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EVIDENCE FOR METABOLISM OF 3-METHYLINDOLE BY PROSTAGLANDIN H SYNTHASE AND MIXED-FUNCTION OXIDASES IN GOAT LUNG AND LIVER MICROSOMES

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Abstract—3-Methylindole (3MI) is the causative agent of a naturally occurring lung disease in cattle. The effects of 3MI on the prostaglandin H synthase (PHS) and mixed-function oxidase (MFO) systems in lung and liver microsomes were investigated. Addition of 3MI in goat lung microsomes resulted in a pronounced increase in PHS activity as indicated by both the initial rate and total oxygen consumption. The effect of 3MI on PHS activity was dependent on arachidonic acid and inhibited by indomethacin. PHS was capable of activating [^{14}C]3MI to a reactive intermediate as indicated by the covalent binding of 3MI to microsomal protein. [^{14}C]3MI was converted to water-soluble 3MI metabolites in the PHS system. Biosynthesis of total prostaglandins was enhanced 69% with 0.5 mM 3MI. PHS-catalyzed co-oxidation of 3MI was shown to be independent of the MFO-catalyzed metabolism of 3MI. PHS and MFO enzyme activities were found to exist in both goat lung and liver microsomes. PHS activity was significantly higher in lung microsomes than in liver microsomes. Pulmonary MFO activity, unlike hepatic MFO activity, was enhanced with addition of 3MI. This indicates a tissue selectivity for the metabolism of 3MI. Lung microsomal PHS and MFO systems were capable of activating 3MI to a greater extent than liver microsomes. Thus, the combined effects of PHS and MFO systems in the activation of 3MI, the selectivity of pulmonary MFO to metabolize 3MI, and the capacity for altered prostaglandin biosynthesis in lung tissue may explain the tissue specificity of 3MI-induced toxicity.

Prostaglandin H synthase (PHS), a microsomal enzyme complex containing both cyclooxygenase and hydroperoxidase activities, catalyzes the insertion of two molecules of oxygen into the arachidonic acid (AA) forming prostaglandin G_2 (PGG_2) which is reduced subsequently to PGH_2. Many xenobiotics can serve as reducing co-substrates for the hydroperoxidases of the enzyme complex and thus potentiate the biosynthesis of prostaglandins [1]. PHS-catalyzed metabolism of the reducing co-substrate has been implicated as a mechanism by which a wide variety of inert toxins and carcinogens, such as acetaminophen, phenetidine and benzidine, are metabolically activated to form ultimate toxic or carcinogenic metabolites [2–4]. Microsomal NADPH-dependent cytochrome P-450 mixed-function oxidase (MFO) catalyzes the metabolism of many drugs and toxins using NADPH and molecular oxygen as cofactors. MFO-catalyzed oxidation has also been implicated as a mechanism for metabolic activation of xenobiotics. In many cases, the arachidonic acid-dependent PHS system has been shown to complement the NADPH-dependent MFO in the initiation of cytotoxicity [2, 5, 6]. PHS-catalyzed metabolic activation would be particularly important in tissues such as the kidney, colon, bladder, and lung [7] which contain low cytochrome P-450 activity and high PHS activity.

3-Methylindole (3MI) is a naturally occurring lung-specific toxin resulting from the ruminal fermentation of tryptophan. Administration of 3MI induces acute pulmonary edema in cattle [8], sheep [9], and goats [10, 11]. 3MI is metabolized by the NADPH-dependent MFO system and excreted in the urine as oxidation products including derivatives of 3-methyloxindole and indole-3-carbinol [12]. The MFO system is known to metabolically activate 3MI to a highly reactive intermediate which is capable of binding to macromolecules in both *in vitro* [13, 14] and *in vivo* [15, 16] systems. This binding has been shown to be organ specific with greater binding in pulmonary tissue than in hepatic tissue [14, 17].

It has been shown that in goat lung both the NADPH-dependent microsomal MFO activities and the quantity of the enzyme are considerably lower than in liver [14], yet the 3MI-induced pathology is confined to the lung. Since various lung cells, such as Clara [6] and alveolar Type II cells [18], contain highly enriched fractions of PHS, it would seem possible that this enzyme system plays a role in 3MI-induced pneumotoxicity. Therefore, the objectives of this study were to determine whether 3MI can be co-oxidized by the arachidonic acid-dependent PHS system in lung tissue and to compare the arachidonic acid-dependent and the NADPH-dependent microsomal metabolism of 3MI in goat lung and liver.

MATERIALS AND METHODS

Chemicals and radiochemicals. [*Methyl*-^{14}C]3-Methylindole (5.17 mCi/mmol, >99% pure) and [^{14}C-U]arachidonic acid (390 mCi/mmol, >99% pure) were purchased from New England Nuclear, Boston, MA.

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Animals. Six healthy male goats weighing 20–30 kg were obtained from local farms and fed a standard diet of mixed hay with pelleted supplement (16% protein) for 2 weeks. The animals were killed by captive bolt gun, and the lungs and livers were removed and stored at −80° until used.

Preparation of microsomes. Microsomal fractions of lung and liver were isolated at 4° by differential centrifugation [19]. Tissue (16 g) was homogenized in 0.01 M sodium phosphate buffer with 0.15 M KCl (microsomal buffer), pH 7.4, and centrifuged at 10,000 *g* for 20 min. The supernatant fraction was centrifuged at 100,000 *g* for 60 min, and the resultant pellet was resuspended in buffer. Microsomal protein was analyzed by the method of Lowry *et al.* [20].

Oxygen consumption studies. Arachidonic acid-dependent and NADPH-dependent oxygen consumption was monitored in a thermostatic chamber equipped with a Clark-type electrode (Yellow Springs Instrument Oxygen Monitor model 53). The oxygen electrode has been used frequently as a tool for monitoring the metabolism of xenobiotics *in vitro* for both PHS [21–23] and MFO [24–26] systems. The incubation mixture contained approximately 9 mg of lung or 27 mg of liver microsomal protein, 0.5 mM 3MI and microsomal buffer to a volume of 2.5 ml. The reaction was preincubated at 37° for 1 min and initiated by addition of 0.3 mM NADPH or arachidonic acid (200 and 400 μM in lung and liver incubations respectively). Various metabolic blanks that contained solvent alone, heat-inactivated enzyme, or indomethacin (100 μM) or piperonyl butoxide (7.5 mM) treated microsomes were carried out as above. Initial oxygen consumption rates were determined from a tangent to the initial portion of the oxygen uptake curve. Calculations were based upon an initial dissolved oxygen content in Guelph atmospheric conditions of 0.463 μmol in the incubation mixture [27]. All indolic compounds, indomethacin, piperonyl butoxide, and arachidonic acid were dissolved in ethanol (<2% of the final incubation volume).

Assay for biosynthesis of prostaglandins. Each reaction mixture was monitored for total oxygen consumption and for the synthesis of prostaglandins from arachidonic acid. The incubation medium contained 9.6 mg of lung microsomal protein and various concentrations of 3MI (0, 0.05 mM, and 0.5 mM) in a total volume of 2.5 ml of microsomal buffer. The total oxygen consumption was monitored for 5 min after initiation of the reaction with 200 μM [^{14}C-U]arachidonic acid (sp. act. 0.085 mCi/mmol). The reaction was incubated at 37° with vigorous stirring. The sample was acidified to pH 3 with 2 N formic acid and extracted with 2 vol. of ethyl acetate. The organic phase was evaporated to dryness under N_2. Reference standards containing 15 μg of the following prostaglandins were added: PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and TxB_2. Residues were dissolved in ethyl acetate and spotted quantitatively on silica gel plates. The plates were developed in ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (11:5:2:10, by vol.) as described by Murota *et al.* [28]. To visualize the spots, the plates were sprayed with a 1% solution of vanillin dissolved in a 1:1 mixture of 95% ethanol and 85% phosphoric acid and heated for 10 min at 110° [29]. The plates were scanned for radioactivity on a thin-layer scanner. The spots indicative of the arachidonic acid and reference prostaglandins were scraped into vials and counted in Scinti Verse scintillation mixture. Treatments were standardized to contain an equal amount of radioactivity (dpm) on each lane for comparison purposes. The amount of each prostaglandin formed was calculated from the specific activity of the radioactive arachidonic acid.

Comparison of PHS and MFO metabolism of [^{14}C]3*MI.* Oxidative metabolism of [^{14}C]3MI catalyzed by the arachidonic acid-dependent PHS or NADPH-dependent MFO systems was determined by comparing the extent of partitioning of the metabolites to the aqueous phase after ethyl acetate extraction. Increased oxidative metabolism of 3MI should increase the water-soluble metabolites. Reaction mixtures contained 4.3 mg of goat lung microsomal protein, and 0.10 mM 3MI (sp. act. 3.5 mCi/mmol) in a total volume of 1.25 ml of microsomal buffer. In some reactions, indomethacin (100 μM) or piperonyl butoxide (7.5 mM) was added to inhibit the metabolism. Reactions were initiated with either arachidonic acid (200 μM), NADPH (0.3 mM) or ethanol as blank and incubated for 5 min at 37° in a shaking water bath. The reaction was terminated by the addition of 66 μl of 100% trichloroacetic acid (TCA) to precipitate the protein. The 3MI parent compound and other lipophilic metabolites were extracted by addition of 1.5 ml of ethyl acetate. The ethyl acetate phase and the aqueous phase were separated by centrifugation at 1200 *g* for 10 min. Aliquots were taken from both phases and counted for radioactivity by liquid scintillation.

Covalent binding study. Both PHS- and MFO-catalyzed covalent binding of [^{14}C]3MI to TCA-precipitable macromolecules were determined with modifications of the method of Rapp *et al.* [30]. The reaction mixtures contained 9 and 27 mg of lung and liver microsomal protein, respectively, [^{14}C]3MI (0.5 mM, sp. act. 0.35 mCi/mmol) and microsomal buffer to a final volume of 2.5 ml. In some incubates, indomethacin (100 μM) or piperonyl butoxide (7.5 mM) was added prior to the microsomes. All incubations were initiated by the addition of arachidonic acid (200 μM in lung and 400 μM in liver) or NADPH (0.3 mM) and incubated at 37° in a shaking water bath. Ethanol was added in place of arachidonic acid or NADPH to determine the non-specific binding of 3MI to protein. Reactions were terminated after 15 min of incubation (except during the time study) by addition of 132 μl of 100% TCA for a final concentration of 5% TCA to precipitate the protein. Samples were vortexed and centrifuged at 1200 *g* for 10 min, and the supernatant fraction was discarded. The precipitates were re-extracted five times with 2.5 ml of 5% TCA. The resulting protein pellet was solubilized in 1.5 ml of 1 N NaOH at 60° for 60 min. The soluble protein was exhaustively extracted by addition of 2.5 ml ethyl acetate to remove all extractable radioactivity (four to six washes). Aliquots were taken for determination of the protein bound radioactivity and for protein quantitation [20]. Radioactivity representing the non-specific binding was subtracted from all reported data.

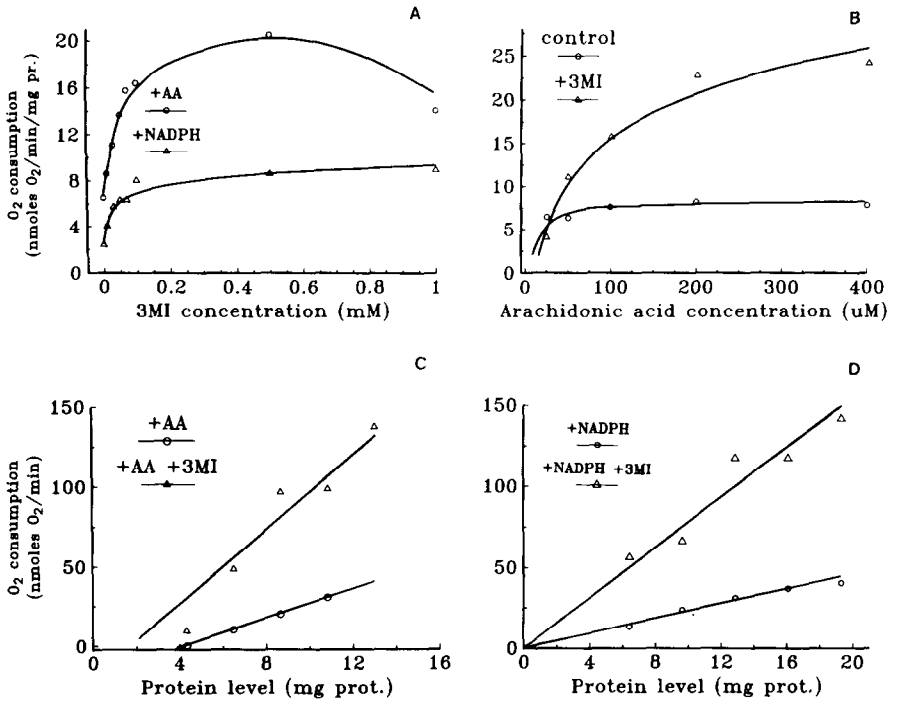

Fig. 1. Characterization of arachidonic acid-dependent and NADPH-dependent oxygen consumption in goat lung microsomes. Microsomes were suspended in 2.5 ml of microsomal buffer in the presence or absence of 3MI. Components were added in the following amounts unless stated otherwise: 9 mg microsomal lung protein, 0.5 mM 3MI, 200 μM arachidonic acid and 0.3 mM NADPH. Incubations were performed at 37° in a cell equipped with a Clark-type electrode. Reactions were initiated by arachidonic acid or NADPH. The initial rate of oxygen consumption was monitored at: (A) various concentrations of 3MI; (B) various concentrations of arachidonic acid; and at various protein levels using (C) arachidonic acid or (D) NADPH as substrate. Results are expressed as the mean of three replicates obtained from different animals.

RESULTS

A series of experiments was carried out to establish if 3MI is a reducing co-substrate for PHS-catalyzed and a substrate for MFO-catalyzed enzymatic reactions (Fig. 1). Figure 1A illustrates that very low concentrations of 3MI (0.01 mM) caused a 2.5- to 3.0-fold increase in both arachidonic acid-dependent and NADPH-dependent oxygen consumption in goat lung microsomes. Maximal oxygen consumption was reached with addition of higher concentrations of 3MI (0.5 mM) in both systems. The rate of oxygen consumption was greater in the arachidonic acid-dependent system than in the NADPH-dependent microsomal system. The arachidonic acid requirement for maximal rate of oxygen consumption was increased with addition of 3MI (Fig. 1B), indicating that 3MI was a reducing co-substrate of PHS system. The oxygen consumption curve was linear up to 20 mg of lung microsomal protein in the presence or absence of 3MI in both PHS (Fig. 1C) and MFO systems (Fig. 1D). The oxygen consumption curve was also linear up to 38 mg of liver microsomal protein in the presence or absence of 3MI in both PHS and MFO systems (data not shown).

Figure 2 summarizes a series of experiments that demonstrate that the PHS-catalyzed metabolism of 3MI was independent of the MFO-catalyzed metabolism of 3MI in lung microsomes. Addition of 3MI significantly ($P < 0.05$) enhanced the rate of oxygen consumption in both the arachidonic acid-dependent and NADPH-dependent systems as shown in the left and center bars of Fig. 2A. With simultaneous addition of arachidonic acid and NADPH into the incubation, the rate of oxygen consumption was equal to the sum of the rates for each system, indicating two independent enzyme systems for the metabolism of 3MI (shown in the right bars of Fig. 2A). Specific inhibitors for PHS and MFO enzymes were used to verify the involvement of these enzymes in the metabolism of 3MI (Fig. 2B). The metabolism of 3MI by lung microsomal PHS was inhibited completely by the cyclooxygenase inhibitor, indomethacin (ID). NADPH-dependent MFO metabolism of 3MI in the lung was also inhibited significantly ($P < 0.05$) by the cytochrome P-450 inhibitor, piperonyl butoxide (BT). There was no cross-inhibition of piperonyl butoxide on PHS activity (left bars of Fig. 2C) or indomethacin on MFO activity (right bars of Fig. 2C), suggesting again that the two enzymes operate independently in the metabolism of 3MI.

To study the tissue specificity of 3MI toxicity, the effect of 3MI on PHS and MFO-dependent oxygen

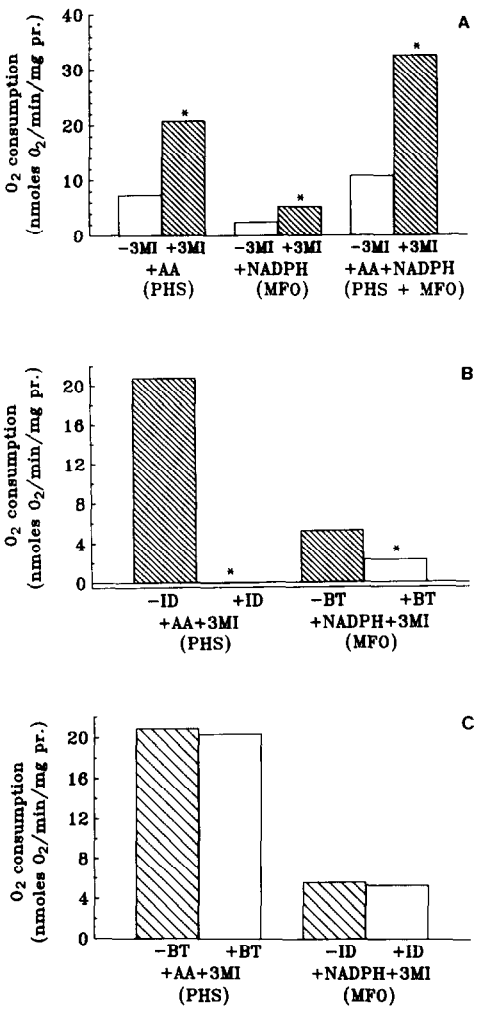

Fig. 2. Metabolism of 3MI by two independent enzyme systems in lung microsomes. Conditions were as in Fig. 1 except that indomethacin (ID, 100 μM) or piperonyl butoxide (BT, 7.5 mM) was added to some incubations. (A) Arachidonic acid-dependent and NADPH-dependent metabolism of 3MI; (B) effect of enzyme specific inhibitors; (C) cross-inhibition of enzyme inhibitors. The data were analyzed using each animal as a block. Results are expressed as the mean of six replicates obtained from different animals. Means with an asterisk are significantly different ($P < 0.05$) as determined by paired-comparison.

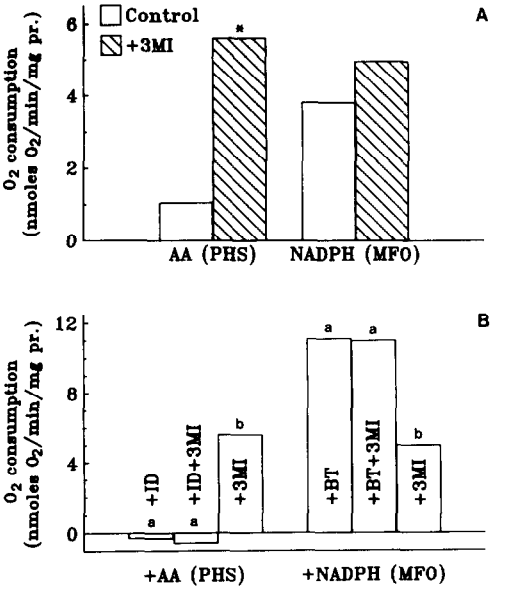

Fig. 3. Liver microsomal metabolism of 3MI. Incubations contained 27 mg of liver microsomal protein in 2.5 ml of microsomal buffer in the presence or absence of 3MI (0.5 mM), indomethacin (ID, 100 μM), or piperonyl butoxide (BT, 7.5 mM) as indicated. Reactions were initiated with either arachidonic acid (400 μM) or NADPH (0.3 mM) and monitored for the initial rate of oxygen consumption as described in Fig. 1. (A) Arachidonic acid-dependent and NADPH-dependent metabolism of 3MI; (B) effect of inhibitors on PHS and MFO metabolism. The data were analyzed using each animal as a block. Results are expressed as the mean of six replicates obtained on different animals. Means with an asterisk are significantly different ($P < 0.05$) from controls as determined by paired-comparison. Means with different letters are significantly different ($P < 0.05$) as determined by the REGWQ multiple range test [31].

consumption in liver microsomes, a nontarget tissue, was investigated, and the results are presented in Fig. 3. Arachidonic acid-dependent oxygen consumption was enhanced 5-fold in the presence of 3MI (Fig. 3A). There was no significant ($P < 0.05$) effect of 3MI on the initial rate of the NADPH-dependent oxygen consumption in liver microsomes. Addition of indomethacin in the incubation completely inhibited the effect of 3MI (compare +3MI and +ID+3MI of Fig. 3B) by total inhibition of PHS activity (+ID). Addition of piperonyl butoxide did not inhibit, but rather enhanced the rate of oxygen consumption in the presence of 3MI (compare +3MI and +BT+3MI of Fig. 3B). Since piperonyl butoxide is a substrate for MFO system, an increase in the initial rate of oxygen consumption would be expected, as shown in the bars of Fig. 3B. It is possible that 3MI competes with piperonyl butoxide for the binding site of the same MFO isozyme in the goat liver microsomes. Thus, addition of 3MI has no further effect on oxygen consumption (compare +BT and +BT+3MI of Fig. 3B).

A comparison of the effect of 3MI on PHS and MFO enzyme activities between goat lung and liver microsomes is shown in Table 1. PHS activity was higher in the lung than in the liver in the presence or absence of 3MI. Since lung has higher PHS activity, the potential for 3MI co-oxidation is greater than in liver. The basal level of MFO activity was significantly ($P < 0.05$) lower in lung microsomes than in liver microsomes. However, addition of 3MI caused an increase in MFO activity specifically in lung microsomes (Fig. 2A and Fig. 3A), demonstrating tissue selectivity in the metabolism of 3MI.

The effect of 3MI on prostaglandin biosynthesis was assessed by measuring the conversion of [^{14}C]arachidonic acid to various prostaglandins in goat lung microsomes. 3MI significantly ($P < 0.05$) enhanced the total biosynthesis of prostaglandins

Table 1. Comparison of the arachidonic acid- and NADPH-dependent rates of oxygen consumption between goat lung and liver microsomes

Incubation mixture*	Oxygen consumption† (nmol O_2/min/mg protein)	
	Lung	Liver
+AA	7.43‡	1.06
+AA + 3MI	20.81‡	5.62
+NADPH	2.50‡	3.82
+NADPH + 3MI	5.27	4.99

* The complete system contained microsomal buffer (pH 7.4), 0.5 mM 3MI, and 8 mg or 26 mg of lung/liver microsomal protein respectively. The reaction was initiated with either arachidonic acid (AA, 200 μM in lung or 400 μM in liver) or NADPH (0.3 mM) and monitored for the initial rate of oxygen consumption.

† Results are expressed as the mean of six replicates obtained from different animals.

‡ Significantly different ($P < 0.05$) from liver as determined by paired-comparison.

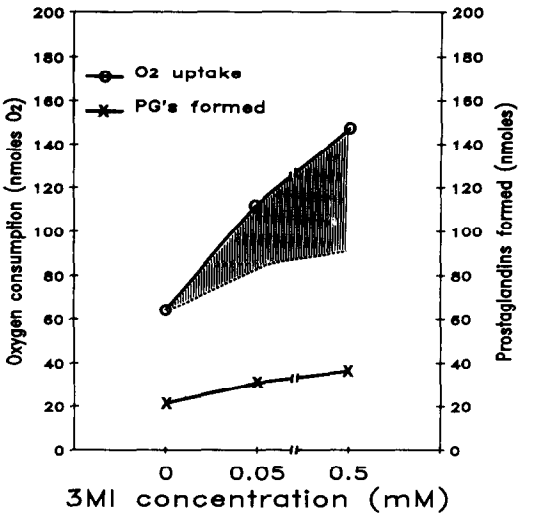

Fig. 4. Relationship of oxygen consumption and prostaglandin formation with various concentrations of 3MI by goat lung microsomal PHS. Prostaglandin biosynthesis and total oxygen consumption were monitored in lung microsomes. Results are expressed as the mean of three replicates obtained on different animals. The calculated oxygen required for the amount of prostaglandins formed is represented by the dotted line (-----). The unaccountable oxygen consumed is represented by the shaded area [/ / / / /].

from [^{14}C]arachidonic acid (Table 2). The formation of both PGI_2 and TxB_2 was induced significantly ($P < 0.05$) with the addition of 0.5 mM 3MI by 119 and 158% respectively. This increase in prostaglandins was 3MI concentration dependent. There was no effect of 3MI on the other prostaglandins assayed.

To establish if the increased oxygen consumption induced by 3MI was entirely due to an increased rate of oxygenation of arachidonic acid to prostaglandins or due, in part, to the oxygenation of 3MI, the relationship between oxygen consumption and prostaglandin formation at various concentrations of 3MI was assessed (Fig. 4). If the oxygen consumed was only responsible for the oxygenation of arachidonic acid during synthesis of prostaglandins, then the number of moles of molecular oxygen consumed should be two times the number of moles of prostaglandins formed, as indicated by the dotted line. The results show that the monitored oxygen consumption was qualitatively greater than the calculated oxygen required for prostaglandin synthesis at the various levels of 3MI (Fig. 4). It would appear that the additional oxygen consumed (shaded area) may have resulted from the oxygenation of 3MI.

Table 2. Effect of 3MI on prostaglandin biosynthesis by goat lung microsomal PHS

Prostaglandin	Amount of prostaglandin formed* (nmol/mg protein)		
	Control	+0.05 mM 3MI	+0.5 mM 3MI
PGI_2	0.41[a]	0.71[b]	0.89[c]
$PGF_{2\alpha}$	0.21[a]	0.23[a]	0.20[a]
TxB_2	0.71[a]	1.27[b]	1.83[c]
PGE_2	0.28[a]	0.39[a]	0.37[a]
PGD_2	0.58[a]	0.60[a]	0.42[a]
Total	2.19[a]	3.20[b]	3.71[c]

Prostaglandins were separated by TLC. Zones indicative of the reference prostaglandins and arachidonic acid were scraped into vials and counted for radioactivity.

* The data were analyzed using a randomized complete block design with each animal representing a block. Results are expressed as the mean of three replicates obtained from different animals. Means across each row having different superscripts are significantly different ($P < 0.05$) as determined by the REGWQ multiple range test [31]. Pooled SEM: PGI_2, 0.02; $PGF_{2\alpha}$, 0.01; TxB_2, 0.03; PGE_2, 0.03; PGD_2, 0.04; and Total, 0.03.

If 3MI is a substrate for PHS-catalyzed co-oxidation, then one should expect it to undergo oxidative metabolism. 3MI has also been shown previously to be metabolized by the MFO system to water-soluble oxidation products [17]. The formation of [^{14}C]3MI water-soluble metabolites was determined from both arachidonic acid-dependent PHS and NADPH-dependent MFO enzyme systems, and the results are presented in Fig. 5. The results indicate that 4% of the total radioactivity was remaining in the water phase as background following ethyl acetate extraction (blank). When arachidonic acid was added as substrate for PHS in the lung microsomal incubation, there was a 2-fold increase in water-soluble 3MI metabolites compared to the blank. The formation of radioactive metabolites was reduced to the background level when indomethacin was added to the incubation (+ID). NADPH-dependent metabolism of 3MI resulted in a 3-fold increase in water-soluble metabolites compared to the blank. This metabolism was inhibited by 30% when piperonyl butoxide (+BT) was added to the incubation. The results indicate that 3MI can be oxidatively metabolized in goat lung microsomes by both PHS- and MFO-catalyzed reactions.

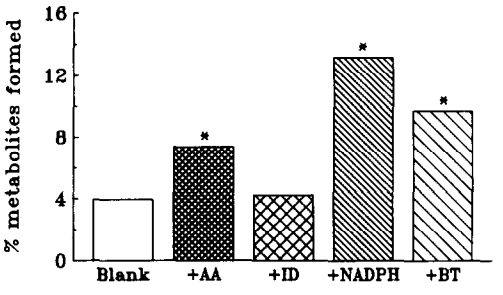

Fig. 5. Oxidative metabolism of [^{14}C]3MI to water-soluble metabolites by PHS- and MFO-dependent systems. Incubations contained microsomal buffer, 4.3 mg goat lung microsomal protein, and 0.10 mM 3MI (sp. act. 3.5 mCi/mmol) in a total volume of 1.25 ml. In some reactions, indomethacin (ID, 100 μM) or piperonyl butoxide (BT, 7.5 mM) was added prior to the microsomes. Reactions were initiated with one of the following: arachidonic acid (200 μM), NADPH (0.3 mM) or ethanol as a blank. 3MI parent compound and lipophilic metabolites were removed from the reaction mixture by ethyl acetate extraction. Oxidative metabolism of [^{14}C]3MI to water-soluble metabolites was measured as the percent of dpm remaining in the aqueous phase compared to the total dpm of the reaction mixture following the ethyl acetate extraction. Results are expressed as the mean of three replicates obtained from different animals. Means with an asterisk are significantly different ($P < 0.05$) from the blank as determined by paired-comparison.

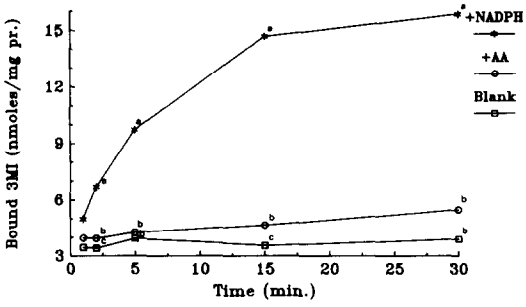

Fig. 6. Time course of arachidonic acid-dependent and NADPH-dependent covalent binding of 3MI to microsomal protein. 3MI (0.05 mM, sp. act. 0.35 mCi/mmol) was incubated with goat lung microsomes (9 mg microsomal protein) in the presence of NADPH (0.3 mM) or arachidonic acid (200 μM). Covalently bound 3MI to protein was determined after exhaustive extraction with various solvents. Non-specific binding was determined by the addition of ethanol as a blank to the reaction mixture. The data were analyzed using a randomized complete block design with animals representing a block. Results are expressed as the mean of three replicates obtained from different animals. Means within various time points having different letters are significantly different ($P < 0.05$) as determined by the REGWQ multiple range test [31]. Pooled SEM: 1 min, 0.44; 2 min, 0.14; 5 min, 0.18; 15 min, 0.14; and 30 min, 0.48.

Covalent binding of radioactivity from a labeled toxin to cellular microsomal protein is often used as an indirect measurement of the formation of reactive intermediates [32]. Tissue specificity can often be explained through tissue differences in covalent binding [32, 33]. To examine and compare the potential of PHS and MFO systems in the activation of 3MI to a reactive intermediate, lung and liver microsomes were incubated with arachidonic acid or NADPH in the presence of [^{14}C]3MI and quantified for covalently bound radiolabeled 3MI metabolites. Figure 6 illustrates a time course study of covalent binding of radiolabeled 3MI derivatives to microsomal protein. NADPH-dependent metabolism of 3MI resulted in a rapid increase in protein-bound 3MI metabolites. Use of arachidonic acid as substrate for PHS resulted in a slight increase in covalently bound derivatives above blank values. These results indicate that the PHS system was capable of activating 3MI to form a reactive intermediate, yet the amount of covalent binding was considerably less than that of the MFO system. A comparison between lung and liver, in both the PHS and MFO systems, of the amount of covalently bound radiolabeled 3MI metabolites is shown in Table 3. The results indicate that covalent binding was higher in lung microsomes than in liver microsomes for both PHS-catalyzed (0.88 vs 0.28) and MFO-catalyzed (9.47 vs 1.74) reactions.

Table 3. Comparison of goat lung and liver microsomal activation of 3MI by PHS and MFO systems

		Covalent binding of [^{14}C]3MI (nmol/mg microsomal protein/15 min)	
Microsomal system	Treatment	Lung	Liver
Arachidonic acid-dependent PHS*	+AA	0.88^{a}	0.28^{b}
	+AA + ID	−0.11^{b}	0.02^{b}
NADPH-dependent MFO*	+NADPH	9.47^{a}	1.74^{b}
	+NADPH + BT	2.31^{b}	2.79^{b}

Both arachidonic acid- (AA) and NADPH-dependent covalent binding of [^{14}C]3MI in goat lung and liver microsomes were determined. Non-specific binding of [^{14}C]3MI to protein was subtracted from all reported results. Abbreviations: ID, indomethacin; and BT, piperonyl butoxide.

* The data were analyzed using a randomized complete block design with each animal representing a block. Results are expressed as the mean of four replications obtained from different animals. Means within each enzyme system having different superscripts are significantly different ($P < 0.05$) as determined by the REGWQ multiple range test [31]. Pooled SEM: PHS, 0.08; MFO, 0.34.

Covalent binding of 3MI to protein was inhibited by either indomethacin or piperonyl butoxide in lung microsomes. These enzyme inhibitors had no effect on covalent binding in liver microsomes.

DISCUSSION

The results of this study firmly established that 3MI can be co-oxidized by PHS in goat lung microsomes. Various criteria were used to verify that 3MI was a substrate for PHS-catalyzed co-oxidation in lung microsomes, i.e. oxygen consumption, biosynthesis of prostaglandins, formation of water-soluble 3MI metabolites, and covalent binding to lung microsomal protein.

Addition of 3MI resulted in a pronounced increase in arachidonic acid-dependent PHS enzyme activity as indicated by both the initial rate (Fig. 1A) and total oxygen consumption (Fig. 4) in lung microsomes. The effect of 3MI on PHS activity was dependent on the presence of arachidonic acid and was inhibited by addition of indomethacin. Since molecular oxygen can be utilized for the oxidation of 3MI to oxidative metabolites such as 3-methyloxindole [12], prostaglandin formation was measured to determine whether the 3MI-dependent oxygen consumption was due solely to an increased rate of oxygenation of arachidonic acid by PHS. Although the synthesis of total prostaglandins was induced 69% by addition of 0.5 mM 3MI (Table 2), it was evident that the increase in prostaglandin biosynthesis alone could not account for the increase in oxygen consumption (Fig. 4). Thus, it is proposed that the unaccounted oxygen that was monitored (shaded area of Fig. 4) is the result of the oxygenation of 3MI during prostaglandin biosynthesis. Using a solvent extraction method to separate the water-soluble 3MI metabolites from the lipophilic 3MI parent compound, it was shown that 3MI undergoes PHS-catalyzed metabolism (Fig. 5). Although the individual metabolites of 3MI were not determined in this study, the results strongly suggest that 3MI is oxygenated by the PHS-dependent reaction. Covalent binding studies of radiolabeled 3MI to microsomal protein (Fig. 6) have established the formation of a reactive intermediate during PHS-catalyzed co-oxidation of 3MI. It can be concluded from this data that 3MI is participating as a reducing cofactor for PHS in goat lung microsomes.

It is evident that at least two enzymatic routes for 3MI metabolism exist in goat lung microsomes, the NADPH-dependent MFO system and the arachidonic acid-dependent PHS system. It was important to demonstrate that the PHS-catalyzed metabolism of 3MI was independent of the MFO-catalyzed metabolism of 3MI. Simultaneous addition of arachidonic acid and NADPH to the microsomal incubation resulted in a rate of oxygen consumption that was equal to the sum of the rates of each system (Fig. 2A). Also, inhibition of the MFO system with piperonyl butoxide did not affect the PHS-catalyzed metabolism of 3MI when arachidonic acid was added to the microsomal system (left bars of Fig. 2C). Similarly, inhibition of the PHS system with indomethacin did not alter the MFO-catalyzed metabolism of 3MI when NADPH was added to the microsomal system (right bars of Fig. 2C). This set of experiments confirmed that each enzyme was metabolizing 3MI independently in goat lung microsomes.

The metabolic activation of 3MI to a reactive intermediate has been proposed to be important in the initiation of 3MI-induced lung disease [15, 34, 35]. Comparison between PHS- and MFO-catalyzed covalent binding of 3MI to microsomal proteins (Table 3) indicated that the NADPH-dependent binding of 3MI was more pronounced than the arachidonic acid-dependent binding. However, it must not be inferred from the results of this *in vitro* study that the MFO system is more significant than the PHS system in the activation of 3MI. *In vitro*, PHS is known to self-inactivate shortly after the initiation of the reaction [36, 37], whereas MFO remains active in the presence of NADPH. The low level of covalent binding resulting from the PHS-catalyzed activation of 3MI may be the result of the self-inactivation property of the PHS enzyme system in an *in vitro* condition. Since the PHS enzyme can be re-synthesized *in vivo*, activation of 3MI by this enzyme system could be biologically significant. Tissue specificity of 3MI toxicosis is partially explained by the high specificity of pulmonary PHS and MFO systems to activate 3MI. This was demonstrated by a greater binding in lung microsomes than in liver microsomes for both PHS- and MFO-catalyzed systems (Table 3).

The roles of PHS and MFO enzyme systems may be complementary in the pathogenesis of 3MI-induced toxicosis. Both PHS and MFO systems are capable of metabolic activation of 3MI to form a reactive intermediate, possibly a free radical. Studies using horseradish peroxidase as a model system for PHS hydroperoxidase have demonstrated the capacity of the peroxidase to catalyze the activation of 3MI to a free radical (unpublished data). In addition to the PHS system, other peroxidases, such as myeloperoxidase, may be involved in 3MI metabolism, since lung tissue can be infiltrated with cells that contain myeloperoxidase. It is most likely that the cytotoxic effects, i.e. lipid peroxidation or protein cross-linking, caused by 3MI free radicals are the consequences of PHS, MFO and other peroxidase systems. In addition to the direct tissue damage initiated by 3MI free radicals, the enhanced and/or altered prostaglandin profile caused by PHS-catalyzed co-oxidation of 3MI may cause various secondary physiological responses in the target tissue. It has been suggested that changes in the prostaglandin profile may be responsible for the promotion of inflammation, edema, and changes in the cardiopulmonary vasculature [38–41]. The high level of PHS activity found in the lung microsomes may be the significant factor for the lung being the target organ for 3MI-induced toxicity.

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