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The Effect of 3-Methylindole on Phospholipid Synthesis in Goat Lung Tissue Slices[1] (41761)

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Abstract. 3-Methylindole (3MI), a ruminal fermentation product of tryptophan, is the causative agent in the development of acute bovine pulmonary edema (ABPE). The disease is dependant on the activation of 3MI by mixed function oxidases (MFO). Electron micrographs have revealed that the lamellar bodies of the type II cells are disrupted in structure and contain neutral lipids (NL) instead of surfactant phospholipids (PL). Goat lung slices were used to investigate the changes in PL metabolism induced by 3MI. Eighteen slices were cut from each lung and divided into control, 3MI (0.57 m*M*), and indole (0.57 m*M*) groups. After a 3-hr pretreatment with these compounds, the slices were incubated with [^{14}C]acetate. The lipids were extracted and separated. 3MI inhibited the incorporation of [^{14}C]acetate into all of the PL studied, but had little effect on its incorporation into NL. Indole displays the same effects on membranes as 3MI, but is not activated by the MFO system and does not induce lung injury. Indole pretreatment had little effect on acetate incorporation in any of the lipid fractions. These results indicate that metabolism of 3MI in lung slices is responsible for the depression of PL synthesis *in vitro*. Increasing the level of unlabeled choline in the medium from 10 μM to 10 m*M* had no effect on the depression of [^{14}C]acetate incorporation into phosphatidylcholine (PC). This suggests that choline uptake is not limiting the synthesis of PC in the 3MI-treated lung slices.

3-Methylindole (3MI), a main fermentation product of tryptophan in the rumen, is the causative agent in the development of the naturally occurring lung disease, ABPE (1). Humans are exposed to 3MI through cigarette smoke (2) and the fermentation of tryptophan in the lower gut (3).

Although 3MI has a direct effect on membrane structure and function *in vitro* (4, 5), the development of the disease is dependent on the metabolism of 3MI by the MFO system, located in the smooth endoplasmic reticulum (SER) (6). Reactive metabolites formed by the MFO metabolism of 3MI have been shown to bind covalently to microsomal proteins (7).

Microscopic studies of the early stages of 3MI induced lung injury in goats demonstrated that the toxic effects are rapid and cell selective. The major effects are on Clara cells and alveolar type I cells. Interstitial and alveolar edema are also present within a few hours after 3MI administration (8). After the initial damage, alveolar type II cells proliferate and electron micrographs reveal some striking abnormalities in these cells. With 3MI treatment the lamellar bodies are devoid of the usual appearance of transverse ribs and the PL in lamellar bodies are replaced by NL (9). These organelles are the main storage and secretory granules of surfactant in Type II cells of the lung (10). These observations suggest that 3MI may depress PL synthesis in the type II cells, causing an insufficient production of surfactant in the lung and/or inhibition of normal membrane repair and turnover. A decrease in either surfactant production or normal maintenance of pulmonary membrane could initiate or potentiate the cellular damage observed in 3MI induced acute pulmonary edema.

The objectives of this study were (i) to investigate the effect of 3MI on the *de novo* synthesis of PL and NL from [^{14}C]acetate using a goat lung tissue slice system, (ii) to investigate the effect of high concentration of choline in the medium on the depression of PL synthesis induced by 3MI treatment.

Materials and Methods. *Animals.* Eight healthy male goats, from 6 to 12 months of age, were used in this study. They were maintained *ad libitum* on alfalfa hay with a con-

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centrate supplement (22% protein) and tap water. The animals were sacrificed with a captive bolt gun, and the lungs removed and placed on ice.

Chemicals. [1-^{14}C]acetic acid (2.4 mCi/mmole) was obtained from New England Nuclear, Boston, Massachusetts; 3MI from Sigma Chemical, St. Louis, Missouri; Silica Gel H from Analabs, North Haven, Connecticut; Fluorescein from Eastman Organic Chemicals, Rochester, N.Y. Other reagents were obtained from Fisher Scientific, Ltd., Toronto, Ontario, Canada.

Experiment 1. Four goats were used in this experiment. Eighteen slices (1 mm thickness) were cut from each lung using two fixed microtome blades. Slices were cut in groups of three from six sites of each lung. The slices from each site were divided into control and two treatment groups. The slices (100–200 mg wet weight) were placed in 25-ml flasks containing 4 ml of modified Krebs Ringer buffer (pH 7.4), with additional glucose (10 m*M*), pyruvate (1 m*M*), and a mixture of 21 amino acids (Table I). The concentrations of amino acids were modified from those used by Eagle (11). A physiological concentration of choline (10 μM), necessary for the *de novo* synthesis of PC and sphingomyelin (SM), was added to the medium. The flasks were placed in a shaking water-bath at 37°C and gased continuously with 95% O_2 and 5% CO_2. After a 3-hr incubation with carrier (15 μl ethanol), indole (0.57 m*M*), or 3MI (0.57 m*M*), 1 μCi of [^{14}C]acetate was added. Incubation was continued for 3 hr. The slices were removed, washed, and homogenized in 5 ml of chloroform:methanol (2:1, vol/vol), using an Ultra-Turrax homogenizer (8-mm diameter). One milliliter of water was added to the tissue homogenate to create a two-phase system. The chloroform fraction was removed and evaporated to dryness under a stream of N_2. The lipids were redissolved in 100 μl of chloroform:methanol (2:1, vol/vol), and spotted onto plates coated with silica gel H (0.50 mm). The plates were developed in a solvent system consisting of chloroform:methanol:acetic acid:water (50:35:6.5:3.25), and sprayed with a saturated solution of fluorescein in methanol:water (1:1, vol/vol). This is a modification of the method of Skipski *et al.* (12). The spots were visualized under UV light after exposure to ammonium hydroxide vapor. The bands containing the various lipid fractions were scraped into vials and resuspended in 1.5 ml of water. Scintiverse counting fluid (Fisher Scientific Ltd.) was added, and radioactivity was measured in a Searle Liquid Scintillation counter. An aliquot of the incubation medium was counted to calculate the uptake of [^{14}C]acetate by the slices. Preliminary experiments have indicated that the incorporation of radioactivity into the PL fractions was linear for 3 hr. The activity in the NL fraction was maximized at 2 hr, as this fraction was utilized in the synthesis of PL.

TABLE I. THE CONCENTRATION OF AMINO ACIDS IN INCUBATION MEDIUM FOR LUNG TISSUE SLICES

Amino acid	Concentration (mg/liter)
L-Arginine · HCl	21.0
L-Cystine	12.0
L-Glutamine	292.0
L-Histidine	8.0
L-Isoleucine	26.0
L-Leucine	26.0
L-Lysine · HCl	36.5
L-Methionine	7.5
L-Phenylalanine	16.5
L-Threonine	24.0
L-Tryptophan	4.0
L-Tyrosine	18.0
L-Valine	23.5
L-Alanine	8.9
L-Asparagine · H_2O	15.0
L-Aspartic acid	13.3
L-Glutamic acid	14.7
Glycine	7.5
L-Proline	11.50
L-Serine	10.50

Experiment 2. Four goats were also used in this experiment. With the following exceptions, the method was unchanged from Experiment 1. The choline concentration in the medium was increased to 10 m*M*, to maximize tissue uptake. 3MI was added either at 0.57 m*M* to compare with the low choline group of Experiment 1, or at 0.19 m*M* to investigate the effects of a lower concentration of 3MI.

Statistics. The level of incorporation of radioactivity into each lipid fraction was averaged across the six slices in each group. The effect of treatments was expressed as the percentage change from the control value, and this difference was assessed in a group of four goats using a paired Student's *t* test (13).

TABLE II. THE EFFECT OF 3-METHYLINDOLE (3MI) ON THE UPTAKE OF [^{14}C]ACETATE BY GOAT LUNG SLICES

	Uptake of ^{14}C-acetate	
	(dpm/mg Tissue) ($\times 10^3$)	(% of depression)
Expt 1		
Control[a]	12.4 ± 1.36	0
3MI (0.57 mM)[a]	10.8 ± 0.837	−12.5 ± 11.6
Control[b]	12.9 ± 1.24	0
Indole (0.57 mM)[b]	12.1 ± 0.991	−6.0 ± 1.3[c]
Expt 2		
Control[a]	11.6 ± 1.50	0
3MI (0.57 mM)[a]	11.0 ± 1.10	−7.6 ± 6.2
3MI (0.19 mM)[a]	10.7 ± 0.724	−5.4 ± 6.9

[a] Values given are the means ± SD from four goat lungs, six slices per lung.

[b] Values given are the means ± SD from three goat lungs, six slices per lung.

[c] The value is significantly different ($P < 0.05$) when the treatment group is compared with its paired control group by the paired Student's t test.

Results. A depression in the uptake of [^{14}C]acetate into the lung slice could lead to decreased incorporation into various lipid fractions. As shown in Table II, 3MI did not cause a significant inhibition of acetate uptake in any of the experiments. Indole did cause a slight depression (−6.0%) in acetate uptake in Experiment 1, but the effect was small.

In the first experiment, when a physiological level of choline (10 μM) was used, 3MI (0.57 mM) caused significant depressions in [^{14}C]acetate incorporation in all of the PL fractions (Table III). While this effect was greatest in the SM fraction (−72%), and the combined phosphatidylserine and phosphatidylinositol (PS + PI) fraction (−63%), incorporation into PC was also decreased by 46%. The incorporation of [^{14}C]acetate into NL was not affected by 3MI treatment.

An equimolar concentration of indole had very little effect ($P > 0.05$) on [^{14}C]acetate incorporation into the various lipid fractions (Table III). The PS + PI fraction is the only PL fraction statistically different ($P < 0.05$) from its paired control.

In the second experiment, when a high concentration of choline (10 mM) was used, 0.57 mM 3MI caused a significant decrease in [^{14}C]acetate incorporation in all PL fractions (Table IV). The incorporation of [^{14}C]acetate into NL was not significantly affected ($P > 0.05$).

The lower level of 3MI (0.19 mM) caused a significant depression of [^{14}C]acetate incorporation into all PL fractions except that of PC.

When 10 mM choline was used, the percentage depression of [^{14}C]acetate incorporation into various lipid fractions induced by 0.57 mM 3MI is similar to that seen with the lower level of choline. The incorporation of [^{14}C]acetate into NL was not significantly decreased, and the depressions in the PL fractions were not significantly different with the two different levels of choline. A lower level

TABLE III. THE EFFECT OF 3MI AND INDOLE ON THE INCORPORATION OF [^{14}C]ACETATE INTO VARIOUS LIPID FRACTIONS[a] OF GOAT LUNG SLICES IN 10 μM CHOLINE MEDIUM

	SM	PC	PS + PI	PE	NL
Control[b]	3770	115,960	9910	22,300	25,660
3MI (0.57 mM)[b]	1030	60,100	3610	13,270	24,400
% Change	−71.9[d]	−46.2[d]	−62.9[d]	−38.6[d]	−4.6
(SD)	(±6.7)	(±12.5)	(±7.8)	(±11.4)	(±30.8)
Control[c]	3480	100,820	9580	20,940	28,440
Indole (0.57 mM)[c]	3210	97,660	8350	19,260	27,380
% Change	−7.2	−2.5	−12.5[d]	−7.9	−2.9
(SD)	(±16.3)	(±3.1)	(±4.0)	(±6.9)	(±11.6)

[a] Lipid fractions are abbreviated as following: SM, sphingomyelin; PC, phosphatidylcholine; PS + PI, phosphatidylserine + phosphatidylinositol; PE, phosphatidylethanolamine; NL, neutral lipid.

[b] dpm/100 mg wet tissue. Values given are from four goat lungs, six slices per goat.

[c] dpm/100 mg wet tissue values given are from three goat lungs, six slices per goat.

[d] Values are statistically different ($P < 0.05$) from the paired control group by the paired Student's t test.

TABLE IV. THE EFFECT OF 3-METHYLINDOLE (3MI) CONCENTRATIONS ON THE INCORPORATION OF [^{14}C]ACETATE INTO VARIOUS LIPID FRACTIONS[a] OF GOAT LUNG SLICES IN 10 m*M* CHOLINE MEDIUM

	SM	PC	PS + PI	PE	NL
Control[b]	4470	118,430	10,040	18,840	17,410
3MI (0.57 m*M*)[b]	1187	60,660	3,831	10,120	13,310
% Change	−74.9[c]	−51.0[c]	−64.2[c]	−47.6[c]	−26.7
(SD)	(±8.6)	(±24.4)	(±11.3)	(±19.3)	(±20.5)
3MI (0.19 m*M*)[b]	2218	79,300	5,945	11,770	13,610
% Change	−52.9[c]	−34.7	−43.9[c]	−38.3[c]	−22.7
(SD)	(±10.9)	(±25.3)	(±16.5)	(±20.7)	(±12.1)

[a] Lipid fractions are abbreviated as following: SM, sphingomyelin; PC, phosphatidylcholine; PS + PI, phosphatidylserine + phosphatidylinositol; PE, phosphatidylethanolamine; NL, neutral lipid.

[b] dpm/100 mg tissue. Values given are means from four goat lungs, six slices per goat.

[c] Values are statistically significant ($P < 0.05$) from the control group by the paired Student's *t* test.

of 3MI (0.19 m*M*) induced similar changes in [^{14}C]acetate incorporation into PL fractions. The depression is less, but not statistically different. Indole has very little effect on the PL or NL synthesis in lung tissue slices.

Discussion. 3MI is lipophilic and has been shown to interact with membrane lipids and alter the activity of membrane bound enzymes *in vitro* (5). The naturally occurring lung lesions are not related to these direct effects, but are caused by reactive 3MI metabolites produced by the MFO system (6). Indole has qualitatively similar *in vitro* effects on membrane lipids and enzymes as 3MI (14). It is not metabolized by the MFO system (15), and does not produce lung lesions when infused in cattle or goats (16). The depression of PL synthesis induced by 3MI is not caused by an equimolar concentration of indole (Table III). This indicates that the changes induced by 3MI are not the result of a direct physical effect, but are due to the formation of reactive intermediates.

Decreased uptake of [^{14}C]acetate by the lung slice could cause the decreased incorporation of [^{14}C]acetate into PL induced by 3MI. The depression of uptake, however, was not significant (Table II). Furthermore, the level of [^{14}C]acetate incorporation into NL was unaffected, suggesting that the ^{14}C-labeled acetate was available in the fatty acids of the diacylglycerol (DAG) pool for incorporation into the PL species. One of the reasons that the NL fraction was unaffected by 3MI may be that the fatty acid synthetase complex, which is responsible for the conversion of acetate to fatty acids, is cytosolic. The incorporation of DAG into the PL classes involves enzymatic reactions on the SER, and these steps may be inhibited by 3MI induced damage within the SER membrane.

The rate of synthesis of PC and SM may depend on the level of choline available in the medium. The level in the first experiment, 10 μM, is close to levels found in serum. 3MI may inhibit the uptake of choline by lung slices, and therefore limit the incorporation of ^{14}C-labeled fatty acids into PC and SM.

Tokmakjian *et al.* (17) showed that the uptake of [^{14}C]choline into rabbit lung slices was linear up to levels above 6 m*M*. The incorporation of [^{14}C]choline into phosphorylcholine, however, leveled off between 1 and 2 m*M*. Thus by increasing the choline concentration to 10 m*M*, the uptake of choline by both the control and 3MI-treated groups was increased to the point that the capacity for incorporation into PC and SM was saturated. If the predominant effect of 3MI was on the uptake of choline, the conditions in the second experiment should have minimized the depression of [^{14}C]acetate incorporation into these fractions. Increasing the choline concentration did not decrease the effect of 3MI on PC and SM synthesis (Tables III and IV). This indicates that the depressed synthesis of PC and SM is not due to a limiting concentration of substrate.

The effect of 3MI at 0.19 m*M* was studied to investigate the response to a lower dose. Although the level of 3MI was decreased by 67%, the response in the PL fractions was only

decreased an average of 30%. This implies that these levels of 3MI are above the threshold of linear response, and that still lower levels may induce similar changes.

In conclusion, this work shows that 3MI treatment of goat lung slices inhibits the incorporation of [^{14}C]acetate into all PL fractions, but does not inhibit its incorporation into NL. Decreased PL synthesis may play a role in initiating or potentiating the cellular damage which leads to the pulmonary lesions of ABPE. PC is the major component of surfactant and a decrease in PC synthesis could lead to a surfactant deficiency or abnormality. As well, PL would be less available for the maintenance and repair of pulmonary membranes. It appears that 3MI may directly inhibit the synthesis of PL by altering enzyme activity. The results indicate that the uptake of choline by the 3MI-treated lung slice is not limiting its capacity to synthesize PC, the most important PL of the lung.

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