Vol. 149, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1987 Pages 73-78

3-METHYLINDOLE INHIBITS LIPID PEROXIDATION

James D. Adams, Jr., Mikel C. Heins, and Garold S. Yost*[1]

Pharmacology/Toxicology Program
College of Pharmacy, Washington State University
Pullman, WA 99164-6510

[1] Department of Pharmacology and Toxicology,
113 Skaggs Hall,
University of Utah,
Salt Lake City, UT 84112

Received October 12, 1987

SUMMARY: The mechanism of pneumotoxicity of 3-methylindole has been postulated to occur via protein alkylation or lipid peroxidation. This report describes the effects of the addition of 3-methylindole to goat lung microsomes to evaluate the possibility that this xenobiotic may increase NADPH-supported lipid peroxidation. Concentrations of malondialdehyde were measured as an index of lipid peroxidation. Instead of a stimulation of lipid peroxidation by 3-methylindole, a complete inhibition of lipid peroxidation was produced by concentrations of 3-methylindole as low as 10 μM. The addition of 3-methylindole to actively peroxidizing microsomes (NADPH-supported) caused an immediate cessation of malondialdehyde production. These results demonstrate that 3-methylindole pneumotoxicity does not proceed by a mechanism of lipid peroxidation, but in fact, this molecule may act as an effective antioxidant to prevent lipid peroxidation in pulmonary tissue.

3-Methylindole (3MI) is a fermentation product of ruminant tryptophan metabolism and is normally found in the intestines of ruminants and other animals, including man (1). 3-Methylindole and indoles derived from the diet, such as indole-3-carbinol (2) and indole-3-carboxylic acid, are commonly found in many animals. The toxicity of 3MI is an area of interest in agriculture since cattle are susceptible to the pulmonary toxicity of 3MI (3), and significant numbers of animals are poisoned each year by this toxin. In addition, exposures to man occur through intestinal absorption of 3MI and from cigarette smoke (4).

The mechanism of toxicity of 3MI is not yet clear. It is known that 3MI depletes pulmonary glutathione by 44% in mice within six hours (5). The pneumotoxin alkylates tissue proteins and forms an adduct (6) with glutathione in microsomal incubations from pulmonary and hepatic tissues (7) from several different species (8). On the other hand, 3MI is also metabolized to produce free radicals (9) and has been postulated to induce lipid peroxidation (9,10). However, the significant in vivo depletion of glutathione by lipid-peroxidizing agents

*To whom reprint requests should be sent.

Abbreviations: 3MI, 3-methylindole; MDA, malondialdehyde.

0006-291X/87 $1.50

such as carbon tetrachloride is usually not observed (11). In addition, 3MI does not stimulate superoxide or hydrogen peroxide production in goat lung microsomes (12). Therefore, it is not clear whether lipid peroxidation is critical to the toxic mechanism of 3MI. This study was designed to examine the ability of 3MI to initiate lipid peroxidation in goat lung microsomes in an attempt to clarify the significance of lipid peroxidation in the mechanism of toxicity of 3MI.

MATERIALS AND METHODS

Chemicals 3MI, ascorbic acid, ferrous ammonium sulfate, NADPH, 2-thiobarbituric acid, and 1,1,3,3-tetraethoxypropane were purchased from Sigma (St. Louis, MO). Other chemicals were analytical grade and were used without further purification.

Preparation of microsomes Adult, male goats (40-50 kg) were purchased from local suppliers and were pretreated with phenobarbital (100 mg/kg day i.p. for 3 days). Microsomes were prepared from lung tissues by the procedure of Guengerich (13), and stored at -70^{o}.

Lipid peroxidation assays Incubations contained 1.5 mg of microsomal protein, 9.0 μmoles of magnesium chloride, 3.0 μmoles of 3MI or less, 0.9 mmole of phosphate buffer (pH 7.4) and 3.0 μmoles of NADPH. 3-Methylindole was dissolved in hot deionized water, cooled to room temperature, and added in a 1.5 ml aliquot to the incubations. The incubations were initiated by addition of NADPH and were 3.0 ml total volume. Some incubations contained 20 μl of CCl_4 instead of 3MI. Following incubation in a shaking water bath at 37^{o} for the appropriate period, the incubations were quenched with acidic thiobarbituric acid solution and assayed for malondialdehyde (MDA) levels via the thiobarbituric acid-reactive substances test using 1,1,3,3-tetraethoxypropane standard curves (14).

Experiments were also performed in which the precise experimental conditions of Kubow, *et al.* (9), were reproduced. In these incubations, goat lung microsomes (48 mg) were added to 0.01 M phosphate buffer (pH 7.4) containing 0.1 mM EDTA with a final volume of 6.0 ml. Concentrations of 3MI and NADPH were 0.063 M and 0.2 μM, respectively. To attain a concentration of 3MI as high as 0.063 M, ethanol (2% of the final volume) was used to solvate 3MI, but 3MI precipitated when the ethanolic solutions were added to microsomal incubations. The mixtures were preincubated at 37^{o} for 5 min before addition of 3MI and aliquots were withdrawn at 3, 6, 12, 30, and 60 min for MDA determination as described above.

Ascorbate/iron incubations Assays with an ascorbate/iron lipid-peroxidizing system were also performed as a means of stimulating lipid peroxidation (15). Incubations contained 1.5 mg of microsomal protein, 3.0 μmoles of 3MI, 1.2 μmoles of ascorbate, 30 μmoles of $Fe(NH_4)_2(SO_4)_2$ and 0.9 mmole of phosphate buffer (pH 7.4) in a total volume of 3.0 ml. Ascorbate/iron solutions were made by quickly dissolving in water and were used within 1 minute. Incubations were performed, quenched, and assayed as above.

RESULTS

3-Methylindole was shown to inhibit lipid peroxidation in NADPH-treated goat lung microsomes. This inhibition is essentially complete and not time-dependent. In other words, no NADPH-stimulated lipid peroxidation occurs in the presence of 3MI (Fig. 1). Carbon tetrachloride is known to induce lipid peroxidation in lung tissue and was used as a positive control to demonstrate the activity of the microsomal preparations (Fig. 1). Carbon tetrachloride increased lipid peroxidation beyond the NADPH-stimulated levels by about 0.1 nmol/mg protein/min.

Normal NADPH-stimulated lipid peroxidation was quenched by the addition of 3MI to actively peroxidizing microsomes (Fig. 2). Addition of 3MI (1.0 mM) at 0, 20 or 45 min totally inhibited additional formation of MDA, and presumably lipid peroxides.

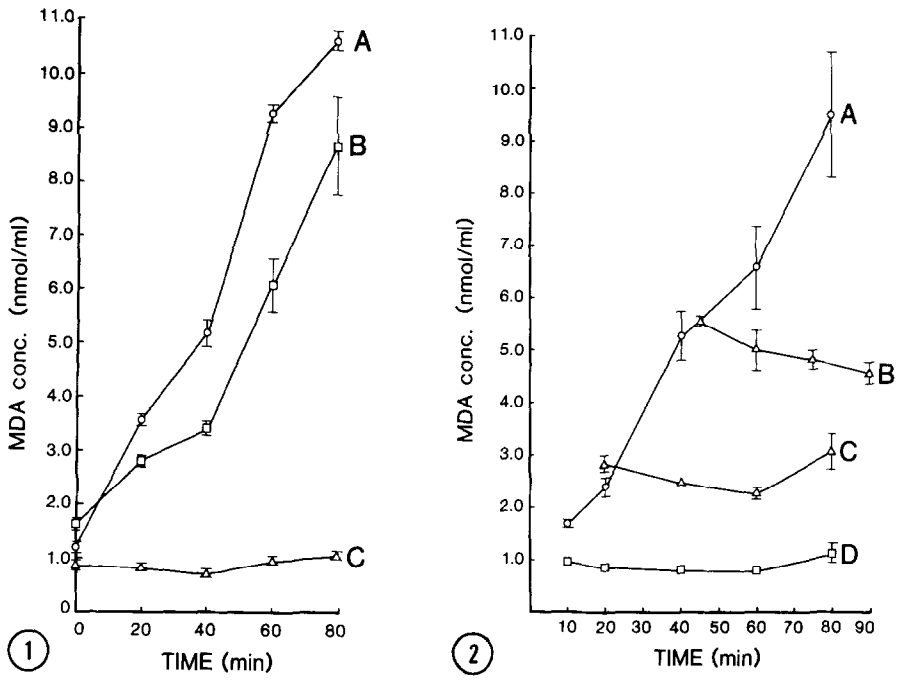

Fig. 1. Time course of lipid peroxidation of goat lung microsomes in the presence of: (A) CCl_4 (0.67 μl/ml) and NADPH (1.0 mM); (B) NADPH (1.0 mM); or (C) 3MI (1.0 mM) and NADPH (1.0 mM).

Fig. 2. Time course of lipid peroxidation of goat lung microsomes in the presence of: (A) NADPH (1.0 mM); (B) NADPH (1.0 mM) and 3MI (1.0 mM) added 45 min after NADPH; (C) NADPH (1.0 mM) and 3MI (1.0 mM) added 20 min after NADPH; and (D) NADPH (1.0 mM) and 3MI (1.0 mM) added together at 0 min.

However, it is possible that the assay conditions may have interfered with the detection of MDA. Interference with the thiobarbituric acid-reactive substances assay was not a factor, since addition of 3MI (1.0 mM) to 1,1,3,3-tetraethoxypropane followed by addition of thiobarbituric acid did not decrease the detection of MDA. These results, when considered with the positive control data above, demonstrate that 3MI does indeed prevent lipid peroxidation.

Concentrations of 3MI between 1.0 mM and 10.0 μM completely prevented lipid peroxidation in goat lung microsomes. Below 10.0 μM, the inhibition by 3MI was decreased (Table 1). These results indicate that 3MI is nearly as effective in the prevention of lipid peroxidation as classic antioxidants such as BHT (16). In fact, the electrochemical anodic oxidation potential of a 2,3-disubstituted indole, 2,3-diphenylindole, is 1.08 V (17), which is lower than the oxidation potential of BHT, 1.21 V (18). Comparisons of the electrochemical oxidation potentials of these molecules should be a relative indicator of the ease of hydrogen atom abstraction and, therefore, of the ability to inhibit lipid peroxidation by a hydrogen radical quenching mechanism.

Ascorbate/iron preparations were used to stimulate lipid peroxidation without the addition of NADPH (15). In these incubations cytochrome P-450 should not be involved in the lipid peroxidation process, since no reducing equivalents were included. Following a 2-hr

Table 1. Concentration Dependence of Lipid Peroxidation Inhibition by 3MI.

3MI (μM)	Percent Inhibition of Lipid Peroxidation
1000	100
100	100
10	100
1.0	52.6
0.1	19.3

incubation, ascorbate/iron stimulated 12.5 $\pm$ 1.5 nmoles of MDA (n=4) in 3.0 ml incubations of goat lung microsomes. The addition of 1.0 mM 3MI to these incubations prior to the addition of ascorbate/iron resulted in 0.9 $\pm$ 0.4 nmoles of MDA (n=4) above the blanks. Blank incubations consisted of microsomes in 0.1 M phosphate buffer (pH 7.4).

Earlier reports of 3MI-induced lipid peroxidation in goat lung microsomes (9) were performed under assay conditions which were different from the conditions presented here in Fig. 1-2 and Table 1. Attempts to repeat the conditions reported earlier were unsuccessful because of the following problems: a) the concentration of 3MI was supersaturating (0.063 M); b) the concentration of NADPH (0.2 μM) was approximately 5,000 times lower than normal microsomal incubation levels; c) ethanol, used as a solvent, inhibited lipid peroxidation; and d) blanks which contained only NADPH produced much more lipid peroxidation than incubations which contained both 3MI and NADPH. The incubations with NADPH (without ethanol) produced 10.9 nmole of MDA/ml in 60 minutes. Incubations with 3MI (solvated with ethanol) and NADPH produced only 7.0 nmole of MDA/ml in 60 minutes, an amount which was very similar to the 6.25 nmole of MDA/ml that was present in control incubations without 3MI and NADPH. Therefore, repetition of the previously reported assay conditions (9) with goat lung microsomes also showed that 3MI blocks NADPH-induced lipid peroxidation.

DISCUSSION

The results presented here demonstrate that 3MI inhibits lipid peroxidation in goat lung microsomal preparations. The goat was chosen for this work since this is the species with the highest susceptibility to 3MI-induced pneumotoxicity (19). 3-Methylindole may block lipid peroxidation by inhibition of both the initiation and propagation stages of lipid peroxidation, since addition of 3MI at 0 min blocked the initiation of lipid peroxidation and the addition of 3MI at 20 or 45 min blocked the propagation of lipid peroxidation. It is possible, however, that 3MI does not block initiation when it is added at 0 min, but rather, effectively blocks propagation such that lipid peroxidation is not detected.

Since 3MI is probably a substrate for cytochrome P-450 (20), which is required for NADPH-stimulated lipid peroxidation, it is possible that 3MI could block the initiation of lipid peroxidation by competitively inhibiting cytochrome P-450 activity. A number of cytochrome

P-450 substrates have been shown to block lipid peroxidation (21) in both NADPH-supported and ascorbate/iron-supported incubations. Thus, these compounds acted as "antioxidants" without concomitant inhibition of P-450. 3-Methylindole, like these other cytochrome P-450 substrates, blocked lipid peroxidation in ascorbate/iron incubations which did not depend upon cytochrome P-450 activity. Therefore, 3MI was also likely acting as an inhibitor of lipid peroxidation without cytochrome P-450 inhibition.

Perhaps the ability to donate a hydrogen atom to lipid radicals (terminating lipid peroxidation), in a manner analogous to the action of vitamin E, is the critical action for cytochrome P-450 substrates which are also antioxidants. 3-Methylindole would most likely donate a hydrogen from the indole nitrogen, forming a nitrogen-centered radical. Such a radical has been implicated by trapping experiments from goat lung microsomal incubations (9). This radical could rearrange to form a methyl carbon-centered radical. The carbon-centered radical has been identified from incubations of indole-3-carboxylic acid and horseradish peroxidase (22). The radical might also donate another hydrogen atom to a lipid radical to form an imine methide, an electrophilic intermediate that has been implicated in 3MI-mediated pneumotoxicity (5,6).

Metabolism of 3MI could occur in incubations containing iron and peroxide-reducing equivalents such as in ascorbate/iron incubations (23). Therefore, in both NADPH-fortified or ascorbate/iron microsomal incubations, oxidation of 3MI might have taken place. It is possible then, that an intermediate produced from 3MI oxidation could be responsible for the antioxidant properties. The metabolite could be a 3MI radical, an imine methide, a phenolic metabolite or some other intermediate. It is also possible that a mechanism occurs wherein 3MI itself is the antioxidant.

In conclusion, we have demonstrated that not only does 3MI lack an ability to stimulate lipid peroxidation in pulmonary microsomes from a susceptible species, but the xenobiotic appears to be an effective antioxidant at low concentrations. Recent findings on the antioxidant properties of indole-3-carbinol (24), coupled with our work on 3MI, may provide a common theme for the antioxidant properties of substituted indoles in biological systems. Certainly, the assumption that lipid peroxidation is the cause of 3MI-mediated pneumotoxicity (9-10) would seem highly doubtful in light of these results.

ACKNOWLEDGEMENTS

This work was supported by Grant HL13645 from the United States Public Health Service, National Institutes of Health. G.S.Y. is a United States Public Health Service Research Career Development Awardee (HL02119).

REFERENCES

1. Yokoyama, M.T., and Carlson, J.R. (1979) Appl. Microbiol. 27, 540-548.
2. Fiala, E.S., Reddy, B.S., and Weisburger, J.H. (1985) Annu. Rev. Nutr. 5, 295-321.
3. Breeze, R.G., and Carlson, J.R. (1982) Adv. Vet. Sci. Comp. Med. 26, 201-231.
4. Wynder, E.L., and Hoffmann, D. (1967) Tobacco and Tobacco Smoke, pp. 374-379. Academic Press, New York, NY.

5. Huijzer, J.C., Adams, J.D., Jr., and Yost, G.S. (1987) Toxicol. Appl. Pharmacol. 90, 60-68.
6. Nocerini, M.R., Yost, G.S., Carlson, J.R., Liberato, D.J., and Breeze, R.G. (1985) Drug Metabol. Dispos. 13, 690-694.
7. Nocerini, M.R., Carlson, J.R., and Yost, G.S., (1985) Toxicol. Appl. Pharmacol. 81, 75-84.
8. Nocerini, M.R., Carlson, J.R., and Yost, G.S. (1985) Tox. Lett. 28, 79-87.
9. Kubow, S., Janzen, E.G., and Bray, T.M. (1984) J. Biol. Chem. 259, 4447-4451.
10. Kubow, S., Bray, T.M., and Janzen, E.G. (1985) Biochem. Pharmacol. 34, 1117-1119.
11. Mitchell, J.R. Smith, C.V., Lauterburg, B.H., Hughes, H., Corcoran, G.B., and Horning, E.C. (1984) Drug Metabolism and Drug Toxicity, pp. 301-319. Raven Press, New York, NY.
12. Laegreid, W.W., and Breeze, R.G. (1985) Res. Commun. Chem. Pathol. Pharmacol. 47, 387-397.
13. Guengerich, F.P. (1982) Principles and Methods of Toxicology, pp. 609-634. Raven Press, New York, NY.
14. Recknagel, R.O., Glende, E.A., Waller, R.L., and Lowry, K. (1982) Toxicology of the Liver, pp. 213-241. Raven Press, New York, NY.
15. Shertzer, H.G., Niemi, M.P., and Tabor, M.W. (1985) Adv. Exp. Med. Biol. 197, 347-356.
16. Trotta, R.J., Sullivan, S.G., and Stern, A. (1983) Biochem. J. 212, 759-772.
17. Cheek, G.T., and Nelson, R.F. (1978) J. Org. Chem. 43, 1230-1232.
18. Ronlan, A. (1971) Chem. Commun. 2, 1643-1645.
19. Dickinson, E.O., Yokoyama, M.T., Carlson, J.R., and Bradley, B.J. (1976) Am. J. Vet. Res. 37, 667-672.
20. Leung, C.T., Carlson, J.R. and Breeze, R.G. (1983) Can. J. Physiol. Pharmacol. 61, 395-402.
21. Miles, P.R., Wright, J.R., Bowman, L., and Colby, H.D. (1980) Biochem. Pharmacol. 29, 565-570.
22. Mottley, C., and Mason, R.P. (1986) J. Biol. Chem. 261, 16860-16864.
23. Kelly, W., and Horning, E.C. (1958) Biochem. J. 70, 13P.
24. Shertzer, H.G., Niemi, M.P., Reitman, F.A., Berger, M.L., Myers, B.L., and Tabor, M.W. (1987) Exper. Molec. Pathol. 46, 180-189.