

ORIGINAL ARTICLE

Effect of Methomyl on the Phenobarbital and Benzo [a] Pyrene Induced Hepatic Microsomal Mixed Function Oxidase System in Rats

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Abstract: Methomyl (Lannate) is a pesticide widely used to control of insects in grape gardens. Methomyl treatment induces significant alteration in mixed function oxidase system. The present work was designed to study the inhibitory effect of methomyl on different forms of cytochrome P₄₅₀ induced by phenobarbital (CYP 2B1, 2B2 and 3A) and benzo[a]pyrene induced (CYP 1A1). Adult male rats were divided into 8 groups of 6 animals each. Microsomes were isolated by calcium precipitation. The levels of electron transport components, CYP₄₅₀, cytochrome b₅, and cytochrome c-reductase were determined using extinction coefficients. Activities of drug-metabolizing enzymes were assayed. Inducers like phenobarbital, benzo[a]pyrene, showed significant induction of mixed function oxidase in rat. The methomyl treatment (4mg/kg) of inducer-(Phenobarbital, benzop [a] pyrene) pretreated rat caused a significant decrease in electron transport components and activities of drug-metabolizing enzymes when compared with treatment of inducer alone. Induction of mixed function oxidase enzymes due to phenobarbital was also altered by the pretreatment of methomyl. Benzo[a]pyrene treatment of methomyl pretreated rats showed significant decreased levels of electron transport components and drug metabolizing enzymes as compared to benzo[a]pyrene treatment alone. These results indicate that the susceptibility of phenobarbital and benzo[a]pyrene induced cytochrome P₄₅₀ isoform (CYP 2B1, 2B2, 3A; and CYP 1A1) to methomyl and also affected in the induction pattern of some of the inducers with respect to CYP₄₅₀ isoforms.

Keywords: Methomyl, cytochrome P₄₅₀, mixed function oxidase, phenobarbital, Benzo[a] pyrene.

Introduction

Microsomal cytochrome P₄₅₀ consists of a multigene family that plays an important role in the metabolism of a wide variety of endogenous compounds and xenobiotics including drugs, carcinogens, toxic chemicals, steroids and fatty acids [1]. The liver microsomal cytochrome P₄₅₀ isoenzymes exhibit broad, often overlapping specificities towards their substrates and are differentially regulated by several factors including sex, age and administration of drugs [2-6]. Various forms of cytochrome P₄₅₀ catalyze the metabolism of a particular substrate at significantly different rates. The available evidence indicates that the presence of different CYP₄₅₀

isozymes in tissues from various sources is primarily responsible for the observed differences in metabolism. The difference in substrate specificity, regiospecificity and stereospecificity of various cytochrome P₄₅₀ isozymes plays an important role in the fate of metabolism of the drug. Thus the relative proportions of various forms of cytochrome P₄₅₀ in a given tissue or individual is an important factor in determining the cytotoxic and carcinogenic action of many toxic compounds [7].

Methomyl is a pesticide widely used to control of insects. It is direct inhibitor of acetylcholine esterase in both insects and mammals. The Inhibition is depend on dose and duration of methomyl treatment. Metabolic pathway for methomyl in the rat includes the displacement of the S-methyl by glutathione and enzymatic transformation to give the mercapturic acid derivative. We have previously observed that methomyl (4 mg/kg, i.p., for 3 days) treatment induces significant decrease in the levels of total cytochrome P₄₅₀ and aminopyrine N-demethylase activity in rats [8]. However, the effect of methomyl on various forms of CYP₄₅₀ is still unknown, Hence the present work is designed to study the inhibitory effect of methomyl on different forms of cytochrome P₄₅₀ (phenobarbital inducible CYP 2B, 3A & benzo[a]pyrene inducible CYP 1A1) [9].

Materials and Methods

2.1 *Animals*: Adult (200-230 g) male Wistar rats were obtained from Haffkine Institute, Mumbai, India. The animals were housed in plastic cages and were given an appropriate standard laboratory diet (Hindustan Lever Ltd, Mumbai) and tap water *ad libitum*.

2.2 *Chemicals*: Reduced nicotinamide adenine dinucleotide phosphate (NADPH), oxidized nicotinamide adenine dinucleotide phosphate (NADP), cytochrome c, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, aminopyrine, aniline hydrochloride, HEPES and benzo[a]pyrene were obtained from Sigma Chemical Co. (St. Louis, MO). Phenobarbital was obtained from John Baker (Colorado Springs, CO), sucrose, phenol, trichloroacetic acid, sodium chloride, potassium chloride, calcium chloride and other chemicals were of analytical grade obtained from Qualigens Chemical (Mumbai).

2.3 *Treatment*: Adult male rats were divided into 8 groups of 6 animals each. Group 1 was injected with 0.9% saline and served as control. Group 2 was treated with methomyl (4 mg/kg in saline, i.p., 3 day). Group 3 was injected with phenobarbital (80 mg/kg in saline, i.p., 3 days). Group 4 received phenobarbital (80 mg/kg in saline, i.p., 3 days) prior to methomyl treatment (4 mg/kg in saline, i.p., 3 days). Group 5 received methomyl (4 mg/kg in saline, i.p. 3 days) prior to phenobarbital treatment (80 mg/kg in saline, i.p., 3 days). Group 6 was treated with benzo[a]pyrene (20 mg/kg in oil, i.p., 2 days). Group 7 was injected with methomyl (4 mg/kg in saline, i.p., 3 days) after benzo[a]pyrene treatment (20 mg/kg in oil, i.p., 2 days). Group 8 was received methomyl (4 mg/kg in saline, i.p., 3 days) prior to benzo[a]pyrene (20 mg/kg in oil, i.p., 2 days). The animals were injected between 8.00 am and 9.00 am. The volume injected into rats of body weight 200 g was 1 ml.

2.4 Preparation of microsomes: The rats used in this study were killed by cervical dislocation 24 h after the last treatment. Livers were perfused *in situ* with ice cold 1.15% KCl solution containing 0.05 mM EDTA, rapidly excised, blotted dry, weighed, minced and homogenized with 2 volumes of ice-cold 0.25 M sucrose solution, in a Potter–Elvehjem type homogenizer. The homogenate was centrifuged at 10,000 x g for 30 min in a refrigerated REMI C-24 centrifuge. Microsomes were isolated by the procedure of Cinti et al. (1972) [10]. The microsomal pellet was washed with 1.15% KCL solution containing 0.05 mM EDTA, resuspended in phosphate buffer (0.1 M, pH 7.4) and the suspension was used for the microsomal enzyme assays. Microsomal protein was estimated by the biuret method using bovine serum albumin as a standard [11].

2.5 Enzyme assays: The levels of microsomal electron transport components, cytochrome P₄₅₀ and cytochrome b₅ were determined using Hitachi UV-visible recording spectrophotometer by the procedure of Omura and Sato (1964) [12]. Cytochrome c reductase activity was determined by the method of Masters et al. (1967) [13]. Aminopyrine N–demethylase activity was assayed according to the procedure of Schenkman et al. (1967) [14]. Formaldehyde liberated during N-demethylation was estimated by the procedure of Nash (1953) [15]. Aniline hydroxylase assay was performed using the procedure reported by Govindwar and Dalvi (1990) [16].

2.6 Analysis of data: Statistical analysis was done by ANOVA and Tuckey- Kramer post test using Graphpad software. The level of significance was set at 0.05.

Results and Discussion

Administration of methomyl showed significant decrease in the electron transport components and drug metabolizing enzymes as we observed in earlier report [8]. The objective was to study the effect of methomyl on the phenobarbital induced CYP 2B, 3A and benzopyren induced Cyt 2A1 [17-18]. Phenobarbital treatment resulted in the significant increase of microsomal protein (50%), electron transport components such as cytochrome b₅ (115%), cytochrome P₄₅₀ (366%), cytochrome c reductase (138%) and drug metabolizing enzyme activities i.e. aminopyrine N-demethylase (40%) and aniline hydroxylase (56%) as compared to control rats (Table & Fig. No.1). The protein levels of some cytochrome P₄₅₀ increase modestly following phenobarbital treatment (e.g. 2-4 fold increase in CYP 2C6), while others are increased dramatically in response to drug exposure (up to 50-100 fold for greater increase of CYP 2B1). CYP 2B3 is not induced by phenobarbital whereas CYP 2B1 and 2B2 are phenobarbital inducible. Our results of induction of microsomal mixed function oxidase system are very similar to those obtained earlier [19-20].

Methomyl treatment of phenobarbital pretreated rats caused significant decrease in the microsomal protein (13%), cytochrome P₄₅₀ (53.81%), cytochrome c reductase (49.91%), cytochrome b₅ (49.53%), aminopyrine N-demethylase (11%) and aniline hydroxylase activity (35%) as compared to phenobarbital treatment alone (Table & Fig. No.1).

Table 1: Alteration in the rat liver microsomal protein, electron transport components and drug metabolizing enzymes due to methomyl (4 mg/kg, i.p., 3 days), phenobarbital (80 mg/kg, i.p., 3 days) treatment

Groups	MP ^a	Cyt. b ₅ ^b	Cyt. P ₄₅₀ ^b	Cyt. c red ^c	AND ^d	AH ^e
Control	14.7±0.13	0.25±0.008	0.35±0.015	34.2±0.14	5.58±0.22	0.86±0.02
Met	16.4±1.05	0.16±0.018	0.20±0.016	27.4±2.20	3.36±0.11*	0.62±0.02 ***
Pb	21.9±1.7**	0.53±0.007 ***	1.65±0.18 ***	81.6±4.20 ***	7.82±1.07*	1.34±0.01 ***
Pb+Met	18.9±1.88	0.27±0.011 [§]	0.76±0.025 ** [§]	47.4±2.10* [§]	6.95±0.67	0.87±0.02 [§]
Met+Pb	17.4±1.47	0.32±0.024 [§]	0.73±0.013* [§]	65.6±3.40 *** [§]	4.71±0.18 [§]	1.1±0.02 [§] ***

Values are means of three experiments ± SEM; six animals in each group.

Met – Methomyl, **Pb** – Phenobarbital, **Pb+Met** – Phenobarbital + Methomyl,

Met+Pb –Methomyl+Phenobarbital **MP** – Microsomal Proteins, **Cyt. b₅** – Cytochrome b₅, **Cyt. P₄₅₀** – Cytochrome P₄₅₀, **Cyt. c red** – Cytochrome c reductase, **AND** – Aminopyrine N-demethylase, **AH** – Aniline hydroxylase

^a mg of protein/g liver, ^b nmole/mg of microsomal protein,

^c nmole cytochrome c reduced/min/mg of microsomal protein.

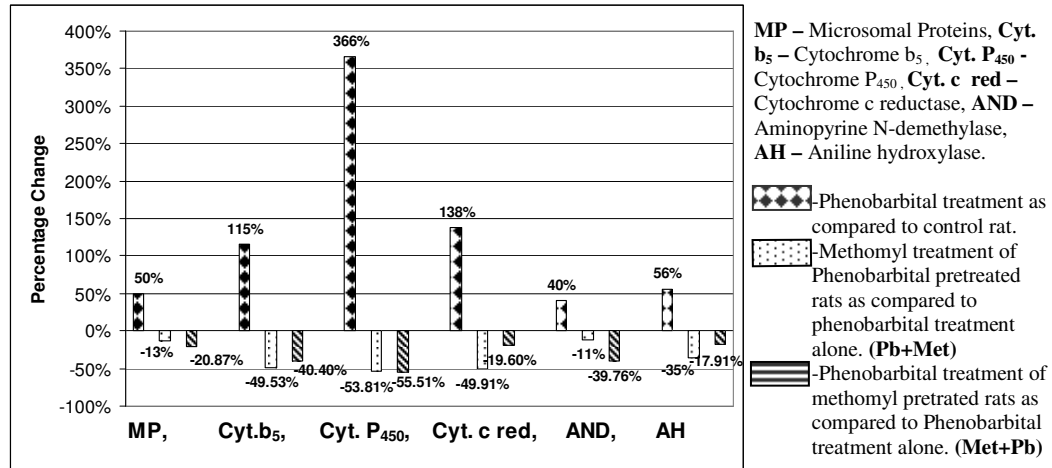
^d nmole formaldehyde liberated/min/mg of microsomal protein.

^e nmole *p*-aminophenol formed/min/mg of microsomal protein.

Significantly different from control value at * P < 0.05, ** P < 0.01,

*** P < 0.001. Significantly different from phenobarbital [§] P < 0.001,

Fig. 1: Percentage change of rat liver microsomal proteins, cytochrome b₅, cytochrome P₄₅₀, cytochrome c reductase, aminopyrine N-demethylase and aniline hydroxylase due to methomyl (4 mg/kg, i.p., 3 days), phenobarbital (80 mg/kg, i.p., 3 days) treatment



Phenobarbital treatment of methomyl pretreated rats caused decrease in the microsomal protein (20.87%), cytochrome P₄₅₀ (55.51%), cytochrome c reductase (19.60%), cytochrome b₅ (40.40%), aminopyrine N-demethylase (39.76%) and aniline hydroxylase activity (17.91%) as compared to phenobarbital treatment alone (Table & Fig. No.1). These results indicate that the CYP 2B1, 2B2 and 3A are susceptible to methomyl or its metabolite. Decreased levels of cytochrome P₄₅₀ may be due to inhibition of protein biosynthesis. OP and carbamates compounds have shown to inhibit the activity and/or alter the expression of various cytochrome isoforms [21].

Benzo[a]pyrene treatment caused significant increase of cytochrome P450 (55.36%), cytochrome c reductase (44.12%), cytochrome b₅ (144%), and aniline hydroxylase activity (59.30%) and decreased microsomal protein (17.82%) and aminopyrine N-demethylase (6.27%) as compared to control group (Table & Fig. No.2). The benzo[a]pyrene treatment results an increase in the levels of cytochrome P₄₅₀ and aminopyrine N-demethylase activity reported in earlier literature [20]. Our results are also consistent to earlier reports. Benzo[a]pyrene specifically induces CYP 1A1, which represents an increase in aryl hydrocarbon hydroxylase activity. Methomyl treatment of benzo[a]pyrene pretreated rats caused significant decrease in the cytochrome P₄₅₀ (21.18%), cytochrome c reductase (48.91%), cytochrome b₅ (35.72%), aminopyrine N-demethylase (52%) and aniline hydroxylase activity (20.43%) as compared to benzo[a]pyrene treatment alone (Table & Fig. No.2). These results indicate the susceptibility of CYP 1A1 towards methomyl or its metabolites. Benzo[a]pyrene treatment of methomyl-pretreated rats caused significant decrease in microsomal protein (10.18%), cytochrome P450 (16.15%), cytochrome c reductase (37.06%), cytochrome b₅ (19.90%), aminopyrine N-demethylase (26.57%) and aniline hydroxylase activity (35%) as compared to benzo[a]pyrene treatment alone (Table & Fig. No.2). These results indicate alteration in the induction pattern of benzo[a]pyrene.

Table 2: Alteration in the rat liver microsomal protein, electron transport components and drug metabolizing enzymes due to methomyl (4 mg/kg, i. p., 3 days), benzo[a]pyrene (20 mg/kg, i. p., 2 days) treatment.

Groups	MP ^a	Cyt. b ₅ ^b	Cyt. P ₄₅₀ ^b	Cyt. c red ^c	AND ^d	AH ^e
Control	14.7±0.13	0.25±0.008	0.35±0.015	34.22±0.14	5.5±0.22	0.86±0.027
Met	16.4±1.05	0.16±0.018	0.20±0.016	27.48±2.20	3.3±0.11*	0.62±0.022 ***
BP	12.0±0.49	0.61±0.008 ***	0.55±0.066	49.32±0.99	5.2±0.10	1.37±0.15 ***
BP+Met	11.4±0.20	0.39±0.05 *** ##	0.43±0.039	25.40±1.28 [#]	2.5±0.04 [#] ***	1.09±0.07 ^{##} ***
Met+BP	10.8±0.47	0.49±0.005 *** #	0.46±0.007	31.04±2.93	3.8±0.19	0.89±0.01 [#] #

Values are means of three experiments ± SEM; six animals in each group.

Met – Methomyl, **BP** - Benzo[a]pyrene, **BP + Met** - Benzo[a]pyrene + Methomyl, **Met + BP** – Methomyl + Benzo[a]pyrene, **MP** – Microsomal Proteins, **Cyt. b₅** – Cytochrome b₅, **Cyt. P₄₅₀** - Cytochrome P₄₅₀,

Cyt. c red – Cytochrome c reductase, **AND** – Aminopyrine N-demethylase, **AH** - Aniline hydroxylase

^a mg of protein/g liver, ^b nmole/mg of microsomal protein,

^c nmole cytochrome c reduced/min/mg of microsomal protein.

^d nmole formaldehyde liberated/min/mg of microsomal protein.

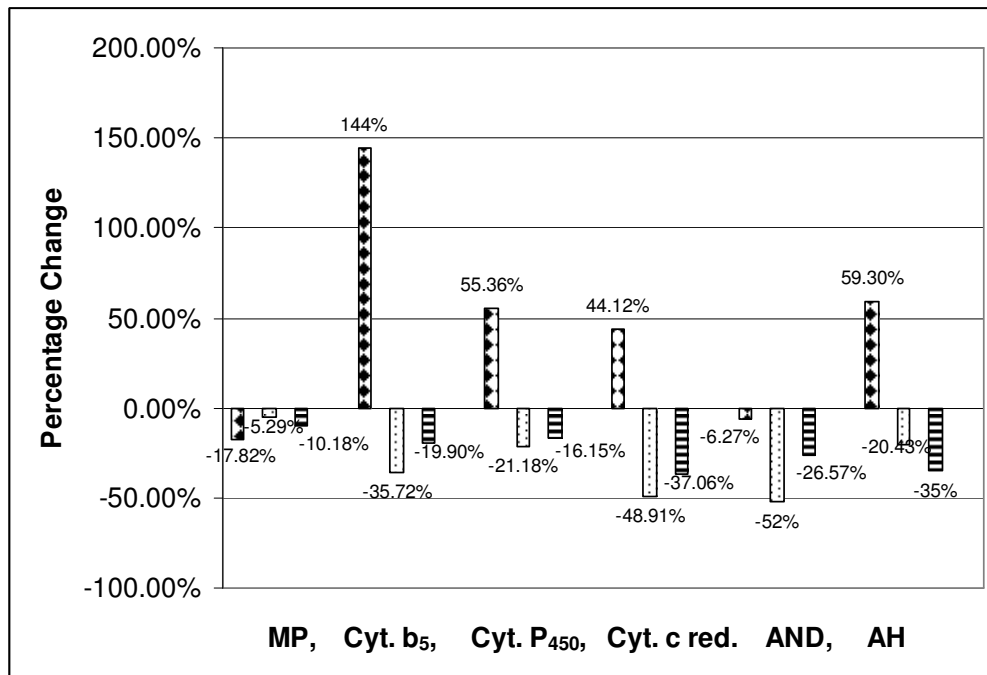
^e nmole *p*-aminophenol formed/min/mg of microsomal protein.

Significantly different from control value at * P < 0.05, ** P < 0.01,

*** P < 0.001. Significantly different from benzo[a]pyrene # P < 0.01,

P < 0.001

Fig. 2: Percentage change of rat liver microsomal proteins, cytochrome b₅, cytochrome P₄₅₀, cytochrome c reductase, aminopyrine N-demethylase and aniline hydroxylase due to methomyl (4 mg/kg, i.p., 3 days), benzo[a]pyrene (20 mg/kg, i. p., 2 days) treatment.



MP – Microsomal Proteins, **Cyt. b₅** – Cytochrome b₅, **Cyt. P₄₅₀** - Cytochrome P₄₅₀,

Cyt. c red – Cytochrome c reductase, **AND** – Aminopyrine N-demethylase,

AH – Aniline hydroxylase.



- Benzo[a]pyrene treatment as compared to control rat.



- Methomyl treatment of Benzo[a]pyrene pretreated rats as compared to Benzo[a]pyrene treatment alone. (**BP + Met**)



- Benzo[a]pyrene treatment of methomyl pretreated rats as compared to Benzo[a]pyrene treatment alone. (**Met + BP**)

In conclusion, this investigation indicates that inducers like, phenobarbital, benzo(a)pyrene, of mixed function oxidase in rodents show induction. Significant decrease in electron transport components and levels of drug-metabolizing enzymes due to methomyl pre and post-treatment indicates the susceptibility of CYP 2B1, CYP 2B2, 3A; CYP 1A1 to methomyl or its metabolites. In addition, decrease in the activity of cytochrome P450 and drug-metabolizing enzymes in this study may be because of inhibition of heme synthesis. Methomyl or its metabolite may inhibit the rate limiting enzymes of heme biosynthesis and decreased heme pool, which may results the decreased activity of cytochrome P₄₅₀ activity.

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