Co-ordinate Regulation of Ethanolamine Kinase and Phosphoethanolamine Cytidylyltransferase in the Biosynthesis of Phosphatidylethanolamine in Rat Liver

EVIDENCE FROM ESSENTIAL-FATTY ACID-DEFICIENT ANIMALS

By JUAN PABLO INFANTE and JOHN E. KINSELLA
*College of Agriculture and Life Sciences, Department of Food Science,
Stocking Hall, Cornell University, Ithaca, NY 14853, U.S.A.*

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Essential-fatty acid deficiency produces a 52% increase in the rate of phosphatidylethanolamine synthesis in rat liver as calculated from results obtained *in vivo* [Trehwella & Collins (1973) *Biochim. Biophys. Acta* **296**, 34–50]. This flux change was used to test the possible regulatory roles of ethanolamine kinase and of phosphoethanolamine cytidylyltransferase, which are rate-limiting enzymes of the cytidine pathway for the synthesis of phosphatidylethanolamine [Infante (1977) *Biochem. J.* **167**, 847–849]. The results show that essential-fatty acid deficiency produces 50% and 53% increases respectively in the specific activity of these enzymes, accounting for the increased rate of phosphatidylethanolamine synthesis produced by this dietary insufficiency. This evidence leads to the conclusion that ethanolamine kinase and phosphoethanolamine cytidylyltransferase have co-ordinated regulatory roles in the flux control of the cytidine pathway, and its sphinganine 1-phosphate lyase branch reaction, for the synthesis of phosphatidylethanolamine.

An enzyme must satisfy two criteria to have a regulatory role in the control of the flux through a metabolic sequence. It must catalyse a rate-limiting reaction, and an observed change in flux through the pathway *in vivo* must be accounted for by a similar change in the specific activity of the enzyme in question (Infante & Kinsella, 1978a). Compliance with the latter criterion shows that the enzyme is in fact responding to a regulatory signal. Ethanolamine kinase (MgATP²⁻–ethanolamine phosphotransferase, EC 2.7.1.32) and phosphoethanolamine cytidylyltransferase (MgCTP²⁻–phosphoethanolamine cytidylyltransferase, EC 2.7.7.14) are the only enzymes of the cytidine pathway for the synthesis of phosphatidylethanolamine that comply with the first requirement (Infante, 1977). However, satisfaction of the second criterion has not been demonstrated.

Essential-fatty acid deficiency increases the biosynthetic flux of some of the phosphatidylcholine species made via the cytidine pathway, i.e. species that contain C_{16:1(9)} and C_{18:1(9)} fatty acids (Trehwella & Collins, 1973). This dietary deficiency has been successfully used to demonstrate a regulatory role for choline kinase (MgATP²⁻–choline phosphotransferase, EC 2.7.1.32) in the control of this pathway (Infante & Kinsella, 1978a). The essential-fatty acid deficiency also induces a net increase in the synthesis of phosphatidylethanolamines, particularly the species that contain C_{22:6(4,7,10,13,16,19)}

fatty acids (Trehwella & Collins, 1973; Pooviah *et al.*, 1976). Therefore this nutritional perturbation was also used to test the possible regulatory roles of the ethanolamine kinase and of phosphoethanolamine cytidylyltransferase in the control of phosphatidylethanolamine synthesis by the criteria proposed above. The results obtained show that the specific activities of these two rate-limiting enzymes in rat liver are significantly increased by essential-fatty acid deficiency, and account for the increased rate of phosphatidylethanolamine synthesis calculated from results obtained *in vivo*.

Experimental Procedures

Materials

The animals and diets have been described by Infante & Kinsella (1978a). ATP, CTP, CDP-ethanolamine and ethanolamine were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [2-¹⁴C]Ethanolamine hydrochloride (sp. radioactivity 50 μ Ci/ μ mol) and phospho[2-¹⁴C]ethanolamine (sp. radioactivity 49 μ Ci/ μ mol) were purchased from Amersham Corp. (Arlington Heights, IL, U.S.A.). Tetramisole [($\pm$)-2,3,5,6-tetrahydro-6-phenylimidazo[2,1-*b*]thiazole hydrochloride] was obtained from Aldrich Chemical Co. (Metuchen, NJ, U.S.A.). A 1.0M-MgCl₂ solution was obtained from Fisher Scientific Co. (Fair Lawn, NJ, U.S.A.). All other chemicals were of reagent grade or of the highest purity commercially available.

Methods

The animals were killed by exsanguination from the abdominal aorta, under light diethyl ether anaesthesia, at a time between 10:00 and 12:00 h. The livers were immediately excised, blotted and homogenized in 3 vol. of ice-cold 0.25M-sucrose. The high-speed supernatants and the microsomal fractions were obtained by differential centrifugation and stored as previously described (Infante & Kinsella, 1976a, 1978b). Ethanolamine kinase was assayed by the MgATP²⁻-dependent phosphorylation of [2-¹⁴C]-ethanolamine (Infante & Kinsella, 1976b). Standard assays were performed in a total volume of 373 μ l, containing 80 mM-Tris/HCl buffer, pH 8.0, 10 mM-MgATP²⁻, 40 μ M-[2-¹⁴C]ethanolamine (sp. radioactivity 50 μ Ci/ μ mol), free 10 mM-Mg²⁺ and 0.15–0.20 mg of high-speed-supernatant protein. Initial velocities were obtained with 10–20 min incubations at 37°C. Phosphoethanolamine cytidyltransferase in the high-speed supernatant was assayed by the MgCTP²⁻-dependent conversion of phospho[2-¹⁴C]-ethanolamine into CDP-[2-¹⁴C]ethanolamine. Standard assays were performed in a total volume of 375 μ l containing 80 mM-4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (Hepes) buffer, pH 7.5, 0.6 mM-MgCTP²⁻, 36 μ M-phospho[2-¹⁴C]ethanolamine (sp. radioactivity 49 μ Ci/ μ mol), free 1.0 mM-Mg²⁺ and 0.30–0.40 mg of protein. The phosphoethanolamine cytidyltransferase that remained associated with the microsomal fraction was assayed with the extra addition of 2.0 mM-tetramisole. The presence of this reactant (apparent K_i 0.1 mM) was necessary to produce a total and specific inhibition of a contaminating phosphatase activity on phosphoethanolamine. Initial velocities were obtained with 10–20 min incubations at 37°C.

Ethanolamine, phosphoethanolamine and CDP-ethanolamine were resolved by paper chromatography as described by Infante & Kinsella (1976b). After identification of the appropriate standards with a ninhydrin spray (0.2%, w/v, in water-saturated butanol), paper strips containing the radioactive substrates and products were cut. Radioactivity was

measured by a Packard (model 3385) scintillation spectrometer with a toluene-based scintillation solution (Infante & Kinsella, 1978b). The radiopurity of ¹⁴C-labelled compounds and the recovery of radioactivity in the assays were routinely checked, and were better than 99%. Protein was determined with the Folin phenol reagent (Lowry *et al.*, 1951), with crystalline bovine serum albumin as standard. Concentrations of the nucleotide-Mg²⁺ complexes and of free Mg²⁺ species were calculated from the appropriate apparent binding constants and the corresponding equilibria equations (Infante & Kinsella, 1976a, 1978a).

Results and Discussion

The essential-fatty acid-deficient animals developed the classical clinical symptoms of this dietary perturbation (Infante & Kinsella, 1978a).

The specific activity of ethanolamine kinase in the essential-fatty acid-deficient animals increased 50% with respect to the control group (Table 1). The specific activity of phosphoethanolamine cytidyltransferase in the high-speed supernatant and in the microsomal fractions increased 49 and 73% respectively, compared with the control values (Table 1). If both fractions are pooled, a 53% increase in the total cytidyltransferase activity was observed in the essential-fatty acid-deficient group, with respect to the controls. A statistical analysis of the data by the Student's *t* distribution (Student, 1908; Fisher, 1925) showed that these results were highly significant (Table 1).

The increased specific activity of the above enzyme accounts for a 52% increase in the net rate of phosphatidylethanolamine synthesis calculated from results obtained *in vivo* from essential-fatty acid-deficient rats (Trehwella & Collins, 1973). This indicates that these two rate-limiting steps comply with the second requirement proposed before, i.e. they accounted for the response observed *in vivo* (Infante, 1977). Consequently, these two enzymes must have regulatory roles in the control of the

Table 1. Specific activities of ethanolamine kinase and phosphoethanolamine cytidyltransferase from livers of controls and essential-fatty acid-deficient rats

Results are expressed as means (nmol of product/min per mg of protein) $\pm$ s.d. Numbers in parentheses denote the numbers of animals per treatment. Means are significantly higher than the controls at $P < 0.025$ (*), $P < 0.001$ (**) and $P < 0.0005$ (***) by the Student's *t* test. The operational term 'high-speed supernatant' was preferred to 'cytosol', since the fact that an enzyme can be found in this homogenate fraction is not conclusive evidence for its cytosolic location *in vivo*.

Diet	Fraction	...	Ethanolamine kinase activity High-speed supernatant	Cytidyltransferase activity	
				High-speed supernatant	Microsomal fraction
Control			0.10 $\pm$ 0.01 (4)	0.85 $\pm$ 0.08 (4)	0.15 $\pm$ 0.05 (4)
Essential-fatty acid-deficient			0.15** $\pm$ 0.02 (12)	1.27*** $\pm$ 0.17 (12)	0.26* $\pm$ 0.08 (11)

cytidine pathway for the synthesis of phosphatidylethanolamine, at least in the flux changes elicited by the essential-fatty acid deficiency. Moreover, the similar relative increments of specific activities of these two enzymes suggests that they are under co-ordinated control.

The regulatory role of ethanolamine kinase can readily be understood since this enzyme catalyses the first committed step of the cytidine pathway for the synthesis of phosphatidylethanolamine. The second control point at the next consecutive step is consistent with a converging branch reaction at the 'stage' of phosphoethanolamine. This phosphorylated base can be synthesized *de novo* by ethanolamine kinase (Wittenberg & Kornberg, 1953; Sung & Johnstone, 1967) as well as by sphinganine 1-phosphate lyase (EC 4.1.2.-; Stoffel & Sticht, 1967; Stoffel & Henning, 1968; Stoffel, 1968, 1973; Henning & Stoffel, 1969; Shimojo *et al.*, 1976). Calculations of rates *in vivo* of phosphatidylethanolamine synthesis from CDP-ethanolamine indicate that its contribution is of the order of 0.06–0.08 $\mu\text{mol}/\text{min}$ per 10g wet wt. of liver (Sundler, 1973). These rates are of the same order of magnitude as the values obtained by using sphinganine as the labelled precursor, i.e. 0.04 $\mu\text{mol}/\text{min}$ per 10g wet wt. of liver (Offenbartl *et al.*, 1973). Therefore, sphinganine 1-phosphate lyase has a significant contribution to the synthesis of phosphoethanolamine. This makes necessary the second regulatory step after this converging branch point so as to control the flux of phosphatidylethanolamine synthesis.

The fact that essential-fatty acid deficiency induces in liver a 3.5-fold increase in the specific activity of choline kinase (Infante & Kinsella, 1978a), whereas the specific activity of ethanolamine kinase is increased by only 50%, suggests that these two phosphorylating activities can be independently regulated. This inference is supported by other lines of evidence, suggesting that these two kinase activities may not necessarily be produced by a single identical active site in the same polypeptide chain, in liver and other tissues (Sung & Johnstone, 1967; Weinhold & Rethy, 1974; Broad & Dawson, 1974; Upreti *et al.*, 1976; Infante & Kinsella, 1976b; Brophy *et al.*, 1977).

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References

- Broad, T. E. & Dawson, R. M. C. (1974) *Biochem. Soc. Trans.* **2**, 1272–1274
- Brophy, P. J., Choy, P. C., Coone, J. R. & Vance, D. E. (1977) *Eur. J. Biochem.* **78**, 491–495
- Fisher, R. A. (1925) *Metron* **5**, 90–104
- Henning, R. & Stoffel, W. (1969) *Hoppe-Seyler's Z. Physiol. Chem.* **350**, 827–835
- Infante, J. P. (1977) *Biochem. J.* **167**, 847–849
- Infante, J. P. & Kinsella, J. E. (1976a) *Int. J. Biochem.* **7**, 483–496
- Infante, J. P. & Kinsella, J. E. (1976b) *Lipids* **11**, 727–735
- Infante, J. P. & Kinsella, J. E. (1978a) *Biochem. J.* **176**, 631–633
- Infante, J. P. & Kinsella, J. E. (1978b) *Biochim. Biophys. Acta* **526**, 440–449
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Offenbartl, K., Wennerberg, J., Sundler, R., Åkesson, B. & Nilsson, Å. (1973) *Biochim. Biophys. Acta* **306**, 460–465
- Pooviah, B. P., Tinoco, J. & Lyman, R. L. (1976) *Lipids* **11**, 194–202
- Shimojo, T., Akino, T., Miura, Y. & Schroepfer, G. J., Jr. (1976) *J. Biol. Chem.* **251**, 4448–4457
- Stoffel, W. (1968) *Hoppe-Seyler's Z. Physiol. Chem.* **349**, 1745–1748
- Stoffel, W. (1973) *Mol. Cell. Biochem.* **1**, 147–155
- Stoffel, W. & Henning, R. (1968) *Hoppe-Seyler's Z. Physiol. Chem.* **349**, 1400–1404
- Stoffel, W. & Sticht, G. (1967) *Hoppe-Seyler's Z. Physiol. Chem.* **348**, 1345–1351
- Student (1908) *Biometrika* **6**, 1–25
- Sundler, R. (1973) *Biochim. Biophys. Acta* **306**, 218–226
- Sung, C. & Johnstone, R. M. (1967) *Biochem. J.* **105**, 497–503
- Trehwella, M. A. & Collins, F. C. (1973) *Biochim. Biophys. Acta* **296**, 34–50
- Upreti, R. K., Samual, G. G. & Krishnan, P. S. (1976) *Arch. Biochem. Biophys.* **174**, 658–665
- Weinhold, P. A. & Rethy, V. B. (1974) *Biochemistry*, **13**, 5135–5141
- Wittenberg, J. & Kornberg, A. (1953) *J. Biol. Chem.* **202**, 431–444