Vol. 114, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 18, 1983 Pages 168-174

THE SPIN-TRAPPING OF ENZYMATICALLY AND CHEMICALLY CATALYZED FREE RADICALS FROM INDOLIC COMPOUNDS

STAN KUBOW[1], C.M. DUBOSE[2], JR., E.G. JANZEN[2], J.R. CARLSON[3], T.M. BRAY[1]

[1]Department of Nutrition, University of Guelph, Guelph, Ontario, N1G 2W1, Canada

[2]Department of Chemistry, Guelph-Waterloo Centre for Graduate Work in Chemistry, University of Guelph, Guelph, Ontario, N1G 2W1, Canada

[3]Department of Animal Science, Graduate Program in Nutrition Washington State University, Pullman, Washington, USA 99164

Received May 7, 1983

A nitrogen-centered free radical was spin-trapped from superoxide-catalyzed oxidation of indolic compounds, using the spin-trap phenyl-t-butyl-nitrone. The hyperfine splitting constants observed were a_N = 13.9 G, a_β^N = 3.6 G and a_β^H = 2.3 G. Incubation of various indolic compounds with goat lung microsomes showed that only 3-methylindole was able to generate a free radical in the NADPH-dependent microsomal system, as tested by spin-trapping. The splitting constants were the same as those seen with superoxide incubations with 3-methylindole. The study demonstrates the generality of formation of a nitrogen-centered free radical from various indolic compounds. Enzymatic radical formation from 3-methylindole suggests a microsomal-activated free radical mechanism for the specificity of 3-methylindole-induced pulmonary toxicity.

3MI* is the main ruminal fermentation product of L-tryptophan (1,2). Oral or intravenous administration of 3MI has been shown to cause acute pulmonary edema and emphysema in cattle (3), goats (4), and sheep (5). The 3MI-induced toxicity is lung specific. 3MI has also been detected in significant amounts in cigarette smoke (6) although the risk of exposure of 3MI to man has not been investigated.

3MI is rapidly metabolized by the MFO and at least 10 metabolites of 3MI have been found in the urine. The majority of these metabolites are oxidation products such as 3-methyloxindole and its hydroxy derivatives (7).

*Abbreviations: 3MI, 3-methylindole; MFO, mixed function oxidases; PBN, α-phenyl-tert-butyl nitrone; KO_2, potassium superoxide.

0006-291X/83 $1.50

Previous work has shown that 3MI is capable of covalent binding to cellular macromolecules of the lung in vivo (8) and in vitro (9). Other indolic compounds such as indole and 3-methyloxindole do not lead to covalent binding to macromolecules (9), and do not cause acute pulmonary injury (10,11).

A growing number of chemicals are known to be metabolized by MFO into highly reactive intermediates such as free radicals which can attack proteins, DNA or other macromolecules and so interfere with the normal function of cells (12). The present study employed the technique of spin-trapping (13) which relies on the interaction of a reactive radical with a substituted nitrone (spin trap) to yield a stable nitroxide radical (spin adduct) that can be studied by ESR. In this report we have demonstrated that (A) KO_2-catalyzed oxidation of various indolic compounds yields a nitrogen-centered radical adduct of PBN, (B) only 3MI was capable of generating a nitrogen-centered free radical trapped by PBN under microsomal incubation conditions. These observations suggest the involvement of the radical in 3MI-induced pulmonary toxicosis.

Materials and Methods

Chemicals and reagents were purchased from the indicates sources: Aldrich Chemical Co.: 1-methylindole; Eastman Kodak Co.: 3-indoleacetic acid; ICN K&K Laboratories, Inc.: KO_2, indole-3-carbinol; Sigma Chemical Co.: NADPH (type 1), 3-methylindole. PBN was available in the Department of Chemistry, University of Guelph or from Aldrich Chemical Co. 3-Methyloxindole was synthesized (13) in the laboratory of J.R. Carlson, Department of Animal Science, Washington State University. To generate free radicals chemically, 250 mg crushed KO_2 was added to a 6 ml hexane solution containing 0.063 M of indolic compound and 0.05 M PBN and was set at room temperature for 2 hours. The mixture was then dried with anhydrous sodium sulfate and concentrated to 450 µl under a stream of N_2.

Microsomes were prepared from lungs and livers of mature male goats (Farr's Farm Service, Guelph, Ontario). In order to maximize cytochrome P-450-dependent MFO activity, animals were pretreated with phenobarbital. Phenobarbital (sodium salt) was injected intraperitoneally for 3 days at a dose of 100 mg^{-1} per day prior to the sacrifice of the animals. The lungs and livers were homogenized in cold 0.01 M potassium phosphate buffer, pH 7.4 (4.0 ml per g of tissue, wet weight). The microsomal fraction (100,000 x g) was isolated by differential centrifugation as described previously (15). The microsomes were resuspended in the phosphate buffer (0.05 M, pH 7.4) that was used throughout the experiment described herein. Protein concentrations were determined by the Lowry method (16). Unless otherwise noted, the reaction mixtures contained 48 mg of microsomal protein, 0.1M PBN, 0.1 mM EDTA, 0.3 µM NADPH, 0.063 M indolic compound in a total volume of 6 ml of 0.05 M phosphate buffer (pH 7.4). The reaction mixtures were preincubated at 37°C for 5 min before addition of indolic compounds. The incubation reaction was stopped at

10 min after addition of indolic compounds by extraction with hexane. Spin adducts formed in the incubation mixtures were extracted with 2 x 6 ml of hexane which was dried with anhydrous sodium sulfate and concentrated under a stream of N_2 to 450 µl.

The ESR spectrum of the extracted spin-trapped radicals was recorded at room temperature with a Varian E-104 spectrometer. The instrument settings were: microwave power 20 mW; modulation amplitude, 0.1-0.4G; time constant 1S; scan range, 100G; and scan time, 8 min.

Results

A number of indolic compounds were incubated with KO_2 in order to generate free radicals (Table 1). The KO_2-catalyzed oxidation of 3MI, indole-3-carbinol, 3-methyloxindole, 3-indoleacetic acid and indole gave an 18-line ESR spectrum with an additional triplet of doublets (Fig. 1A). This 18-line spectrum was analyzed by computer simulation (Fig. 1C) using hyperfine splitting constants of a_N = 13.9 G, a_β^N = 3.6 G and a_β^H = 2.3 G which are characteristic of a nitrogen-centered PBN adduct (17). The triplet of doublets had hyperfine splitting constants of a_N = 13.9 G and a_β^H = 2.0 G which are consistent with literature values for oxygen-centered PBN adducts (18, 19). The oxygen-centered adduct was formed when PBN alone incubated with KO_2 or with microsomes.

Incubation of goat lung microsomes with 3MI, NADPH and PBN at 37°C resulted in the formation of a nitrogen radical adduct of PBN with ESR hyperfine splitting constants identical with those seen from KO_2-catalyzed autoxi-

Table 1. Hyperfine splitting constants of the spin adducts obtained during KO_2 or MFO catalyzed oxidation of indolic compounds

Indolic Compound	KO_2			MFO		
	A_N	A_β^N	A_β^H	A_N	A_β^N	A_β^H
3-Methylindole	13.9	3.6	2.3	13.9	3.6	2.3
Indole	13.9	3.6	2.3		ND	
1-Methylindole		ND			ND	
3-Methyloxindole	13.9	3.6	2.3		ND	
Indole-3-carbinol	13.9	3.6	2.3		ND	
3-Indoleacetic acid	13.9	3.6	2.3		ND	

ND: not detectable

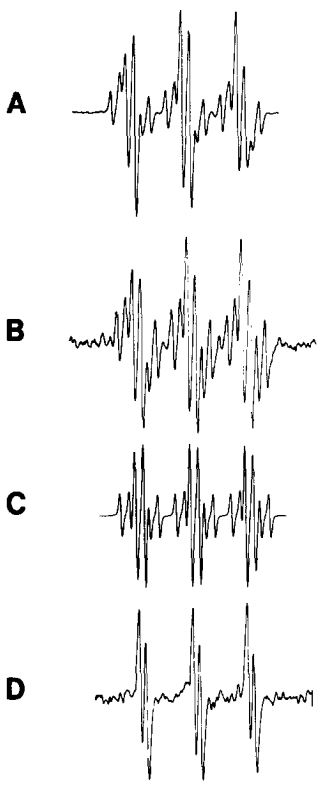

Figure 1. (A) The ESR spectra of 3MI-PBN radical adduct generated by addition of 250 mg crushed KO_2 to a 6 ml hexane solution containing 0.063 M of 3MI and 0.05 M PBN.

(B) The ESR spectra of 3MI-PBN radical adduct obtained from microsomal incubations of 0.063 M 3MI, 0.3 μM NADPH and 0.1 M PBN in a 0.05 M phosphate buffer, pH 7.4.

(C) Computer simulation of 3MI-PBN radical adduct using a_N = 13.8 G, a_β^N = 3.6 G and a_β^H = 2.3 G for the nitrogen-centered adduct and a_N = 13.7 G and a_β^H = 2.0 G for the oxygen-centered adduct.

(D) The ESR spectra obtained upon heat denaturation of microsomes under the same incubation conditions as described in 1(B).

dation of 3MI (Table 1, Fig. 1B). Radical formation in the microsomal incubation of 3MI depended on all three components of the system since omission of NADPH, or 3MI, or thermal inactivation of the microsomes in a steam bath for 20 min, prevented the appearance of the nitrogen free radical signal (Fig. 1D). Replacement of 3MI with any of the other indolic compounds in the microsomal incubations also resulted in the absence of the nitrogen-centered radical signal (Table 1).

Discussion

The results of this investigation show that a 3MI radical intermediate resulting from the metabolism of 3MI is trapped by PBN and the nitroxyl adduct demonstrated by ESR is dependent on the presence of 3MI, NADPH and microsomes. The spectrum of the spin adduct was identical to that observed when 3MI was incubated with KO_2 indicating that a nitrogen-centered 3MI radical was trapped in the microsomal incubations.

Although a nitrogen-centered radical was generated chemically from indole, indole-3-carbinol, 3-methyloxindole, and 3-indoleacetic acid, no radical was observed in the microsomal incubations of these compounds. Indole, like 3MI, has been shown in in vitro systems to disrupt biological membranes (20, 21), cause hemolysis of erythrocytes (22) and cause the release of lysosomal enzymes from rabbit lung cells (23). Although indole and 3MI have qualitatively similar effects on biological membranes and similar chemical properties, only 3MI causes pulmonary injury. This difference in pulmonary toxicity between 3MI and indole could be explained by the differences in microsomal metabolism seen in the present study where indole does not produce a free radical from microsomal metabolism whereas 3MI does. A free radical produced from 3MI may be of significance as free radical intermediates have been shown to be of importance in many examples of tissue injury that require metabolic activation of the primary toxic agent (24).

The products of the major and minor pathways of 3MI metabolism are postulated to be 3-methyloxindole and indole-3-carbinol, respectively (25). Pulmonary injury does not result from infusion of 3-methyloxindole or indole-3-carbinol discounting these metabolites as the cause of 3MI-induced lung injury (11). Investigations into covalent binding of radioactivity from labelled substrates in in vitro microsomal systems, widely used as an indication of the formation of reactive intermediates (26, 27), showed that 3-methyloxindole and indole did not lead to covalent binding in microsomal suspensions (9). The present study supports the above findings as microsomal metabolism of 3-methyloxindole, indole and indole-3-carbinol were not observed to generate

free radicals. 1-Methylindole does not cause disruption of biological membranes (20) and did not generate a nitrogen-centered free radical by KO_2 or microsomal incubations. This may be explained by the fact that the nitrogen in the indole ring is blocked from radical formation by the methyl group.

The exact relationship of radical formation and toxicity of 3MI is not known. However, the ESR spin trapping data presented here is the first evidence for the formation of a nitrogen-centered 3MI radical by the lung microsomal system and provides strong support for the hypothesis that such 3MI radicals are involved in the 3MI-induced pulmonary toxicity.

Acknowledgments

This work is supported by National Science and Engineering Research Council, grant No. A6632 to T.M. Bray and by Ontario Ministry of Agriculture and Food. All correspondences and requests for reprints should be addressed to T.M. Bray, Department of Nutrition, University of Guelph, Guelph, Ontario, N1G 2W1, Canada.

References

1. Yokoyama, M.T. and Carlson, J.R. (1974). Appl. Microbiol. 27, 540-548.
2. Yokoyama, M.T., Carlson, J.R. and Dickinson, E.O. (1975). Am. J. Vet. Res. 36, 1349-1352.
3. Carlson, J.R., Dickinson, E.O., Yokoyama, M.T. and Bradley, B.J. (1975). Am. J. Vet. Res. 36, 1341-1347.
4. Dickinson, E.O., Yokoyama, M.T., Carlson, J.R. and Bradley, B.J. (1976). Am. J. Vet. Res. 37, 667-672.
5. Bradley, B.J., Carlson, J.R. and Dickinson, E.O. (1978). Am. J. Vet. Res. 39, 1355-1358.
6. Hoffman, D. and Rathkamp, G. (1970). Anal. Chem. 42, 366-370.
7. Hammond, A.C., Carlson, J.R. and Willett, J.D. (1979). Life Sci. 25, 1301-1306.
8. Bray, T.M. and Carlson, J.R. (1979). Am. J. Vet. Res. 40, 1268-1272.
9. Bray, T.M. and Carlson, J.R. (1979). Fed. Proc. 38, 1329.
10. Hammond, A.C., Carlson, J.R. and Breeze, R.G. (1980). Vet. Rec. 107, 322-325.
11. Potchoiba, M.J., Carlson, J.R. and Breeze, R.G. (1981). Am. J. Vet. Res. 43, 1418-1423.
12. Gillette, A.R., Mitchel, J.R. and Brodie, B.B. (1974). Annu. Rev. Pharmacol. 14, 271.
13. Janzen, E.G. (1980). In: Free Radicals in Biology (Pryor, W.A., ed.), Vol. 4, p. 115, Academic Press, New York.
14. Hinman, R.L. and Bauman, C.P. (1964). J. Org. Chem. 29, 1206-1215.
15. Mazel, P. (1971). Experiments illustrating drug metabolism in vitro. In: Fundamentals of Drug Metabolism and Disposition (LaDu, B.N., Mandel, H.B., and Way, E.L., eds.), p. 206, Williams and Wilkins, Baltimore.
16. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.D. (1951). J. Biol. Chem. 193, 265-275.
17. Ledwith, A. and Russell, P.J. (1974). J. Chem. Soc., Perkin Trans. II, 582-591.
18. Ohto, N., Niki, E. and Kamiya, Y. (1977). J. Chem. Soc., Perkin Trans II, 1770-1774.

19. Ledwith, A. and Russell, P.J. (1972). J. Chem. Soc., Perkin Trans II, 1925-1928.
20. Bray, T.M., Magnuson, J.A. and Carlson, J.R. (1974). J. Biol. Chem. 249, 914-918.
21. Bray, T.M., Sandberg, H.E. and Carlson, J.R. (1975). Biochim. Biophys. Acta 382, 534-541.
22. Bray, T.M. and Carlson, J.R. (1975). Proc. Soc. Exp. Biol. Med. 148, 875-879.
23. Yang, N.Y.J. and Carlson, J.R. (1977). Proc. Soc. Exp. Biol. Med. 154, 269-273.
24. Mason, R.P. (1979). In: Reactions in Biochemical Toxicology (Hodgson, E., Bend, J.R. and Philpot, R.M., eds.), Vol. 1, pp. 151-200, Elsevier/ North-Holland, Amsterdam and New York.
25. Carlson, J.R. and Bray, T.M. (1983). Nutrition and 3-methylindole-induced lung injury. In: Advances in Nutritional Research (Draper, H.H., ed.), Vol. 5, pp. 31-55, Plenum Publishing Corporation.
26. Dutcher, J.S. and Boyd, M.R. (1979). Biochem. Pharmacol. 28, 3367.
27. Gillette, J.R. (1979). Drug Metab. Reviews 10, 59.