Involvement of Free Radicals in the Mechanism of 3-Methylindole-Induced Pulmonary Toxicity: An Example of Metabolic Activation in Chemically Induced Lung Disease

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3-Methylindole (3-MI) is a metabolite of tryptophan which causes acute pulmonary edema and emphysema in ruminants when administered orally or intravenously. 3-MI is metabolized by mixed-function oxidases to a reactive intermediate which may play a role in 3-MI-induced pneumotoxicity. Electron spin-trapping techniques have been used to investigate the in vitro and in vivo formation of free radicals during 3-MI metabolism by goat lung. A nitrogen-centered free radical of 3-MI has been generated from 3-MI in goat lung microsomal incubations. Although a nitrogen-centered free radical can be generated chemically from most of the indolic compounds, only the 3-MI free radical can be generated enzymatically. The formation of the nitrogen-centered 3-MI free radical was followed by the appearance of a carbon-centered lipid radical in microsomal preparations. The findings that an identical carbon-centered free radical was generated by FeSO₄ in the microsomal system in the absence of 3-MI and that malonaldehyde formation is stimulated by 3-MI in microsomes led to the conclusion that 3-MI metabolism induces lipid peroxidation of microsomal membranes. The formation of 3-MI-induced lipid radicals was inhibited by vitamin E and glutathione. A carbon-centered radical was spin trapped in vivo in the lungs of goats infused with 3-MI. This radical had the same splitting constants as the carbon-centered lipid radical trapped in microsomal incubations containing 3-MI. This finding indicates that the metabolism of 3-MI in goat lung in vivo generates a lipid radical. When lung glutathione levels were depressed by pretreatment with diethylmaleate, tissue concentrations of the carbon-centered lipid radical were increased and 3-MI-induced pulmonary toxicity became more severe. These studies support the hypothesis that free radicals are involved in 3-MI-induced pneumotoxicity and that tissue glutathione plays an important role in the defense of the lung against 3-MI toxicity.

Introduction

Although the major role of the lung is in external gas exchange, it is also a metabolic organ of extreme complexity. As a consequence of its location, its architecture, and the metabolic activities of more than 40 types of cells, the nonrespiratory functions of the lung play important roles in maintaining general health. Damage to lung tissue can result from exposure to a variety of chemicals. Many pulmonary toxins share a common mechanism of action by which the parent compounds induce lung injury as a result of metabolic activation to reactive intermediates (1). 3-Methylindole (3-MI, skatole)-induced lung disease is an excellent example of pulmonary toxicity in which formation of free radicals by metabolic activation is involved in the initial step of pulmonary toxicosis.

3-MI is a microbial fermentation product of tryptophan in the rumen of cattle (2–3). 3-MI is also present in the feces of pigs, rats and man as a result of microbial fermentation of dietary tryptophan in the lower gastrointestinal tract (4–6). Another source of 3-MI is cigarette smoke; each cigarette generates 4 to 50 μg of 3-MI as a result of pyrolysis of tryptophan in tobacco leaves (7). The most prominent feature of 3-MI toxicity is its consistency in inducing a lung disease experimentally in ruminants with selective damage to specific lung cells. Oral or intravenous administration of 3-MI can induce acute pulmonary edema and emphysema in cattle (8), goats (9), and sheep (10). 3-MI is considered a major etiological factor in naturally occurring acute bovine pulmonary edema and interstitial emphysema, an important respiratory disease of grazing cattle (8). The possible risk of exposure of 3-MI to man has not been assessed. The extent of the risk of 3-MI to man would depend on the source and the route of exposure to 3-MI as well as the characteristics of the metabolic en-
zymes associated with activation and detoxification. Elucidation of the primary mechanism of action and the pathological changes associated with 3-MI-induced pulmonary injury in ruminants would provide a basis for comparative studies on the risk of 3-MI toxicity in other species, including man.

**Metabolism of 3-Methylindole**

3-MI, as the parent compound, has been shown to adversely affect biological membrane systems and to alter membrane function in vitro (11-13). The development of the disease, however, is not due to these direct effects on membranes, but is dependent on the metabolism of 3-MI by microsomal mixed-function oxidases (MFO) (14). A shorter half-life of 3-MI in the plasma of goats treated with phenobarbital (an MFO inducer) and a longer half-life in goats treated with piperonil butoxide (an MFO inhibitor) clearly indicate that compounds known to alter MFO activity can change the rate of 3-MI metabolism. Goats pretreated with phenobarbital developed more severe clinical signs and pulmonary lesions following 3-MI infusion. Pretreatment with piperonil butoxide prevented the development of clinical signs and pulmonary lesions. The enhanced pulmonary injury in phenobarbital-treated goats and the protection by piperonil butoxide implicate metabolites of 3-MI formed by the MFO system in the mechanism of pulmonary injury (14).

Two pathways of 3-MI metabolism have been proposed. 3-MI is rapidly metabolized by MFO and excreted in the urine mainly as oxidation products, 3-methyloxindole and its hydroxyl derivatives (15). A minor pathway leads to the formation of indole-3-carboxylic acid and conjugated products (16). Incubation of [14C]-3-MI with goat lung microsomal preparations results in covalent binding of radioactivity to microsomal protein. The degree of covalent binding is NADPH- and O2-dependent and is organ specific (16,17). A reactive intermediate generated from 3-MI at a metabolic step prior to the formation of 3-methyloxindole is indicated since 3-methyloxindole does not lead to covalent binding in microsomal suspensions (16,17) or to pneumotoxicity in vivo (18). Although these studies did not define the exact mechanism of 3-MI toxicity, the production of free radicals from 3-MI may be of significance.

**Evidence of 3-MI Radical Formation**

To demonstrate that free-radical metabolism is involved in the initiation of 3-MI-induced pulmonary toxicity, the following criteria have to be satisfied. First, the 3-MI molecule must be capable of generating a free radical and the free-radical metabolite of 3-MI must be demonstrated to exist. Second, specific and predictable interactions between the 3-MI free radical and cell components must be identified, such as covalent binding to macromolecules or lipid peroxidation products. Third, compounds known to modulate free-radical scavenging defense systems must alter the production of 3-MI free radicals and the toxic effect of 3-MI.

Electron spin resonance (ESR) spin-trapping techniques have been utilized to detect free radicals generated during 3-MI metabolism. ESR spin trapping is a unique method which detects free radicals selectively among many types of metabolites in a reaction mixture, and this method has been extensively applied to detect free radicals in biological systems (19). This technique consists of using a spin trap, a nitroso or nitrone compound, which reacts covalently with an unstable radical to form a stable nitroxide (spin adduct). The unstable radical is, therefore, "trapped" as a long-lived species which can be observed at room temperature using conventional ESR equipment. Since the relatively stable spin adduct accumulates, spin trapping is an integrative method for measuring free radicals and is inherently more sensitive than measures which detect only instantaneous, or steady-state, levels of free radicals. The hyperfine splitting of the spin adduct provides information which can aid in the identification and quantitation of the original radical. Spin trapping has been used in a microsomal system to detect free radicals formed in the metabolism of various toxins including nitrosoamines (20), halocarbons such as carbon tetrachloride (21) and halothane (22), and hydrazines (23).

In order to demonstrate that the 3-MI molecule is capable of producing a free radical, 3-MI was incubated with KO2 and irradiated with ultraviolet light (24,25). A nitrogen-centered 3-MI free radical was detected by using the spin trap phenyl-tert-butyl nitrone (PBN). The hyperfine splitting constant of its 18-line spectrum was $a_N = 13.9$ G, $a_N^L = 3.6$ G and $a_N^H = 2.3$ G. It has also been possible to use PBN to trap a nitrogen-centered 3-MI free radical with identical hyperfine splitting constants which is produced enzymatically in a lung microsomal preparation after a 3 min incubation with a NADPH generating system. The nitroxyl adduct was dependent on the presence of 3-MI, NADPH, O2, and microsomes (24,25).

A nitrogen-centered radical can also be generated from indole, indole-3-carbinol, 3-methyloxindole, and indole-3-acetic acid when treated with KO2. No free radical has been observed, however, in microsomal preparations of these indolic compounds (24), and they do not cause pneumotoxicity in vivo (18). Indole and 3-MI have qualitatively similar effects on biological membranes and similar chemical properties (11). 3-Methyloxindole and indole-3-carbinol are postulated to be the products of the major and minor pathways of 3-MI metabolism (15). 1-Methylindole does not cause disruption of biological membranes (11) and does not generate a nitrogen-centered free radical when treated with KO2 or incubated with microsomes (24). The methyl group on the nitrogen atom in the indole ring of 1-methylindole inhibits abstraction of a hydrogen from the nitrogen to form a radical. The exact relationship between radical formation and 3-MI toxicity is not known. However, the...
formation of a nitrogen-centered 3-MI radical by the lung microsomal system provides strong support for the hypothesis that such 3-MI radicals are involved in 3-MI-induced pulmonary toxicity.

3-Methylindole Free Radical and Lipid Peroxidation

It is possible to predict the potential reactions of a reactive 3-MI free radical in a biological system. The 3-MI radical metabolite can bind covalently to cellular components such as proteins and nucleic acids. The 3-MI radical metabolite can initiate the formation of alkyl and peroxy free radicals derived from membrane lipids. To follow the sequence of events of 3-MI free radical-initiated reactions in a biological system, a time series experiment was carried out to follow the appearance of free radical signals in a microsomal system over an incubation period of 60 min (25). ESR spectra of the PBN spin adduct extracted after various incubation intervals are shown in Figure 1. Incubation of 3-MI with lung microsomes for 3 min in the presence of NADPH and PBN gave a very weak multiline ESR spectrum (Fig. 1A) which developed into a strong signal after 6 min (Fig. 1B). Incubation of 3-MI with microsomes for 12 min resulted in a composite spectrum of nitrogen- and carbon-centered radical adducts (Fig. 1D). After 30 and 60 min incubations (Fig. 1D,E), a six-line spectrum with hyperfine splitting constants \( a_H = 14.4 \) G and \( a_N = 3.2 \) G typical of a carbon-centered free radical adduct of PBN was detected. However, the carbon-centered radical trapped in the microsomal system was shown not to be a 3-MI radical and is likely derived from membrane lipids. To test the hypothesis that the carbon-centered free radical is a 3-MI-induced lipid peroxidation product, the ESR spectra from the 30 and 60 min microsomal incubations were compared with those obtained from a FeSO\(_4\)-induced lipid peroxidation system (25). In the absence of 3-MI, a carbon-centered radical adduct of PBN with the same splitting constants was obtained after the addition of FeSO\(_4\) to microsomes. These data indicate that the initially formed nitrogen radicals of 3-MI preferentially react with some membrane component, probably lipids, and that the resulting carbon-centered lipid radicals are then trapped by PBN to give the carbon-centered radical adduct. Malonaldehyde, an index of lipid peroxidation, also increased in the lung microsomal incubation system containing 3-MI during the 60-min time-course study (Fig. 2) (25). The stimulation of malonaldehyde production by 3-MI supports the concept that the metabolism of 3-MI by the microsomal MFO system to its free radical intermediate initiates lipid peroxidation. Formation of the carbon-centered lipid radicals \textit{in vitro} can be inhibited by free-radical scavenging agents such as vitamin E and glutathione (24). Spin trapping of fatty acid radicals in microsomal preparations has also been reported in various studies (26–28). Recently, McCay and co-workers (29) reported the presence of a carbon-centered lipid adduct of PBN in microsomal incubations containing CC\(_14\) with the same splittings as the carbon-centered radical found in 3-MI incubations.

3-MI-Induced Free-Radical Production and Pulmonary Toxicity

Before the 3-MI free radical and its interaction with cellular components can be implicated as the cause of pulmonary toxicosis, it is essential to demonstrate the
existence of 3-MI-induced free radicals in vivo. Spin trapping of free radicals in an in vivo system has been attempted only recently (21, 22, 25, 27–29). Although the spin trap, PBN, has been used successfully to trap free radicals induced by toxins in vivo, the organ and cellular distribution, metabolism, and toxicity of the spin trap itself have not been studied. Furthermore, the appropriate time for the measurement of concentrations of spin adduct after administration of the spin trap and the toxin to the animal needs to be determined. Since spin trapping is an integrative technique, the spin adduct accumulates over time. However, the spin adduct may also decompose, be metabolized, or be excreted from the body. Thus, the time of maximal concentrations of the spin adduct is dependent on the relative contributions of these factors.

PBN has been used in vivo to trap free radicals generated from liver metabolism of halothane (22, 29) and CCl₄ (21, 28) and from lung metabolism of 3-MI (25). These studies indirectly suggest that PBN is capable of trapping free radicals generated from MFO metabolism of foreign compounds in both lung and liver. Albano et al. (27) have shown that PBN at concentrations of 5 and 50 mM inhibited CCl₄-stimulated lipid peroxidation, ¹⁴C-CCl₄ binding to microsomal protein and aminopyrene demethylase activity in the rat. PBN acts as a substrate in the MFO system and gives Type 1 binding spectra. These findings suggest that PBN is capable of reaching the site of the MFO enzyme system where toxins are metabolized to free radicals. However, when PBN was used to trap free radicals induced by 3-MI in in vitro microsomal incubations, PBN did not show any inhibition on metabolic activation of 3-MI by MFO of goat lung at concentrations up to 0.1 M. The LD₅₀ of PBN has not been determined; however, infusion of 0.1 g PBN/kg of body weight over 1 hr to goats did not induce any visible toxic effect.

The methods of tissue sample preparation and spin adduct extraction are still in the developmental stage. As the PBN adduct is lipid-soluble, extraction procedures have focused upon chloroform–methanol extraction of total lipids and then concentration of the CHCl₃ layer (21, 28). However, this indiscriminate extraction of total lipids from the organs of large animals results in a viscous extract which is difficult to concentrate. Another method is to extract spin adducts more selectively by using hexane, which yields a less viscous lipid extract that can be concentrated (25).

An attempt was made to spin trap in vivo either the primary radical of 3-MI or the secondary lipid radicals (25). Goats were infused with the spin trap PBN and 3-MI in propylene glycol for 1.0 hr. The dose of 3-MI used had been shown previously to induce moderate to severe pulmonary lesions in goats. Control goats received an infusion of PBN in propylene glycol. All goats were killed immediately after the infusion, and the lungs as well as the liver were analyzed for tissue concentrations of spin adducts. The ESR signal (Fig. 3A) obtained from the lungs of goats which had been infused with 3-MI had splitting constants identical to those of the carbon-centered radical obtained from lung microsomes incubated with 3-MI (Fig. 3C). This radical was demonstrated to be a lipid peroxidation product (Fig. 3B). These findings provide evidence that the metabolism of 3-MI in goat lung in vivo generates a lipid radical. No ESR signal was observed in the livers of goats following administration of 3-MI.

If the carbon-centered lipid free radical induced by 3-MI is related to the initiation of pulmonary injury, then manipulation of the free-radical-scavenging defense system should alter the production of 3-MI-induced free radicals and the toxic effect of 3-MI. This hypothesis is supported by an experiment in which tissue levels of vitamin E and glutathione were manipulated by administration of vitamin E, cysteine (a glutathione inducer), and diethyl maleate (a glutathione-depleting agent) in order to observe the effect of these variables on levels of free radicals generated by 3-MI in the lung of intact goats (30). Prior to intrajugular infusion of 3-MI, the goats were given one of four pretreatments: (i) vitamin E + cysteine; (ii) vitamin E + diethyl maleate; (iii) cysteine; (iv) diethyl maleate. The amount of free radicals obtained from the lungs after these various pretreatments, was expressed as picomoles of PBN-trapped radicals per lung (Fig. 4). Animals pretreated with vitamin E + cysteine or cysteine had the lowest concentrations of the trapped radical. However, when tissue glutathione levels were depressed by diethyl maleate pretreatment, higher concentrations of the PBN-trapped radical were detected, regardless of vitamin E pretreatment. The effect of these pretreatments on the severity of 3-MI-induced lung lesions was also investigated. The severity of the disease was scored by clinical signs, lung to body weight ratio, moisture
**FIGURE 3.** Comparison of ESR spectra obtained from in vivo and in vitro studies: (A) ESR spectrum of a hexane extract from lung of a goat infused with 3-MI in propylene glycol together with PBN; (B) ESR spectrum produced by incubation of 0.05 M FeSO₄, 2.5 μM NADPH, and 0.1 M PBN after a 60-min incubation; (C) ESR spectrum of a 60-min microsomal incubation containing approximately 16 mg of microsomal protein/mL, 0.1 M PBN in 0.05 M phosphate buffer (pH 7.4), 0.063 M 3-MI, and 0.2 μM NADPH at 37°C.

content of the lung, respiration rate and microscopic lesions. The results showed that the severity of the disease followed the same trend observed in the concentrations of the carbon-centered free radicals. The severity of the disease and the concentrations of free radicals both increased with decreasing lung glutathione content. These findings agree with the hypothesis that the metabolism of 3-MI to a free radical is the initial step in the sequence of events leading to lung toxicity and that tissue glutathione is an important defense mechanism against 3-MI-induced free radicals.

**Possible Mechanisms of Pulmonary Toxicity of 3-MI**

The enzymatic formation of reactive 3-MI free radicals may lead to a number of toxic effects. A hypothetical scheme is presented in Figure 5. A possible NADPH-mediated, cytochrome P-450-dependent mechanism for the activation of 3-MI to a nitrogen-centered free radical and the chemical reactions that may be of importance in the toxicity of this compound are depicted. A highly reactive 3-MI free radical could abstract a hydrogen atom from an unsaturated fatty acid to form lipid carbon-centered free radicals which react with molecular oxygen, thereby initiating lipid peroxi-
Lipid peroxidation may be associated with alterations of membrane function resulting in changes in membrane permeability or inactivation of integral enzymes (31).

A second possible reaction is the covalent binding of the 3-MI-induced radicals to proteins and nucleic acids. The nitrogen-centered free radical of 3-MI may covalently bind to proteins which are crucial in the regulation of cellular metabolism, such as enzymes which control phospholipid synthesis and affect surfactant production in the lung (32,33).

The third important reaction is the reaction of sulphydryl groups of cysteine and reduced glutathione with the 3-MI free radical. Glutathione and cysteine have been shown to inhibit the in vitro formation of the nitrogen-centered 3-MI free radical as well as carbon-centered fatty acid radicals induced by 3-MI (24). These thiol compounds also dramatically decrease covalent binding of 3-MI metabolites to protein (16,34) and drastically reduce the cytotoxic effect of 3-MI in vivo (35,36).

In spin-trapping studies, higher concentrations of carbon-centered lipid radicals were seen in animals with reduced lung tissue levels of glutathione compared to animals with induced lung glutathione levels (30). Pulmonary toxicity due to 3-MI was also more severe when tissue concentrations of glutathione in the lung were depleted (35,36). If 3-MI toxicity is due to covalent binding and peroxidative damage by a free radical intermediate, sulphydryl groups may decrease its toxicity by functioning as hydrogen donors, since thiol compounds are very reactive towards carbon-, oxygen-, and nitrogen-centered radicals (37).

These studies support the hypotheses that 3-MI-induced lung damage results from activation of 3-MI by the mixed-function oxidase system in the lung to a free radical which can covalently bind to protein and induce lipid peroxidation and that tissue glutathione plays an important role in the lung’s defenses against 3-MI-mediated lipid peroxidation.

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