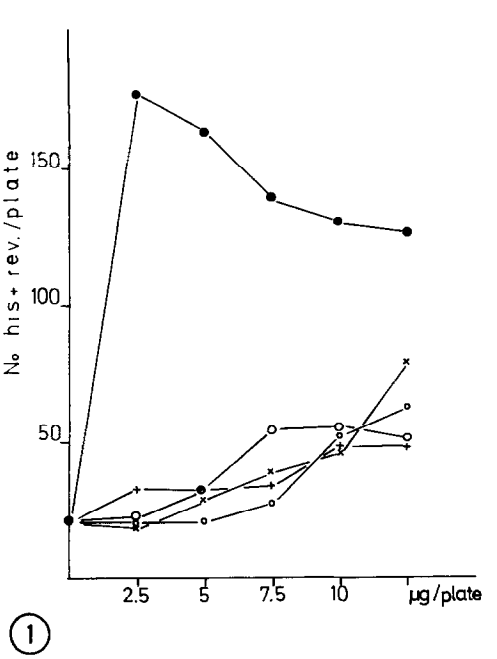
In the strictly defined Michaelis-Menten conditions, at very low concentration of both proteins (4.3 µg/ml) and BP (from 0.05 up to 2 µM) the computerized V_{max} and K_M of liver microsomal BP hydroxylase from 3MC treated rat are respectively 2.4 times higher and 10 times lower than the same kinetic parameters of control enzymatic preparation (table 1).
This effect of 3MC is rather specific since, as compared with control microsomes, it is not produced by PB or 2FAA.
In strain TA1538 of S.typhimurium, the number of his^+ revertants resulting from the metabolic (S-9) activation of BP was directly related to the kinetic parameters of BP hydroxylase. In complete incubations containing cofactors, BP, S-9 from the variously pretreated rats, only the 3MC induced and modified enzyme preparation had an increased capacity to activate BP to mutagenic intermediates (Fig.1).
The effect of 3MC was evident already at the lowest concentration of BP (2.5 µg/plate or approximately 5.10^{-6}M) It seemed to decrease when the concentration of BP was increased.
The computer program used in this study allows a complete ana-

Table 1 . Effect of various pretreatments in vivo on the kinetic parameters of rat liver microsomal BP hydroxylase.

PRETREATMENT	CONTROL	3MC	PB	2FAA
CONCENTRATION IN THE INCUBATION MEDIUM				
BP $.10^{-6}$M	0.5 to 5	0.05 to 2	0.5 to 5	0.5 to 5
Cyt P_{450} . 10^{-9}M	4.47	6.86	7.03	4.1
Proteins µg/ml	3.9	4.3	3.4	6.1
KINETIC PARAMETERS				
V_{max} nmoles/min.mg prot.	3.43 ± 0.46	8.22 ± 0.14	3.49 ± 0.31	3.20± 0.14
K_M . 10^{-6}M	2.60 ± 0.6	0.27 ± 0.015*	2.70 ± 0.19	1.51± 0.33

V_{max} and K_M are computerized value given ± S.D.

* Significantly different from control by T test ($p<0.01$)



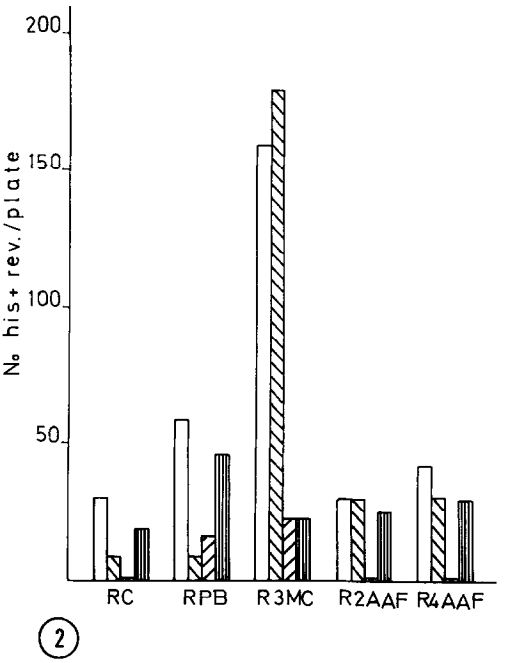


Figure 1.

Mutagenicity assay of benzo(a)pyrene with strain TA1538 in the presence of S-9 mix. Pretreatments of the rats : O controls ; ● 3-methylcholanthrene ; X phenobarbital ; + 2-fluorenylacetamide ; o 4-fluorenylacetamide.

Figure 2.

Effects of SKF525A, miconazole ^{R} and benzoflavone on the mutagenicity of benzo(a)pyrene towards strain TA1538 in the presence of S-9 mix. Doses of benzo(a)pyrene/plate : 12.5 µg with RC, RPB, R2FAA, R4FAA ; 2.5 µg with R3MC.
The numbers of his + rev./plate (19-21/plate) corresponding to the spontaneous reversion rate were subtracted.
In the range of the experimental doses of BP, no significant toxic effect was detected ; in every case, the bacterial survival was close to 100 %.


No his + rev./plate in the absence of inhibitors


No his + rev./plate in the presence of SKF525A


No his + rev./plate in the presence of miconazole


No his + rev./plate in the presence of benzoflavone

lysis of all types of inhibitions of the enzymatic catalysis including the choice of the most probable kinetic model and the quantitative estimation of the parameters V_M, K_M and K_i. The classical inhibitor of the mixed function oxidase, SKF525A, competitively inhibits (table 2) the BP hydroxylase of microsomes from control and PB treated rats but not the activity of enzymatic preparations isolated from 3MC induced or 2FAA pretreated animals.

7,8 benzoflavone, as reported previously [7-11], acts as a specific competitive inhibitor (table 2) of the cytochrome P_{448} dependent monooxygenase. Miconazole ^{R} which is an imidazole derivative, inhibits the BP hydroxylase of all the tested enzymatic preparations. However, it acts as a non competitive inhibitor on control or PB microsomes and as a competitive inhibitor on 3MC and 2FAA preparations.

In strain TA1538 of S.typhimurium, SKF525A (5×10^{-6}M) significantly reduced (fig.2) the enzyme mediated mutagenicity of BP when S-9 from control or PB pretreated rats were used ; it did not affect the metabolic activation of the pro-mutagen when S-9 from 3MC induced or 2FAA pretreated animals were added. Miconazole ^{R}(5×10^{-6}M) significantly reduced (fig.2) the mutagenicity mediated by all the enzyme preparations tested.

7-8 benzoflavone specifically lowered (fig.2) the number of his^+ revertants resulting from the metabolic activation of BP by S-9 from 3MC pretreated rats without affecting the other enzyme-mediated mutagenicities.

Pretreatment of the rat with 4FAA, the non carcinogenic isomer of 2FAA, dit not increase the number of revertants resulting from the metabolic activation of BP by liver S-9 (fig.1). The pattern of reduction of the number of revertants by the various inhibitors of the mixed function oxidase was essentially the same as after pretreatment with 2FAA (fig.2).

DISCUSSION

It is well demonstrated that 3MC pretreatment induces the liver microsomal BP hydroxylase activity [1,2,4] and increases the enzyme mediated formation of mutagenic metabolites of BP towards strain TA1538 of Salmonella typhimurium [14,20]. These effects of 3MC have been related to the appearance of cytochrome P_{448} [4,20]. By analy-

Table 2 Patterns of inhibition of rat liver microsomal BP hydroxylase by various chemicals.

PRETREATMENT	CONTROL	3MC	PB	2FAA
SKF525A Conc. $.10^{-6}$M Type of inhibit. K_i . 10^{-6}M	0.05 to 1 Competitive 0.17 ± 0.02	No inhibition	0.05 to 0.3 Competitive 0.35 ± 0.05	No inhibition
7,8 Benzoflavone Conc. $.10^{-6}$M Type of inhibit. K_i . 10^{-6}M	No inhibition	4.0 to 8.0 Competitive 3.37 ± 0.40	No inhibition	No inhibition
Miconazole ®  Conc. $.10^{-6}$M Type of inhibit. K_i . 10^{-6}M	0.2 to 3 Non competitive 2.32 ± 0.45	1.5 to 3 Competitive 0.59 ± 0.10	0.15 to 0.3 Non competitive 1.10 ± 0.12	0.1 to 0.4 Competitive 0.18 ± 0.02

The K_i are computerized value given ± S.D.
The type of inhibition is the statistically most probable model.

zing the rat liver microsomal BP hydroxylase activity in strictly defined Michaelis-Menten conditions, we have confirmed that the effect of 3MC is not only an increased V_M (induction) but also a decreased K_M (modification). We have suggested that both the enzyme induction (increased activity) and the enzyme modification (increased affinity) could be essential in interpreting the effect of inducers like 3MC[3]. The present report confirms the specificity of the effects of 3MC when compared to PB [4,20], it demonstrates that pretreatment by 2FAA does not induce or modify rat liver microsomal BP hydroxylase and consequently does not increase the S-9 mediated mutagenicity of BP.

The dose-response curve of fig.1 indicates that the 3MC-induced enzyme-mediated mutagenicity is highest at the lowest concentration of BP. These results emphasize the importance of both enzyme induction and enzyme modification in explaining the effect of 3MC since, at low concentration of the substrate, an increase in the affinity is probably more important than an increase in V_M in explaining an increased formation of the product.

3MC-induced microsomal BP hydroxylase is selectively and competitively inhibited by 7,8 BF [7-11]. This compound also selectively inhibits the 3MC-induced S-9 mediated mutagenicity of BP. SKF525A both competitively inhibits the BP hydroxylase activity and reduces the mutagenicity mediated by enzymatic preparation from control or PB treated rats without interfering with the activity of the other preparations (3MC, 2 or 4FAA). The inhibitory effect of 1-alkylimidazoles on the microsomal oxidation *in vitro* and *in vivo* has been reported [21]; MN^R, such a derivative, acts as an inhibitor of all the tested microsomal preparations; it also reduces the ability of all the tested S-9 mix to catalyse the formation of mutagenic metabolites of BP.

It must be pointed out, however, that the effect of MN^R on control and PB microsomes is of the non-competitive type while it is competitive on both 3MC and 2FAA treated enzymes.

Rat liver microsomal BP hydroxylase induction and modification correlate with the S-9 mediated mutagenicity of BP towards TA1538 of *S.typhimurium*. The production of a derivative catalyzed by BP hydroxylase activity measured in the defined experimental conditions might thus be an early step in the metabolic activation of BP.

Microsomal BP hydroxylase from 3MC treated rat is a different enzyme (as compared to control animals) : its activity and its affinity are increased, its sensitivity to various inhibitors is markedly modified. 2FAA, an inducer and modifier of microsomal arylamine and arylamide N-hydroxylase [12], does not induce or modify BP hydroxylase ; it however changes its sensitivity to both SKF525A (no inhibitory effect) and MN ^{R} (from non competitive to competitive inhibition). Since it has been reported [22] that 2FAA pretreatment does not cause the appearance of a modified cytochrome P_{450}, these results suggest that other mechanisms such as for example a change in the cytochrome-phospholipid interaction within the endoplasmic reticulum membranes, [5] could be important in modulating the catalytic properties of the various P_{450} dependent microsomal mixed function oxydases.

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