SUBSTRATE SPECIFICITIES IN ETHER LIPID BIOSYNTHESIS. METABOLISM OF POLYUNSATURATED FATTY ACIDS AND ALCOHOLS BY RAT BRAIN MICROSOMES

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Received October 3,1977

SUMMARY

Reduction of fatty acids having one to four double bonds per molecule to the corresponding alcohols, and the utilization of such alcohols for alkyl dihydroxyacetone phosphate (alkyl DHAP) synthesis was measured with microsomal preparations from 19-day-old rat brain. While alkyl DHAP formation proceeded well with octadecenol, octadecadienol, octadecatrienol and eicosatetraenol, fatty acids with more than one cis-double bond were not readily reduced to the corresponding alcohols.

INTRODUCTION

It is now well established that fatty alcohols are precursors of the alkyl and alk-1-enyl groups of mammalian phosphoglycerides (1). Previous work from our laboratory has shown that a variety of substituted (2-7) or polyunsaturated (8) primary alcohols injected into developing rat brain can be incorporated into the corresponding alkyl (2-7) and alk-1-enyl (7-9) moieties. However, the free alcohols (10-12) as well as the constituent alkyl and alk-1-enyl groups of most mammalian tissues (13), including rat brain, are saturated and monounsaturated. It can, therefore, be assumed that the reduction of fatty acids to alcohols exhibits greater substrate specificity than the utilization of fatty alcohols for ether lipid biosynthesis.

In this communication we report that the presence of more

ISSN 0006-291X

than one cis-double bond in fatty acids markedly decreases their reduction to the corresponding alcohols by rat brain microsomes, but that polyunsaturated alcohols are readily incorporated into alkyl dihydroxyacetone phosphate (alkyl DHAP), the first intermediate in ether lipid synthesis.

EXPERIMENTAL

[1-^{14}C]cis-9-Octadecenoic (oleic) acid, [1-^{14}C]cis,cis,cis-9,12,15-octadecatrienoic (linolenic) acid and [1-^{14}C]cis,cis,cis,cis-5,8,11,14-eicosatetraenoic (arachidonic) acid were purchased from Dhom Products, Ltd., North Hollywood, CA; [1-^{14}C] cis,cis-9,12-octadecadienoic (linoleic) acid was from Applied Science Laboratories, Inc., State College, PA. Fatty alcohols were prepared by $LiAlH_4$ reduction of the corresponding fatty acid methyl esters. Before use as a substrate each fatty acid and alcohol was checked for radiopurity by thin-layer chromatography (TLC; Silica Gel H, Merck; hexane-diethyl ether-acetic acid, 80:20:1) and gas-liquid chromatography (GLC; 10% EGSS-X on 100-120 mesh Gas Chrom P, Applied Science Laboratories, Inc.) of the methyl ester or acetate, respectively. They were purified by TLC to a radiopurity of better than 98% if necessary. Unlabeled fatty acids were obtained from Nu-Chek Prep, Inc., Elysian, MN. The polyunsaturated fatty alcohols were prepared by $LiAlH_4$ reduction of the corresponding fatty acid methyl esters as described above.

1-Hexadecanoyl DHAP was prepared according to Hajra and Agranoff (14) via 1-hexadecyloxy-3-diazoacetone (15) and stored as the cyclohexylamine salt. It was regenerated by HCl treatment (14). ATP, Coenzyme A and fatty acid poor bovine serum albumin (fraction V) were from Sigma Chemical Co., St. Louis, MO; NADPH and dithiothreitol were from P-L Biochemicals, Milwaukee, WI; palmitoyl chloride, glycolic acid and oxalyl chloride were from Aldrich Chemical Company, Inc., Milwaukee, WI.

Brains from 19-day-old rats (male albino, Sprague Dawley, Madison, WI) were homogenized in 0.25 M sucrose (10 ml/g tissue) with a Potter Elvehjem homogenizer. The homogenate was centrifuged at 1000 x g for 10 min and the supernatant at 22,000 x g for 30 min in a Sorvall RC2-B centrifuge to remove the crude mitochondrial fraction. The post-mitochondrial supernatant and one washing of the crude mitochondrial pellet were pooled and centrifuged at 105,000 x g for 1 hr in a Beckman L5-75 ultracentrifuge to obtain the microsomal fraction (16). All the operations were carried out between 0 - 4°C and only fresh, unwashed microsomes were used for the assay. Protein was determined according to Lowry et al. (17) with bovine serum albumin as standard.

Incubations were carried out for 1 hr at 37°C with continuous shaking. The substrates and cofactors (Tables I and II) were sonicated in the appropriate buffer solution for 30 sec (Cole-Parmer Ultrasonic Cleaner, Model 8845-3) and were preincubated

TABLE I. Incorporation of radioactivity from fatty acids into alcohols by rat brain microsomes

Fatty acid[a]	Spec. act (dpm x 10^{-3}/nmol)	Radioactivity (dpm x 10^{-5} per incubation)	Radioactivity[b] (dpm) per incubation in alcohol		Alcohol[c] formed (nmol/h/mg protein)
			I	II	
18:1	5.09	5.27	1847	1585	0.52
18:2	5.31	5.56	215	164	0.06
18:3	6.64	7.04	196	159	0.04
20:4	6.91	7.30	273	293	0.06

[a] Each incubation also contained 10 µl ethanol; CoA (0.63 mM); ATP (11.3 mM); $MgCl_2$ (11.3 mM); NADPH (2.5 mM); dithiothreitol (5 mM); bovine serum albumin (1 mg); 0.2 M phosphate buffer, pH 7.4; and rat brain microsomes (0.69 mg protein in Experiment I and 0.59 mg protein in Experiment II) in a total volume of 0.8 ml.

[b] The values are the average of two determinations minus the blanks obtained with boiled enzyme controls.

[c] The values are the average of Experiments I and II.

for 5 min at 37°C. Reactions were started by adding microsomal protein and terminated by adding chloroform-methanol, 1:2 (v/v), and the lipids were extracted (18).

The conversion of fatty acids to alcohols was assayed after alkaline hydrolysis of the total lipids: The lipid extract was dried under a stream of nitrogen, 1 ml methanol and 0.1 ml of 0.4M NaOH were added and the mixture was kept at room temperature for 20 min. It was then neutralized with 0.1 M HCl, the lipids extracted (18) and 50 µg of unlabeled fatty alcohol was added. The lipids were fractionated by TLC using hexane-diethyl ether-acetic acid, 70:30:1, the alcohol fraction was scraped off and its radioactivity was measured with Permablend in toluene (84 - 86% counting efficiency) in a Packard Tri-Carb liquid scintillation spectrometer.

Alkyl DHAP formation was assayed after fractionation of the acidic (19) lipid extract (18) by TLC using chloroform-methanol-ammonium hydroxide, 65:35:8 as described (20) or layers of Silica Gel H containing EDTA (1% by weight) with chloroform-acetone-methanol-acetic acid-water, 50:15:30:10:8, as solvent. Radioactivity was measured in Aquasol (69 - 71% counting efficiency).

TABLE II. Incorporation of radioactivity from fatty alcohols into alkyl DHAP by rat brain microsomes

Alcohol[a]	Spec. act. (dpm x 10^{-3}/nmol)	Radioactivity (dpm x 10^{-5} per incubation)	Radioactivity[b] (dpm) per incubation in alkyl DHAP		Alkyl DHAP[c] formed (nmol/h/mg protein
			I	II	
18:1	4.17	5.50	7932	6495	2.32
18:2	2.90	3.72	7028	6476	3.17
18:3	2.26	3.07	4851	4726	2.80
20:4	2.15	2.47	1997	1623	1.10

[a]Each incubation also contained hexadecanoyl DHAP (0.058 mM); ATP (7.5 mM); $MgCl_2$ (7.5 mM); NaF (7 mM); dithiothreitol (3.33 mM); bovine serum albumin (1 mg); 0.1 M Tris-HCl buffer, pH 7.2, and rat brain microsomes (0.78 mg protein in Experiment I and 0.71 mg in Experiment II) in a total volume of 1.2 ml.

[b]The values are the average of two determinations minus the blanks obtained in the absence of acyl DHAP.

[c]The values are the average of Experiments I and II.

RESULTS AND DISCUSSION

When microsomal preparations from 19-day-old rat brain were incubated with [1-^{14}C]labeled fatty acids and the cofactors listed in Table I, it was found that two or more double bonds present in the fatty acid made its reduction to the corresponding alcohol considerably more difficult. In contrast, polyunsaturated alcohols were found to be excellent substrates for alkyl-DHAP formation as demonstrated by the data listed in Table II. The presence of fluoride ions in the incubation mixture apparently inhibited the hydrolysis of acyl or alkyl DHAP and, therefore, labeled alkyl DHA could not be detected.

Since polyunsaturated structures are not found among the free alcohols (10-12) or the alkyl and alk-1-enyl moieties of

ether lipids (12), it has long been assumed that the acyl-CoA reductase (21,22) which produces fatty alcohols in mammalian systems does not act upon polyunsaturated substrates, but experimental evidence has not been published so far. In the present work we used rat brain microsomes which were previously shown (22) to reduce palmitic, stearic and oleic acids to the corresponding alcohols in the presence of NADPH, CoA, ATP and Mg^{2+}. We have found (23) that this system also reduces other saturated fatty acids ranging in chain length from 12 to 22 carbon atoms. Our present data show that only minimal amounts of polyunsaturated alcohols were formed under the same conditions; in fact, the levels of radioactivity in the reaction products were too low to permit unequivocal structure determinations by GLC. Microsomal preparations from developing rat brain are known (24) to contain both fatty acid-CoA ligase (EC 6.2.1.3) and acyl-CoA hydrolase (EC 3.1.2.2). At the age of 18-20 days the ligase appears to be rather active for a variety of fatty acids (24) and the hydrolase is inhibited by bovine serum albumin; we can therefore assume that the lack of reduction in our system was not due to a lack in acyl-CoA formation.

It is interesting that even the tetraunsaturated alcohol was rather efficiently incorporated into alkyl DHAP; the di- and triunsaturated alcohols were even more effective precursors of alkyl DHAP than the naturally occurring octadecenol. It has been noted that dietary octadecadienol is incorporated as such into ether lipids of rat intestine (25) and liver (26), and intracerebrally injected octadecadienol and -trienol were found to be incorporated into alkyl and alk-1-enyl moieties at rates comparable to octadecenol (5).

ACKNOWLEDGMENTS

This investigation was supported by PHS Research Grant No. NS 10013, PHS Research Grant No. HL 08214 from the Program Projects Branch, Extramural Programs, National Heart, Lung and Blood Institute, and by the Hormel Foundation.

REFERENCES

1. Wykle, R. L., and Snyder, F. (1976) in The Enzymes of Biological Membranes (Martonosi, A., ed.) Vol. 2, pp. 87-117, Plenum Press, New York.
2. Muramatsu, T., and Schmid, H. H. O. (1971) J. Lipid Res. 12, 740-746.
3. Muramatsu, T., and Schmid, H. H. O. (1973) Biochim. Biophys. Acta 296, 265-270.
4. Chang, N., Muramatsu, T., and Schmid, H. H. O. (1973) Biochim. Biophys. Acta 306, 437-445.
5. Su, K. L., and Schmid, H. H. O. (1975) Biochim. Biophys. Acta 380, 119-126.
6. Chang, N., and Schmid, H. H. O. (1975) J. Biol. Chem. 250, 4877-4882.
7. Schmid, H. H. O., Bandi, P. C., Chang, N., Madson, T. H., and Baumann, W. J. (1975) Biochim. Biophys. Acta 409, 311-319.
8. Su, K. L., and Schmid, H. H. O. (1972) J. Lipid Res. 13, 452-457.
9. Schmid, H. H. O., Bandi, P. C., Madson, T. H., and Baumann, W. J. (1977) Biochim. Biophys. Acta 488, 172-178.
10. Takahashi, T., and Schmid, H. H. O. (1970) Chem. Phys. Lipids 4, 243-246.
11. Blank, M. L., and Snyder, F. (1970) Lipids 5, 337-341.
12. Natarajan, V., and Schmid, H. H. O. (1977) Lipids 12, 128-130.
13. Horrocks, L. A. (1972) in Ether Lipids. Chemistry and Biology (Snyder, F., ed.) Academic Press, New York and London.
14. Hajra, A. K., and Agranoff, B. W. (1968) J. Biol. Chem. 243, 1617-1622.
15. Schlenk, H., Lamp, B. G., and De Haas, B. W. (1952) J. Am. Chem. Soc. 74, 2550-2552.
16. Natarajan, V., and Sastry, P. S. (1974) J. Neurochem. 23, 187-192.
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
18. Bligh, E. G., and Dyer, W. J. (1959) Can. J. Biochem. Physiol. 37, 911-917.
19. Hajra, A. K. (1974) Lipids 9, 502-505.
20. Natarajan, V., and Sastry, P. S. (1975) Ind. J. Biochem. Biophys. 12, 340-350.
21. Snyder, F., and Malone, B. (1970) Biochem. Biophys. Res. Commun. 41, 1382-1387.
22. Natarajan, V., and Sastry, P. S. (1976) J. Neurochem. 26, 107-113.
23. Natarajan, V., and Schmid, H. H. O. (1977) Fed. Proc. 36, 851.
24. Brophy, P. J., and Vance, D. E. (1976) Biochem. J. 160, 247-251.
25. Bandi, Z. L., Mangold, H. K., Hølmer, G., and Aaes-Jørgensen, E. (1971) FEBS Lett. 12, 217-220.
26. Bandi, Z. L., Aaes-Jørgensen, E., and Mangold, H. K. (1971) Biochim. Biophys. Acta 239, 357-367.