Biochemical Pharmacology, Vol. 23, pp. 2467–2469. Pergamon Press, 1974. Printed in Great Britain.

Relationship between reduced nicotinamide adenine dinucleotide phosphate-dependent lipid peroxidation and drug hydroxylation in rat liver microsomes

(*Received* 10 *December* 1973; *accepted* 1 *March* 1974)

WE HAVE demonstrated that the NADPH-dependent peroxidation of endogenous microsomal lipid, promoted in the presence of ferric ion complexed by ADP or other pyrophosphates, is promoted by the activity of the microsomal flavoprotein, NADPH-cytochrome c reductase.[1] A model NADPH-dependent lipid peroxidation system in which a purified bromelain-solubilized preparation of NADPH-cytochrome c reductase promotes peroxidation of extracted microsomal lipid was also described, and evidence was presented which suggests that NADPH-dependent lipid peroxidation in intact microsomes involves an additional electron transport component whose function in lipid peroxidation can be replaced by ferric ion chelated by EDTA. Microsomal lipid peroxidation has been of interest to other investigators because of its apparent relationship to the microsomal electron transport system which supports cytochrome P-450-catalyzed drug hydroxylation, which also involves the activity of NADPH-cytochrome c reductase,[2] and the degradation of cytochrome P-450 which occurs during lipid peroxidation.[3] We have attempted to establish whether there are other unidentified microsomal electron transport components involved in both NADPH-dependent lipid peroxidation and drug hydroxylation. The implied relationship between lipid peroxidation and drug hydroxylation has previously been based on the observed inhibition of NADPH-dependent lipid peroxidation by drug hydroxylation substrates included in the peroxidation reaction mixture.[4–7] It was shown that several hydroxylation substrates effectively inhibited NADPH-dependent peroxidation but failed to inhibit ascorbate-promoted peroxidation.[4–7] In addition, the inhibition of lipid peroxidation by these substrates could be reversed by the presence of carbon moxoxide, an inhibitor of drug hydroxylation,[6] and it was therefore concluded that lipid peroxidation and drug hydroxylation compete for reducing equivalents derived from the oxidation of NADPH. This communication demonstrates that the inhibition of lipid peroxidation by drugs which are not antioxidants, evidenced by their inability to inhibit ascorbate-promoted peroxidation, is caused by the metabolite formed from the substrate in the presence of NADPH.

Microsomes from the livers of male rats (250 g), fed water containing 0·1% phenobarbital 10 days prior to being killed, were prepared as previously described.[8] The assays for the peroxidation of microsomal lipid were performed in reaction mixtures containing 0·05 M Tris–HCl (pH 7·5 at 37°), 2 mM ADP, 0·12 mM $Fe(NO_3)_3$ and 0·5 microsomal protein/ml. The reaction mixtures were incubated in a Dubnoff shaker at 37° under air and the reactions were initiated by the addition of either an NADPH-generating system, containing 7 mM $MgCl_2$, 2 mM D,L-isocitrate, 0·1 mM $NADP^+$, and 0·05 unit of $NADP^+$-isocitrate dehydrogenase/ml, or 0·2 mM ascorbate. The extent of lipid peroxidation was assayed, using the thiobarbituric acid assay to measure the formation of malondialdehyde as previously described.[1] The reproducibility of the assay is within 5 per cent. Microsomes containing either 3,4-benzpyrene or metapyrone were prepared by adding the compounds, in a small aliquot of acetone, to microsomes at a concentration of 20 mg protein/ml. This produced a finely divided suspension of the compound which was rapidly absorbed by the microsomes. These microsomes were then used in those incubations involving these compounds. 3,4-Benzpyrene was obtained from the Aldrich Chemical Co., Milwaukee, Wis., and metapyrone [(1,2-bis-3-pyridyl)-2-methyl-1-propanone] was obtained from Ciba Pharmaceutical Co., Summit, N.J.

A drug hydroxylation substrate which inhibited NADPH-dependent lipid peroxidation but not ascorbate-promoted peroxidation was 3,4-benzpyrene. As is shown in Table 1, the addition of 20–50 nmoles 3,4-benzpyrene/mg of microsomal protein resulted in almost complete inhibition of NADPH-dependent peroxidation, but caused only slight inhibition of ascorbate-promoted peroxidation. The metabolite formed during the oxidation of 3,4-benzpyrene by liver microsomes is 8-hydroxy-3,4-benzpyrene.[9] Since the inhibition of NADPH-dependent peroxidation could be due to the antioxidant properties of the phenolic metabolite rather than to competition for reducing equivalents, the effect of 3,4-benzpyrene hydroxylation on ascorbate-promoted peroxidation was examined. As shown in Table 2, the presence of benzpyrene inhibited ascorbate-promoted peroxidation of microsomal lipid only when NADPH was included in the reaction mixture. Furthermore, the addition of metapyrone, an inhibitor of cytochrome P-450-catalyzed reactions,[10] reversed the inhibition by benzpyrene of both NADPH and ascorbate-promoted peroxidation. Therefore, the inhibition of NADPH-dependent lipid peroxidation by drug hydroxylation substrates is likely due to the antioxidant properties of either the substrate or its metabolite and not to competition for reducing equivalents.

TABLE 1. INHIBITION BY 3,4-BENZPYRENE OF ASCORBATE AND NADPH-PROMOTED MICROSOMAL LIPID PEROXIDATION*

Concn of benzpyrene (nmoles/mg microsomal protein)	% Inhibition NADPH	Ascorbate
0		
10	10	4
20	81	4
40	98	17
50	98	24

* Either an NADPH-generating system (see text) or 0·2 mM ascorbate was used to promote microsomal lipid peroxidation in the presence of increasing amounts of 3,4-benzpyrene. The peroxidation was measured in reaction mixtures containing 0·5 mg microsomal protein/ml.

As was originally suggested by other investigators,[4–7] microsomal drug hydroxylation and NADPH-dependent lipid peroxidation involve a common electron transport component, NADPH-cytochrome c reductase,[1] but the results in this communication have demonstrated that the original reason for this conclusion was misinterpreted. The possible involvement of additional drug hydroxylation remains unresolved. Coon *et al.*[11] have shown that the purified, bromelain-solubilized reductase will also support drug hydroxylation in a reconstituted system containing a partially purified preparation of cytochrome P-450 and added phosphatidyl choline. But they also found that the purified reductase failed to support hydroxylation when a more purified preparation of cytochrome P-450 was used. We obtained samples of these preparations of cytochrome P-450 from Dr. M. J. Coon at the University of Michigan and attempted to reconstitute microsomal lipid peroxidation. However, these preparations of cytochrome P-450 and a purified preparation of cytochrome b_5 did not obviate the requirement for ferric ion chelated by EDTA when using the purified preparation of the reductase and extracted microsomal lipid.* The purified reductase also failed to promote lipid peroxidation by microsomes treated with *N*-ethylmaleimide to inactivate endogenous reductase unless ferric ion chelated by EDTA was also present.[1] Therefore, the eventual identification of all the microsomal components involved in NADPH-dependent lipid peroxidation will apparently require purification of a detergent-solubilized form of the reductase and determination of the conditions required for re-coupling the purified reductase with the normal electron transport system.

TABLE 2. INHIBITION BY 3,4-BENZPYRENE OF ASCORBATE-PROMOTED LIPID PEROXIDATION IN THE PRESENCE OF NADPH*

Description	Malondialdehyde formed (nmoles/min/ml) Control	+ Benzpyrene	% Inhibition
Control microsomes			
Plus NADPH	2·78	0·05	98
Plus ascorbate	6·42	6·35	1
Plus ascorbate and NADPH	6·20	0·12	98
Microsomes with metapyrone			
Plus NADPH	2·62	2·10	20
Plus ascorbate and NADPH	6·10	4·30	29

* Where indicated, the reaction mixtures contained 2 μmoles metapyrone and 20 nmoles 3,4-benzpyrene/mg of microsomal protein. All other conditions are the same as those described in Table 1.

* Unpublished observations.

Acknowledgement—This research was supported in part by NIH Training Grant G.M. 1091 from the National Institute of General Medical Sciences and by National Science Foundation Grant GB-40711.

Department of Biochemistry,
Michigan State University,
East Lansing, Mich. 48824, *U.S.A.*

THOMAS C. PEDERSON
STEVEN D. AUST

REFERENCES

1. T. C. PEDERSON, J. A. BUEGE and S. D. AUST, *J. biol. Chem.* **248,** 7134 (1973).
2. B. S. S. MASTERS, J. BARON, W. E. TAYLOR, E. L. ISAACSON and J. LASPALLUTO, *J. biol. Chem.* **246,** 4143 (1971).
3. W. LEVIN, A. Y. H. LU, M. JACOBSON, R. KUNTZMAN, J. L. POYER and P. B. MCCAY, *Archs Biochem. Biophys.* **158,** 842 (1973).
4. S. ORRENIUS, G. DALLNER and L. ERNSTER, *Biochem. biophys. Res. Commun.* **14,** 329 (1964).
5. T. E. GRAM and J. R. FOUTS, *Archs Biochem. Biophys.* **114,** 331 (1965).
6. L. ERNSTER and K. NORDENBRAND, in *Methods in Enzymology* (Eds. R. W. ESTABROOK and M. E. PULLMAN), Vol. X, p. 574. Academic Press, New York (1967).
7. E. D. WILLS, *Biochem. J.* **113,** 333 (1969).
8. T. C. PEDERSON and S. D. AUST, *Biochem. Pharmac.* **19,** 2221 (1970).
9. A. H. CONNEY, E. C. MILLER and J. A. MILLER, *J. biol. Chem.* **228,** 753 (1957).
10. A. G. HILDEBRANDT, M. R. FRANKLIN, I. ROOTS and R. W. ESTABROOK, *Chem. Biol. Interact.* **3,** 276 (1971).
11. M. J. COON, H. W. STROBEL and R. F. BOYER, *Drug Metab. Dispos.* **1,** 92 (1973).

Biochemical Pharmacology, Vol. 23, pp. 2469–2472. Pergamon Press, 1974. Printed in Great Britain.

Metabolism of N^6-(Δ^2-isopentenyl)adenosine in rat liver

(*Received* 10 *November* 1973; *accepted* 28 *February* 1974)

N^6-(Δ^2-ISOPENTENYL)ADENOSINE (IPAR) is a modified ribonucleoside which has been found in tRNA of plants, micro-organisms and mammalian tissues.[1] In rats treated with toxic doses, IPAR produces transient lymphoid depression and hepatoxicity as well as antiproliferative effects in intestinal mucosa, bone marrow and regenerating liver.[2] Although the antitumor activity of IPAR is slight, complete but short-lasting bone marrow remissions have been obtained with IPAR in one patient with acute promyelocytic leukemia.[3] Studies designed to correlate cytotoxic effects of IPAR with early biochemical lesions induced by the drug indicate that the hepatoxicity in rats may be related to the rapid and prolonged inhibition of incorporation of ^{14}C-phenylalanine into liver protein.[4] This inhibition appeared to be relatively specific for liver, since spleen, thymus and small intestine were almost unaffected.[4]

IPAR is a substrate for adenosine kinase[5] and is also an inhibitor of cellular multiplication in culture.[6] In mammalian systems such as leukemia L1210, sarcoma 180, mammary carcinoma TA3 and canine kidney, IPAR has been reported to be phosphorylated to the 5′-monophosphate level[6,7] and is suggested to exert its inhibitory effects in that form.[6,8]

This report describes a study of the levels of phosphorylation of IPAR-8-^{14}C in rat liver.

A DEAE-cellulose column chromatographic method[9] has been employed for the separation of liver ribonucleotides present in the acid-soluble fraction of a rat treated i.v. with labeled adenosine. The results of such a chromatographic separation are shown in Fig. 1. In this chromatogram, four sharp and symmetrical ^{14}C-labeled ribonucleotide peaks were identified as IMP, AMP, ADP and ATP. The results also show the presence of one large labeled nucleoside peak, AR (Fraction 11–22), and two other peaks not yet identified (Fractions 5–10 and 24–34). No detectable labeling of guanosine nucleotides was observed. Other peaks shown in this chromatogram which are not ^{14}C-labeled are eluted in the general area of pyrimidine nucleotides. Identification of labeled peaks was based on their distinct 280/260 nm absorbance