The effect of essential-fatty-acid deficiency on the activity of liver phosphatidate phosphatase in rats

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The activities of the soluble and microsomal phosphatidate phosphatases (EC 3.1.3.4) are greater in the livers of essential-fatty-acid-deficient rats than in rats fed diets containing linoleic acid

Essential-fatty-acid-deficient rat livers secrete triacylglycerol into blood plasma more rapidly than do rats fed an adequate amount of linoleic acid (Sinclair & Collins, 1968; Huang & Williams, 1980). In contrast, Fukazawa *et al.* (1970), in studies of rat livers perfused with oleate or linoleate, found that the triacylglycerol concentration was greater in perfusates of control than of essential-fatty-acid-deficient rats. This finding may have been due to liver damage during perfusion (De Pury & Collins, 1972) or to differences between the blood perfusing the liver *in vivo* and the perfusion medium *in vitro*.

If hepatic glycerolipid synthesis is increased in livers of essential-fatty-acid-deficient rats, it may be due to increased availability to the liver of fatty acid from lipolysis of triacylglycerol in adipose tissue, decreased hepatic oxidation of fatty acids or increased hepatic lipogenesis (Huang & Williams, 1980). Increased triacylglycerol synthesis may also result from increased activity of hepatic enzymes in the triacylglycerol synthesis pathway (Brindley, 1978). The activity of one of these enzymes, phosphatidate phosphatase, appears more closely correlated with changes in the rate of triacylglycerol synthesis observed *in vivo* than are the activities of other enzymes in the pathway (Fallon *et al.*, 1977; Brindley, 1978).

The present paper describes the effect of an essential-fatty-acid deficiency on the activity of phosphatidate phosphatase in the cytosolic and microsomal fractions of liver.

Experimental

Materials

[1-¹⁴C]Palmitic acid was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Sodium phosphatidate and phosphatidylcholine from egg, dithiothreitol, DL-glycerophosphate, Tris/maleate, CoA (grade III-s) and bovine serum

albumin (fraction V) were from Sigma Chemical Co., St. Louis, MO, U.S.A. Disodium ATP was from Calbiochem-Behring Corp., La Jolla, CA, U.S.A. Cellex-D was from Bio-Rad Laboratories, Richmond, CA, U.S.A.

Animals

Male weanling Sprague-Dawley rats (Simonsen Farm, Gilroy, CA, U.S.A.) weighing approx. 50 g were housed in suspended, galvanized, screen-wire cages with tap water supplied *ad libitum*. One group of 10 rats was fed a 20% casein/sucrose diet (Williams *et al.*, 1972) containing 5% hydrogenated coconut oil that is lacking essential fatty acids. A second group of 10 rats was fed the same diet with 5% corn oil replacing the 5% hydrogenated coconut oil. A third group of 10 rats, as an additional control, was fed a pelleted natural ingredient stock ration with a crude fat content of 4.5% (Purina Rat Chow; Ralston Purina Co., St. Louis, MO, U.S.A.). The rats were maintained on these dietary treatments for 7 weeks. The fatty acid composition of liver phospholipids was determined as described below. The triene/tetraene ratio of liver phospholipids of the hydrogenated-coconut-oil-fed rats was greater than 3, indicating essential-fatty-acid deficiency (Friedman, 1980).

Preparation of [¹⁴C]phosphatidate

Membrane-bound phosphatidate was prepared by incubating liver microsomes from stock controls with [¹⁴C]palmitate and appropriate substrate cofactors and fluoride as described by Brindley & Bowley (1975). Lipids were extracted from the microsomes by the method of Sánchez *et al.* (1971). Phosphatidic acid was purified on DEAE-cellulose by the method of Rouser *et al.* (1967) and stored in chloroform at -20°C. The specific radioactivity of the phosphatidate preparation was determined by separating phosphatidate from other phospholipids

by the method of Skipski *et al.* (1964) and measurement of phospholipid phosphorus (Ames, 1966). Radioactivity was measured as described for the phosphatase assay below.

Preparation of phosphatide/phosphatidylcholine emulsions

Appropriate amounts of phosphatidic acid and phosphatidylcholine solutions were dried and dispersed in deionized water. Emulsions were formed by sonication at half power with a Sonifier (model w140D) sonicator (Ultrasonic Inc., Plainview, NY, U.S.A.) for 2 min under N₂ in a tube on ice.

Preparation of microsomal and particle-free supernatant fractions

Rats were decapitated between 09:00 and 10:00 h. The right lobe of the liver was removed, weighed, minced with scissors and homogenized by three strokes of a plastic/glass hand homogenizer (Wesley Coe, Cambridge, U.K.). The homogenate was centrifugally fractionated as described by Mangiapane *et al.* (1973). The particle-free supernatant (100 000 g) was dialysed against 1 litre of 0.3 M-sucrose, pH 7.4, containing 1 mM-dithiothreitol for 20 h at 0–4°C.

Phosphatide phosphatase assays

Phosphatide phosphatase was assayed by measurement of the conversion of phosphatidic acid into diacylglycerol and phosphate. Each assay contained, in 0.25 ml, 20 mM-Tris/maleate buffer, pH 6.4, 1 mM-dithiothreitol, 2 mM-MgCl₂, 0.9 mM-potassium phosphatide and 0.6 mM-phosphatidylcholine. The soluble-enzyme assay contained 120–140 µg of protein. Triplicate assays were incubated at 37°C for 10 min. The reaction was stopped with 0.25 ml of 0.6 M-trichloroacetic acid, cooled on ice for 1 h and centrifuged at 2°C and 4000 g for 5 min in an SM 24 rotor of the RC2-B centrifuge. The phosphate concentration of a 0.4 ml portion of the supernatant was determined by the method of Ames (1966). The reaction was linear with time for 15 min.

The microsomal assay contained the same reagents except that 6.3 nCi of potassium [¹⁴C]phos-

phatide was added in preparation of the emulsion and 120–140 µg of microsomal protein was added in place of the soluble protein. The reaction was stopped with 0.94 ml of chloroform/methanol (1:2, by vol.). Lipids were extracted by the method of Sánchez *et al.* (1971) and separated on t.l.c. plates of Kiesegel 60 (Merck, Darmstadt, Germany). The developing solvent was light petroleum (b.p. 35–60°C)/diethyl ether/acetic acid (77:23:1, by vol.). The spots were detected visually under u.v. light after spraying with 0.4% 2,4-dichlorofluorescein in ethanol. Spots corresponding to diacylglycerols were scraped into counting vials. Radioactivity was measured in a Beckman LS-100 scintillation counter with toluene containing 0.5% 2,5-diphenyloxazole as scintillation fluid. The reaction was linear with time for 15 min.

Fatty acid analysis

Lipids were extracted by the method of Folch *et al.* (1957) with 0.58% NaCl solution added to the chloroform/methanol extract and were separated by t.l.c. as described above. Bands corresponding to phospholipids were scraped into screw-cap tubes containing 0.15 M-H₂SO₄ in methanol, sealed and transmethylated for 2 h at 85°C. Methyl esters were extracted with light petroleum. Gas chromatography of the methyl esters was performed on a Varian Aerograph model 2100 chromatograph equipped with hydrogen flame detectors and silanized columns packed with 10% SP-216-PS on 100/120 Supelcoport (Supelco, Bellefonte, PA, U.S.A.). Methyl esters were identified by comparison of their relative retention times with those of authentic methyl ester standards (Applied Science Labs., St. College, PA, U.S.A.). Protein was determined by the method of Lowry *et al.* (1951) as described by Layne (1957). Statistical significance of results was calculated by one-way analysis of variance and Duncan's (1955) multiple range test. The α level was set at 0.05.

Results

Body weight gains, food intakes and liver weight per body weights are shown in Table 1. The rats fed

Table 1. *Effect of diet on body weight gain, food intake and liver weight per body weight*

Male rats were fed hydrogenated-coconut-oil, corn-oil or stock diets for 7 weeks. Results are means $\pm$ S.E.M. for 10 rats per diet group. Values not sharing a common superscript are significantly different ($P < 0.05$). N.D., not determined.

Diet	Food intake (g/day)	Weight gain (g)	Food intake [g/(mean body wt.) ^{0.75} per day]		100 $\times$ (Liver wt./body wt.)
			First 3 weeks	Second 3 weeks	
Coconut oil	14.7 $\pm$ 2.5 ^a	203 $\pm$ 41 ^a	0.39 $\pm$ 0.03 ^a	0.56 $\pm$ 0.05 ^a	5.2 $\pm$ 0.3
Corn oil	16.2 $\pm$ 1.6 ^a	258 $\pm$ 35 ^b	0.36 $\pm$ 0.01 ^a	0.52 $\pm$ 0.04 ^a	5.0 $\pm$ 0.4
Stock	N.D.	332 $\pm$ 35 ^c	N.D.	N.D.	5.0 $\pm$ 0.3

Table 2. *Effect of diet on the soluble and microsomal activity of phosphatidate phosphatase*

Enzyme activity (mean $\pm$ S.E.M.) was measured in the particle-free supernatant (soluble 100000g) and microsomal fractions as described in the Experimental section. The number of animals in each group is shown in parentheses. Values within each fraction not sharing a common superscript are significantly different ($P < 0.05$).

Fraction	Phosphatidate hydrolysed (nmol/min per mg of protein)			
	Diets ...	Coconut oil	Corn oil	Stock
Soluble		7.5 ± 2.4 (6) ^a	2.7 ± 0.5 (6) ^b	1.5 ± 2.0 (6) ^b
Microsomal		4.87 ± 0.65 (6) ^a	2.17 ± 0.66 (7) ^b	1.94 ± 0.98 (7) ^b

hydrogenated coconut oil gained less weight than the corn-oil- or stock-fed rats but did not eat significantly less food than the corn-oil-fed rats either in absolute amount or per (body weight)^{0.75}. The proportion of liver weight to body weight was similar for all groups.

The effects of diet on the activity of soluble and microsomal phosphatidate phosphatase are summarized in Table 2. The activity of the enzyme in both fractions was significantly greater in rats fed the coconut oil diet than in rats fed the corn oil or stock diets.

Discussion

The activity of hepatic phosphatidate phosphatase parallels the rate of triacylglycerol synthesis (Fallon *et al.*, 1977; Brindley, 1978) in rats on various diets. Phosphatase activity and triacylglycerol synthesis are greater in rats fed diets containing 75% fructose compared with 75% glucose (Lamb & Fallon, 1974) and also are greater in rats fed ethanol than in rats fed an isocaloric amount of glucose (Pritchard *et al.*, 1977). Hepatocytes incubated with 1 mM-ethanol for 24 h have greater phosphatase activity (Wood & Lamb, 1979). Substitution of 20% lard for 20% starch in a diet also containing 5% corn oil resulted in greater soluble phosphatase activity (Glenny & Brindley, 1978) suggesting that increased saturation and content of fat in the diet increases triacylglycerol synthesis. These findings agree with those of the present study, which show that feeding a coconut-oil diet, and the resultant essential-fatty-acid deficiency, causes increased specific activity of soluble and microsomal phosphatidate phosphatase relative to corn-oil-fed controls. This treatment also results in greater activity of glucose 6-phosphate dehydrogenase and fatty acid synthetase (Williams *et al.*, 1972). These changes may contribute to the greater triacylglycerol secretion observed by Huang & Williams (1980).

The body weight gains of the corn-oil-fed rats were less than those of the stock-fed group. This difference indicates that the diet of the corn-oil-fed

group was suboptimal in some way. The cause of the difference in weight gain and its effect on phosphatidate phosphatase is not known.

Greater phosphatidate phosphatase activity occurs during starvation, stress and diabetes, which also are associated with greater plasma non-esterified fatty acid concentration (Glenny & Brindley, 1978). This relationship may occur during essential-fatty-acid deficiency. Several groups have reported that plasma fatty acid concentration is greater in essential-fatty-acid-deficient rats than in controls (DePury & Collins, 1965; Bergstrom & Carlson, 1965; Sinclair & Collins, 1968). The stock-fed rats had lower phosphatase activity. Although the stock diet contained similar proportions of fat, protein and carbohydrate as the semi-purified diets, the dissimilar types of nutrients, such as sucrose in the semi-purified diet and starch in the stock diet, prevent explanation of these differences.

A mechanism by which phosphatidate phosphatase specific activity may be increased in essential-fatty-acid-deficient rat liver is suggested by the findings of Brindley and his co-workers. Actinomycin D blocks the rise in activity after partial hepatectomy, indicating that the increase in activity may be due to synthesis of enzyme (Mangiapanne *et al.*, 1973). Brindley *et al.* (1979) have reported that various diets that increase serum corticosterone also increase hepatic phosphatase activity and triacylglycerol synthesis.

Several methods have been used to measure phosphatidate phosphatase. An emulsion of phosphatidate and phosphatidylcholine was used as substrate for the microsomal and soluble enzymes in the present study. Sturton & Brindley (1980) found similar results when the activities of the soluble and microsomal phosphatases were determined with phosphatidate in either an emulsion or membrane-bound. The microsomal phosphatase activity has also been determined with endogenously labelled phosphatidate as substrate (Lamb & Fallon, 1974). It is unknown whether this system might yield different results from those found in the present study. Sturton & Brindley (1978) have warned that measurement of phosphate release from phosphati-

date overestimates the microsomal activity. Phosphate is produced not only by the phosphatase but also by dephosphorylation of glycerol phosphate formed by deacylation of phosphatidate. This method was used by Iritani & Fukuda (1980) to measure the effect of increasing dietary corn oil from 0.5% to 10% of the diet on hepatic microsomal phosphatidate phosphatase. Although triacylglycerol synthesis decreased with increasing dietary corn oil, no effect was observed on phosphatidate phosphatase.

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References

- Ames, B. N. (1966) *Methods Enzymol.* **8**, 115–118
- Bergstrom, S. & Carlson, L. A. (1965) *Acta Physiol. Scand.* **64**, 479–480
- Brindley, D. N. (1978) in *Regulation of Fatty Acid and Glycerolipid Metabolism* (Dils, R. & Knudsen, J., eds.), pp. 31–40, Pergamon Press, New York and Oxford
- Brindley, D. N. & Bowley, M. (1975) *Biochem. J.* **148**, 461–469
- Brindley, D. N., Cooling, J., Burditt, S. L., Pritchard, P. H., Pawson, S. & Sturton, R. G. (1979) *Biochem. J.* **180**, 195–199
- DePury, G. G. & Collins, F. D. (1965) *Biochim. Biophys. Acta* **106**, 213–214
- DePury, G. G. & Collins, F. D. (1972) *J. Lipid Res.* **13**, 268–275
- Duncan, D. B. (1955) *Biometrics* **11**, 1–42
- Fallon, H. J., Lamb, R. G. & Jamdar, S. C. (1977) *Biochem. Soc. Trans.* **5**, 37–40
- Folch, J., Lees, M. & Sloane Stanley, G. M. (1957) *J. Biol. Chem.* **226**, 497–506
- Friedman, Z. (1980) *Am. J. Dis. Child.* **134**, 394–408
- Fukazawa, T., Privett, O. S. & Takahashi, Y. (1970) *J. Lipid Res.* **11**, 522–527
- Glenny, H. P. & Brindley, D. N. (1978) *Biochem. J.* **176**, 777–784
- Huang, M. T. & Williams, M. A. (1980) *Am. J. Physiol.* **238**, E499–E505
- Iritani, N. & Fukuda, E. (1980) *J. Nutr.* **110**, 1138–1143
- Lamb, R. G. & Fallon, H. J. (1974) *Biochim. Biophys. Acta* **348**, 179–188
- Layne, E. (1957) *Methods Enzymol.* **3**, 447–528
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mangiapane, E. H., Lloyd-Davies, K. A. & Brindley, D. N. (1973) *Biochem. J.* **134**, 103–112
- Pritchard, P. H., Bowley, M., Burditt, S. L., Cooling, J., Glenny, H. P., Lawson, N., Sturton, R. F. & Brindley, D. N. (1977) *Biochem. J.* **166**, 639–642
- Rouser, G. G., Kritchevsky, G. & Yamamoto, A. (1967) *Lipid Chromatogr. Anal.* **1**, 99–162
- Sánchez, M., Nicholls, D. G. & Brindley, D. N. (1971) *Biochem. J.* **132**, 697–706
- Sinclair, A. J. & Collins, F. D. (1968) *Biochim. Biophys. Acta* **152**, 498–510
- Skipski, V. F., Peterson, R. F. & Barclay, M. (1964) *Biochem. J.* **90**, 374–378
- Sturton, R. G. & Brindley, D. N. (1978) *Biochem. J.* **171**, 263–266
- Sturton, R. G. & Brindley, D. N. (1980) *Biochim. Biophys. Acta* **619**, 494–505
- Williams, M. A., Tamai, K. T., Hincebergs, I. & McIntosh, D. J. (1972) *J. Nutr.* **102**, 847–856
- Wood, C. K. & Lamb, R. G. (1979) *Biochim. Biophys. Acta* **572**, 121–131