Biochem. J. (1978) **176**, 631–633
Printed in Great Britain

Control of Phosphatidylcholine Synthesis and the Regulatory Role of Choline Kinase in Rat Liver

EVIDENCE FROM ESSENTIAL-FATTY ACID-DEFICIENT RATS

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(*Received* 4 *September* 1978)

Choline kinase and phosphocholine cytidylyltransferase catalyse the rate-limiting steps of the cytidine pathway for the synthesis of phosphatidylcholine [Infante (1977) *Biochem. J.* **167**, 847–849]. Essential-fatty acid deficiency induces a 3.5-fold increase in the specific activity of choline kinase, whereas the specific activity of the cytidylyltransferase remains unchanged in rat liver. This change in specific activity accounts for the calculated increase in flux through the cytidine pathway produced *in vivo* by the same dietary state [Trewhella & Collins (1973) *Biochim. Biophys. Acta* **296**, 34–50], thus confirming the fact that choline kinase has a regulatory role in the cytidine pathway for the synthesis of phosphatidylcholine.

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 (Rolleston, 1972; Newsholme & Crabtree, 1973; Ferdinand, 1976*a*,*b*), which indicates that the enzyme has the potential to affect the flux, and an observed change in flux through the pathway *in vivo* must be accompanied by a commensurate change in activity of the enzyme (Newsholme & Gevers, 1967). Satisfaction of the latter requirement shows that the enzyme is, in fact, regulating the flux through the pathway. Choline kinase ($MgATP^{2-}$–choline phosphotransferase, EC 2.7.1.32) and cytidylyltransferase ($MgCTP^{2-}$–phosphocholine cytidylyltransferase, EC 2.7.7.15) satisfy the first criterion (Infante, 1977). However, compliance with the second requirement remained to be demonstrated.

Flux changes generated by long-term control mechanisms (such as the ones induced by a chronic dietary perturbation) are generally brought about by signals that affect the enzyme activity at the translation level, i.e. net synthesis of enzyme (Lynen, 1970; Schimke, 1969, 1973). This results in a higher specific activity of the regulatory enzymes, which can be detected by assays *in vitro*.

The state of essential-fatty acid deficiency is a chronic dietary perturbation that offers a useful tool for the study of the long-term regulation of phosphatidylcholine metabolism. Trewhella & Collins (1973*a*) observed that the livers of essential-fatty acid-deficient rats had a smaller pool of the phosphatidylcholine species containing $C_{18:2}$ and $C_{20:4}$ fatty acids, whereas the pool sizes and specific radioactivities of the $C_{16:1}$ and $C_{18:1}$ phosphatidylcholines were enlarged. From the observed changes in pool size and specific radioactivities of the latter phosphatidylcholines, it can be calculated that their biosynthetic flux increased 3.8-fold. As these phosphatidylcholine species are chiefly synthesized *de novo* by the cytidine pathway (Åkesson, 1970; Åkesson *et al.*, 1970; Rytter *et al.*, 1968; Trewhella & Collins, 1973*b*; van Golde *et al.*, 1969), it can be inferred that the activation of this metabolic sequence should be responsible for the increased biosynthetic flux of the $C_{16:1}$ and $C_{18:1}$ phosphatidylcholines. Because choline kinase and the cytidylyltransferase catalyse the only rate-limiting steps of this pathway (Infante, 1977), the observed flux increase should be coupled to a concomitant increase in the specific activity of at least the first and most rate-limiting enzyme (choline kinase) if it plays a regulatory role in the pathway in question.

On the basis of the above premise, we have compared the specific activities of choline kinase and the cytidylyltransferase from the livers of essential-fatty acid-deficient rats with those of animals fed on a balanced diet. Results show that the specific activity of choline kinase from the livers of essential-fatty acid-deficient rats is increased significantly, whereas the specific activity of the cytidylyltransferase remains unchanged by the same dietary perturbation.

Materials and Methods

Essential-fatty acid-deficient animals

Weanling male Sprague–Dawley rats (Blue Spruce Farms, Altamont, NY, U.S.A.) were housed in individual stainless-steel cages in a room maintained at 23±2°C. Food and tap water were available

ad libitum. Four control animals were fed on a balanced rat chow diet (Charles River, Country Foods, Syracuse, NY, U.S.A.) for 28 weeks. Eight experimental animals were fed on an essential-fatty acid-deficient diet for the same period. The diet was made essential-fatty acid-deficient by the use of hydrogenated coconut oil (ICN Pharmaceuticals, Life Sciences Group, Cleveland, OH, U.S.A.) as the only source of fat. This hydrogenated oil contained a residual 0.3% *cis*-linoleic acid. The diet contained the following components (%, w/w): 5% hydrogenated coconut oil, 20% vitamin-free casein, 4% cellulose, 64.6% sucrose, 0.2% choline chloride, 4% salt mix (Jones & Foster, 1942) and 2% vitamin mix (ICN Pharmaceuticals). This vitamin mix contained the following amounts of vitamins per kg: vitamin A, 902000 i.u.; vitamin D, 100000 i.u.; *all-rac*-α-tocopherol, 1.5g; ascorbic acid, 45g; inositol, 1.0g; choline chloride, 75g; menadione, 2.2g; *p*-aminobenzoic acid, 5.0g; niacin, 4.5g; riboflavin, 1.0g; pyridoxine hydrochloride, 1.0g; thiamin hydrochloride, 1.0g; calcium pantothenate, 3.0g; biotin, 20mg; folic acid, 90mg; vitamin B_{12}, 1.4mg.

Chemicals

ATP, CTP, CDP-choline, phosphocholine and choline bromide were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. [*Me*-^{14}C]-Choline (30μCi/μmol) and phospho[*Me*-^{14}C]choline (57μCi/mol) were purchased from New England Nuclear, Boston, MA, U.S.A. Tetramisole {(±)-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-$MgCl_2$ solution (lot 766151) was obtained from Fisher Scientific, Fair Lawn, NJ, U.S.A. All common chemicals were of reagent grade or of the highest purity commercially available.

Enzyme preparation

The animals were killed by exsanguination from the abdominal aorta, under light ether anaesthesia, between 10:00 and 12:00h. The livers were immediately excised, blotted and homogenized in 3vol. of ice-cold 0.25M-sucrose. The high-speed supernatant and microsomal fractions were subsequently obtained by differential centrifugation and stored as previously described (Infante & Kinsella, 1976*a*).

Enzyme assays

Choline kinase was assayed by the $MgATP^{2-}$-dependent phosphorylation of [*Me*-^{14}C]choline (Infante & Kinsella, 1976*a*). Standard assays were performed in a total volume of 375μl, containing 80mM-Tris/HCl buffer, pH8.0, 2.2mM-$MgATP^{2-}$, 30μM-[*Me*-^{14}C]choline (5μCi/μmol), 1.0mM free Mg^{2+} and 0.1–0.2mg of supernatant protein. Initial velocities were obtained with 10–20min incubations at 36°C.

The cytidylyltransferase was assayed by the $MgCTP^{2-}$-dependent conversion of phospho[*Me*-^{14}C]choline into CDP-[*Me*-^{14}C]choline (Infante & Kinsella, 1978). Standard assays were performed in a total volume of 375μl containing 80mM-Hepes[4-(2-hydroxyethyl-1-piperazine-ethanesulphonic acid] buffer, pH7.5, 0.6mM-$MgCTP^{2-}$, 27μM-phospho[*Me*-^{14}C]choline (57μCi/mol), 1.0mM free Mg^{2+} and 0.1–0.2mg of supernatant protein. The cytidylyltransferase, which remained bound to the microsomal fraction, was assayed in the presence of 2.0mM-tetramisole. This compound produced a total and specific inhibition of a contaminating phosphatase activity on phosphocholine. Initial velocities were obtained with 20–40min incubations and were directly proportional to protein concentration, up to 0.4mg of protein/375μl.

Analytical

Substrates and products were resolved by paper chromatography as previously described (Infante & Kinsella, 1973, 1976*b*, 1978). Paper strips containing the appropriate spots were cut out and their radioactivity was measured with a Packard (model 3385) scintillation spectrometer in a toluene-based scintillation solution (Infante & Kinsella, 1973). Radiopurity of ^{14}C-labelled compounds and recovery of radioactivity in the assays were routinely checked, and both were better than 98%.

Calculations of the concentrations of $MgATP^{2-}$, $MgCTP^{2-}$ and free Mg^{2+} were made from the apparent binding constants of 20000M^{-1} for $MgATP^{2-}$ (Nørby, 1970) and 10200M^{-1} for $MgCTP^{2-}$ (Walaas, 1958) and the corresponding equilibria equations (Infante & Kinsella, 1976*a*). Protein was determined by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin as the standard.

Results and Discussion

The essential-fatty acid-deficient animals developed the classical symptoms of decreased growth rate, development of scaly paws, skin and tail, and fatty livers. The specific activity (nmol of phosphocholine/min per mg of protein) of choline kinase increased from 0.20 in the controls to 0.69 in the deficient animals (Table 1). However, the specific activities of the cytosolic and microsomal cytidylyltransferases remained unchanged under the same dietary treatment (Table 1). These two cytidylyltransferases have been established to be immunologically identical (Choy *et al.*, 1978).

Table 1. *Specific activities of choline kinase and the cytidylyltransferase from livers of essential-fatty acid-deficient rats*
Results are expressed as means (nmol of product/min per mg of protein) ± S.D. The numbers in parentheses denote the numbers of animals per treatment.

Diet	Choline kinase	Cytidylyltransferase	
		Cytosolic	Microsomal
Control	0.20 ± 0.05 (4)	0.07 ± 0.01 (4)	0.07 ± 0.01 (4)
Essential-fatty acid-deficient	0.69* ± 0.08 (8)	0.07 ± 0.01 (8)	0.09[ns] ± 0.01 (8)

* Values are significantly higher than the controls at $P<0.001$ by Student's t test.
[ns] Not significant at $P<0.10$.

As the flux through the cytidine pathway for the synthesis of phosphatidylcholine is almost totally dependent on the forward velocity of choline kinase (Infante, 1977), it can be inferred that the essential-fatty acid deficiency induced a 3.5-fold increase in flux through this pathway. This predicted flux change accounts for the 3.8-fold increase, which can be calculated from the data obtained *in vivo* by Trewhella & Collins (1973*a*), for rats on essential-fatty acid-deficient diets. Therefore choline kinase, which catalyses the first and most rate-limiting step of the sequence, also complies with the second requirement of accounting for the observed change in flux *in vivo*. Consequently, this enzyme must have a regulatory role in the flux control of the cytidine pathway for the synthesis of phosphatidylcholine.

The observed lack of change in specific activity of the cytidylyltransferase suggests that this enzyme, although rate-limiting, does not have a regulatory role in the synthesis of phosphatidylcholine, at least in the flux change induced by the essential-fatty acid deficiency. Thus the flux increase generated by choline kinase may be transmitted to the next step via non-covalent interactions, such as an increase in phosphocholine and free Mg^{2+} concentration. Since the forward velocity of choline kinase *in vivo* has been calculated to be more than 3.7×10^3-fold the velocity of the back reaction (Infante, 1977), the concentration of its products can be increased many-fold without any significant inhibition of the forward flux through that step (Infante & Kinsella, 1978).

The dramatic increase in specific activity of choline kinase may be used as a sensitive enzymic alternative to the $C_{20:3}/C_{20:4}$ ratio (Holman, 1960) commonly used as biochemical evidence for the essential-fatty acid deficiency. The diagnostic validity of the triene/tetraene ratio is vitiated when $C_{18:3}$ and *trans*-fatty acids are present in the diet (Holman, 1971; Privett *et al.*, 1977).

To what extent a higher net rate of enzyme synthesis or covalent modification of existing enzyme is involved, as well as the metabolic signals responsible for the increased specific activity of choline kinase, remain to be determined.

The technical assistance of Ms. Lorna S. Evey is gratefully acknowledged. This work was supported by the National Science Foundation Grant no. PCM 75-19123.

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