Effects of fasting on phosphatidylcholine biosynthesis in hamster liver: regulation of cholinephosphotransferase activity by endogenous argininosuccinate

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The control of phosphatidylcholine biosynthesis in the hamster liver was examined. Livers of hamsters fasted for 24 and 48 h were perfused with labelled choline. Under both fasting conditions, the incorporation of labelled choline into phosphatidylcholine was reduced. After 48 h of fasting, the 52% reduction in phosphatidylcholine biosynthesis was caused by changes in several factors including a diminishing rate of choline uptake and severe reductions in the pool sizes of ATP and CTP (to 33–37% control values) which resulted in a decrease in the pools of choline-containing metabolites. The activation of cytidylyltransferase after 48 h of fasting might be regarded as a compensatory mechanism for the maintenance of phosphatidylcholine biosynthesis. After 24 h of fasting, a 25% reduction in phosphatidylcholine biosynthesis was observed. The ATP and CTP levels were decreased but the reduction was not severe enough to affect the choline uptake or the labelling of the phosphocholine fraction. The activities of the cytidylyltransferase remained unchanged but an accumulation of labelled CDP-choline was detected. Although cholinephosphotransferase activity was not changed in the microsomes, the enzyme activity was attenuated in the postmitochondrial fraction. Further analysis revealed that cholinephosphotransferase in the liver was inhibited by an endogenous inhibitor in the cytosol which was later identified as argininosuccinate. The level of argininosuccinate was elevated during fasting and the change quantitatively accounted for the attenuation of cholinephosphotransferase activity. The inhibition of cholinephosphotransferase by argininosuccinate, coupled with a substantial decrease in the diacylglycerol level, would provide the hamster liver with an immediate mechanism for the transient modulation of phosphatidylcholine biosynthesis during short-term fasting.

INTRODUCTION

Phosphatidylcholine is the principal membrane phospholipid in the eukaryotic cell. In mammalian tissues, the majority of phosphatidylcholine is synthesized *de novo* via the CDP-choline pathway (Hatch et al., 1989; Vance, 1990). Choline is first phosphorylated to phosphocholine by the action of choline kinase (EC 2.7.1.32). Phosphocholine is then converted into CDP-choline by the action of CTP:phosphocholine cytidylyltransferase (EC 2.7.7.15). The final step in this pathway is the formation of phosphatidylcholine from diacylglycerol and CDP-choline, catalysed by CDP-choline:1,2-diacylglycerol cholinephosphotransferase (EC 2.7.8.2). Although the first committed step in the CDP-choline pathway is catalysed by choline kinase, the rate-limiting step of this pathway is catalysed by CTP:phosphocholine cytidylyltransferase (Vance and Choy, 1979; Zelinski et al., 1980; Vance, 1989). The enzyme is located in both microsomal and cytosolic fractions; the microsomal enzyme is regarded as the more active form whereas the cytosolic enzyme requires lipid for full activity (Sleight and Kent, 1983; Vance, 1989). In the last decade, the translocation of the enzyme between the microsomal and cytosolic compartments has been generally accepted as an important mechanism for the modulation of the cytidylyltransferase activity and consequently phosphatidylcholine biosynthesis (Vance, 1989). In spite of the advances in the regulation of the cytidylyltransferase activity, it is not clear if the modulation of the other enzymes in the CDP-choline pathway may also affect phosphatidylcholine biosynthesis. In addition, the co-ordination of phosphatidylcholine biosynthesis via the CDP-choline pathway with other major metabolic pathways in the liver has not been defined.

The liver has been shown to undergo rapid adaptive changes in order to maintain the level of plasma glucose during fasting. Changes in the activities of the glycolysis and gluconeogenesis pathways with alterations in pool sizes of the major metabolites during fasting have been well documented (Foster and Rubenstein, 1991). Alterations in the levels of the metabolites during fasting may affect the rate of phosphatidylcholine biosynthesis. Hence the liver of an animal during fasting is a useful model in which to study the regulation of phosphatidylcholine biosynthesis under extreme physiological changes. In addition, the possible co-ordination of the biosynthesis of phospholipids and other metabolic pathways can be examined. In previous studies, the biosynthesis of phospholipids has been shown to be affected in the liver of the fasting rat (Park et al., 1972; Groener et al., 1979; Tijburg et al., 1988). In this study, we confirm that phosphatidylcholine biosynthesis was reduced in the livers of 24 h- and 48 h-fasted hamsters. The reduction in phosphatidylcholine biosynthesis during 24 h of fasting resulted entirely from the attenuation of cholinephosphotransferase activity by an endogenous inhibitor which was later identified as argininosuccinate.

MATERIALS AND METHODS

Materials

CDP-choline and 1,2-diacylglycerol (pig liver) were purchased from Serdary Research Laboratories (London, Ont., Canada).

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[*Me*-^{3}H]Choline (80.0 mCi/mmol) and CDP-[*Me*-^{14}C]choline (42.4 mCi/mmol) were obtained from NEN Division, Dupont Co. (Dorval, Que., Canada). Amino acid standards and amino acid derivatives were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Centriflo membrane cones (type CF25A) were obtained from Amicon Division of W. R. Grace & Co. (Beverly, MA, U.S.A.). Mono Q HR 5/5 column for f.p.l.c. was the product of Pharmacia LKB Biotechnology (Dorval, Que., Canada). Thin-layer silica plates (Sil-G25) were obtained from Brinkmann Instrument (Rexdale, Ont., Canada) and thin-layer cellulose plates were purchased from Mandel Scientific Co. (Edmonton, Alberta, Canada). Dowex AG 1-X8 ion-exchange resins were obtained from Bio-Rad Laboratories (Mississauga, Ont., Canada). All other chemicals were reagent grade and were obtained through the Canlab Division of Travenol Canada Inc. (Winnipeg, Man., Canada). Phospho[*Me*-^{3}H]choline was synthesized enzymically from [*Me*-^{3}H]choline and ATP with yeast choline kinase (Vance et al., 1981).

Perfusion of the hamster liver

Male Syrian golden hamsters (110 ± 10 g) were maintained on Agway rodent chow and tap water *ad libitum* in a light- and temperature-controlled room. Hamsters were anaesthetized by an intraperitonal injection of sodium pentobarbital (60 mg/kg) with 700 units of heparin. An inflow cannula was placed in the portal vein and an outflow cannula was placed in the thoracic segment of the inferior vena cava. The hepatic artery and the inferior vena cava above the renal vein were ligated. The liver was perfused with Krebs–Henseleit buffer saturated with 95% O_2/5% CO_2 at a flow rate of 2 ml per min. After an initial period of stabilization (the liver was perfused with Krebs–Henseleit buffer for 10 min), the liver was perfused for 40 min with Krebs–Henseleit buffer containing 50 μM [*Me*-^{3}H]choline (1 μCi/ml). All perfusions were carried out at 37 °C.

Analyses of phospholipids and choline-containing metabolites

Subsequent to perfusion with labelled material, the liver was washed by perfusion with 10 ml of Krebs–Henseleit buffer and then homogenized in chloroform/methanol (1:1, v/v) to yield a 15% homogenate. The homogenate was centrifuged at 1000 ***g*** for 10 min and the resulting pellet was washed twice with chloroform/methanol (1:1, v/v) and re-centrifuged. The supernatants were pooled and a portion was taken for the determination of total uptake of radioactivity. Phase separation of the pooled tissue extract was achieved by adding chloroform and water. Phosphatidylcholine in the organic phase was separated from the other phospholipids by t.l.c. with a solvent system containing chloroform/methanol/acetic acid/water (35:15:1:2, by vol.). The choline-containing metabolites in the aqueous phase were analysed by t.l.c. with a solvent system containing methanol/0.6% NaCl/ammonium hydroxide (10:10:1, by vol.) (Zelinski et al., 1980). In order to separate CDP-choline from betaine, after t.l.c. the sample containing CDP-choline was applied to a Norit A charcoal column equilibrated with 2% ethanol (O et al., 1989). Betaine was eluted from the column with 2% ethanol, and CDP-choline was subsequently eluted with 10 ml of 40% ethanol containing 1% ammonium hydroxide.

Analyses of other metabolites

A portion of the aqueous phase was used for the estimation of argininosuccinate content in the tissue. The volume of the sample was reduced, and a portion was applied to a thin-layer cellulose plate. Another sample was mixed with a known amount of argininosuccinate (internal standard) and the mixture was applied to the plate. The plate was developed in butanol/formic acid/water (15:3:2, by vol.), and the fractions on the plate, after development, were visualized with ninhydrin spray. The content in each fraction was determined by using a Shimadzu 910 autoscanner on high resolution at 600 nm. The pool sizes of ATP and CTP were determined from tissue extracts by h.p.l.c. on a Partisil SAX-10 column as previously described (Choy, 1982). The pool sizes of phosphocholine and CDP-choline were estimated by the procedure of Zelinski et al. (1980). Phosphocholine and CDP-choline were isolated by column chromatography, and then converted into choline by enzymic hydrolysis. The amount of choline in each sample was determined by enzymic conversion of choline into phosphocholine using labelled ATP. The amount of diacylglycerol in each sample was determined by g.l.c. subsequent to isolation (Tardi et al., 1992).

Determination of the activities of phosphatidylcholine-biosynthetic enzymes

Hamster liver was homogenized in a solution containing 0.25 M sucrose- and 10 mM Tris/HCl (pH 7.4) to yield a 20% (v/w) homogenate. Subcellular fractions were obtained by differential centrifugation as described previously (Zelinski et al., 1980). Choline kinase activity in the cytosol was determined with labelled choline (Ishidate et al., 1980). CTP:phosphocholine cytidylyltransferase activity in the microsomal and cytosolic fractions was determined as described by Vance et al. (1981) with labelled phosphocholine. CDP-choline:1,2-diacylglycerol cholinephosphotransferase activity was determined as described previously (O et al., 1989) with labelled CDP-choline.

Isolation and identification of the cholinephosphotransferase inhibitor

Hamster liver cytosol (25 ml) was incubated at 100 °C for 10 min and subsequently centrifuged at 5000 ***g*** for 30 min. The resultant supernatant was filtered through an Amicon Centriflo membrane cone (type CF 25A). A portion of the filtered sample (containing 2.06 mg of protein) was loaded on to a f.p.l.c. Mono Q HR 5/5 anion-exchange column, equilibrated with 10 mM Tris/HCl (pH 9.5). The column was first washed with 10 mM Tris buffer (pH 9.5), and, subsequently, a linear pH gradient (pH 9.5–pH 7.5) of 10 mM Tris/HCl was applied. A flow rate of 1 ml per min was maintained. Fractions (1 ml) were collected and a sample was used to test the inhibitory activity of cholinephosphotransferase. The fractions containing inhibitory activity were pooled and the volume was reduced by freeze-drying. A sample of the freeze-dried sample was applied to a cellulose t.l.c. plate with authentic amino acids and amino acid derivatives as standards. The plate was developed in the solvent containing butanol/formic acid/water (15:3:2, by vol.). The locations of the sample fractions and standards were visualized with a 2% ninhydrin spray reagent. In another study, the sample lane on the t.l.c. plate, after solvent development, was divided into 0.5 cm fractions. The content in each fraction was eluted with 0.5 ml of water, and a 0.2 ml sample was analysed for its ability to inhibit choline-phosphotransferase activity.

In order to confirm the identity and purity of the inhibitor, the material was extracted three times from the cellulose gel with 3 ml of water. The pooled extract was filtered and freeze-dried. The freeze-dried sample was redissolved in 0.5 ml of 0.2 M lithium citrate buffer (pH 2.8) and a portion was applied to an

automatic amino acid analyser equipped with a high-resolution Ultrapac 8 resin cation-exchange column. The column was washed with a series of the eluting buffers in the following order: (1) buffer I: 0.2 M lithium citrate, pH 2.80 (0–17 min); (2) buffer II: 0.3 M lithium citrate, pH 3.0 (17–65 min); (3) buffer III: 0.6 M lithium citrate, pH 3.02 (65–93 min); (4) buffer IV: 1 M lithium citrate, pH 3.45 (93–141 min); (5) buffer V: 1.65 M lithium citrate, pH 3.55 (141–204 min). Post-column modification of the amino groups with ninhydrin was utilized for sample detection. Absorbance of the modified sample was monitored at 440 and 570 nm. The retention profile of the sample containing the inhibitor was compared with that obtained for standard argininosuccinate and its anhydrides.

Other determinations

Radioactivity was determined by liquid-scintillation counting and the efficiency of counting was estimated by the channels' ratio calibration method. Protein was determined by the method of Lowry et al. (1951). The Student's t test was used for statistical analysis. The level of significance was set at $P < 0.05$.

RESULTS

Effect of fasting on choline uptake and phosphatidylcholine biosynthesis in the isolated hamster liver

Livers of hamsters fasted for 24 and 48 h were perfused with Krebs–Henseleit buffer containing 50 μM [*Me*-^{3}H]choline (1 μCi/ml) for 40 min. Livers of unfasted hamsters were also perfused and served as controls. Subsequent to perfusion, the liver was homogenized in chloroform/methanol (1:1, v/v) and a portion of the tissue extract was taken for determination of total choline uptake. The tissue extract was separated into aqueous and organic phases by the addition of water and chloroform. The labelling of phosphatidylcholine in the organic phase and the labelling of other choline-containing products in the aqueous phase were determined. As depicted in Table 1, choline uptake was not significantly changed in 24 h-fasted animals but was markedly decreased in 48 h-fasted animals. However, the incorporation of label into phosphatidylcholine was progressively reduced during fasting. After 24 h of fasting, phosphatidylcholine labelling was reduced to 75% of the control, whereas after 48 h of fasting this value had been further reduced to 48% of the control.

After 24 h of fasting, labelling in the choline and phosphocholine fractions of the liver were not significantly changed when compared with the control, but the labelling of CDP-choline was markedly increased. After 48 h of fasting, there was a general decrease in the labelling of choline and phosphocholine, and to a lesser extent, the labelling of CDP-choline in the hamster liver. In another set of experiments, the pool sizes of metabolites in the CDP-choline pathway were determined (Table 2). The phosphocholine and CDP-choline pools were not significantly changed during 24 h of fasting but were substantially reduced after 48 h of fasting. The levels of ATP and CTP were reduced to 61–66% of control values after 24 h of fasting, and to 33–37% of the control values after 48 h. Substantial reductions in total hepatic diacylglycerol levels and diacylglycerol contents in the microsomal fraction were also detected during fasting.

Table 1 Effect of fasting on the uptake of labelled choline and its incorporation into choline-containing metabolites in the rat liver

Hamster livers were perfused with Krebs–Henseleit buffer containing 50 μM [*Me*-^{3}H]choline. Subsequent to perfusion, the liver was extracted with chloroform/methanol (1:1, v/v) and a portion of the extract was used for the determination of total choline uptake. The extract was separated into two phases by the addition of chloroform and water. The radioactivity associated with the phosphatidylcholine fraction (in the lower phase) and other choline-containing metabolites (in the upper phase) was determined. Each value represents the mean ± S.D. of at least five separate experiments. *$P < 0.05$ when compared with the control value.

	10^{-3} × Uptake (d.p.m./g wet weight)		
	Control	24 h-fasted	48 h-fasted
Total uptake	440 ± 48	436 ± 57	213 ± 69*
Phosphatidylcholine	48 ± 4	36 ± 10*	23 ± 8*
Choline	80 ± 16	76 ± 21	44 ± 18*
Phosphocholine	279 ± 41	284 ± 59	125 ± 43*
CDP-choline	7.4 ± 1.4	14.8 ± 3.5*	5.6 ± 4.3

Effect of fasting on hepatic enzyme activities

The effect of fasting on the activities of the enzymes in the CDP-choline pathway was examined (Table 3). When the enzymes were assayed under optimal conditions, no significant change in activities was detected between the control and the 24 h-fasted animals. Since CDP-choline labelling was found to accumulate after 24 h of fasting, the activity of cholinephosphotransferase was analysed in a more detailed fashion. There was no change in the microsomal cholinephosphotransferase activity when assayed in the presence or absence of exogenous diacylglyerol. However, the enzyme activity was found to be inhibited in the liver postmitochondrial fraction of the 24 h-fasted animal. The pattern of enzyme activities in the CDP-choline pathway after 48 h of fasting was different from that obtained for the 24 h-fasted animal. Choline kinase activity remained unchanged but the activity of the cytidylyltransferase in the cytosol was reduced with a corresponding increase in the microsomes. No change in cholinephosphotransferase activity was observed when the enzyme was assayed in the presence or absence of exogenous diacylglycerol, but an attenuation of enzyme activity in the postmichondrial fraction was again detected. Taken together, it appears that fasting (for 24 and 48 h) produced a non-microsomal factor in the liver which caused the attenuation of the cholinephosphotransferase activity.

Presence of a cholinephosphotransferase inhibitor in hamster cytosol

The livers of the 24 h-fasted hamsters provided us with an excellent model for location and identification of the cholinephosphotransferase inhibitor produced during fasting and for examination of the role of this inhibitor in the regulation of phosphatidylcholine biosynthesis. Since inhibition of enzyme activity was observed in the postmitochondrial fraction but not in the microsomes, the inhibitor might be present in the cytosolic fraction. The existence of a cholinephosphotransferase inhibitor was studied by incubating the microsomal enzyme with liver cytosols from control or 24 h-fasted animals. As an additional control, enzyme activity was also determined in the presence of dialysed cytosols. Cholinephosphotransferase activity was inhibited by cytosols from both animal groups, but the cytosol from fasted animals produced greater inhibitory effects than the control. In the presence of 0.6 ml of cytosol from the control

Table 2 Effect of fasting on the pool sizes of metabolites for the biosynthesis of phosphatidylcholine in hamster liver

The pool sizes of the metabolites in the hamster livers are given. The average weights of the control, 24 h-fasted and 48 h-fasted hamster livers were 5.50, 4.75 and 4.32 g respectively. Each value represents the mean ± S.D. of at least three separate experiments. *$P < 0.05$ when compared with the control value.

	Pool size (μmol/g wet weight)		
	Control	24 h-fasted	48 h-fasted
Phosphocholine	1.70 ± 0.44	1.45 ± 0.38	0.91 ± 0.32*
CDP-choline	0.08 ± 0.02	0.07 ± 0.02	0.05 ± 0.02
ATP	0.86 ± 0.10	0.53 ± 0.11*	0.32 ± 0.14*
CTP	0.09 ± 0.01	0.06 ± 0.02*	0.03 ± 0.02*
Diacylglycerol			
Total	2.10 ± 0.38	1.41 ± 0.26*	0.57 ± 0.22*
Microsomal (nmol/mg of microsomal protein)	10.5 ± 1.25	6.4 ± 1.37*	6.1 ± 1.86*

Table 3 Effect of fasting on the activities of hepatic enzymes for the biosynthesis of phosphatidylcholine

The activities of the enzymes in the CDP-choline pathway were assayed in the microsomal or cytosolic fractions of the hamster liver. The cytosolic CTP:phosphocholine cytidylyltransferase was assayed in the presence of 1 mg/ml total lipid extracted from the liver. Each value represents the mean ± S.D. of three separate experiments. *$P < 0.05$ when compared with the control value.

	Enzyme activity (nmol of product formed/min per mg of protein)		
Enzyme	Control	24 h-fasted	48 h-fasted
Choline kinase	0.69 ± 0.09	0.68 ± 0.08	0.74 ± 0.10
CTP:phosphecholine cytidylyltransferase			
Cytosolic	1.14 ± 0.26	1.67 ± 0.32	0.83 ± 0.08*
Microsomal	0.33 ± 0.05	0.32 ± 0.14	0.78 ± 0.16*
Cholinephosphotransferase			
Microsomal	0.88 ± 0.14	0.80 ± 0.11	0.81 ± 0.08
Postmitochondrial	0.25 ± 0.02	0.19 ± 0.03*	0.16 ± 0.02*
Microsomal (no exogenous diacylglycerol) (nmol of product formed/15 min per mg of protein)	0.054 ± 0.006	0.048 ± 0.005	0.046 ± 0.007

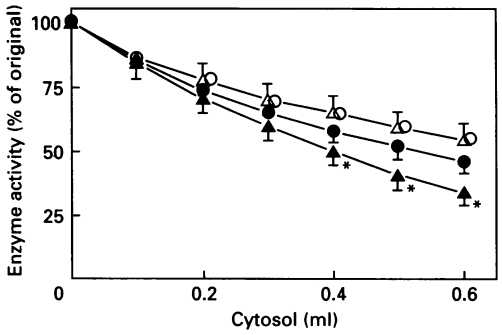

Figure 1 Inhibition of cholinephosphotransferase activity by liver cytosol of the fasted hamster

Cholinephosphotransferase activity in hamster liver was determined in the presence of 0.1–0.6 ml of liver cytosol from control (●) or 24 h-fasted (▲) hamsters. In a separate experiment, the cytosol from the control or fasted hamsters was dialysed (○, △) in 500 vol. of 0.25 M sucrose/10 mM Tris/HCl (pH 7.4), and the inhibitory effect of the dialysed cytosol was determined. Cytosolic protein concentrations were 16.6 mg/ml for the control and 16.8 mg/ml for the fasted hamster. Enzyme activity (0.8 nmol/min per mg) without the addition of cytosol is expressed as 100%. Each point is the mean of three to five separate determinations and the vertical bar is the S.D. *Value obtained from 24 h-fasted hamsters is significantly different ($P < 0.05$) from the controls.

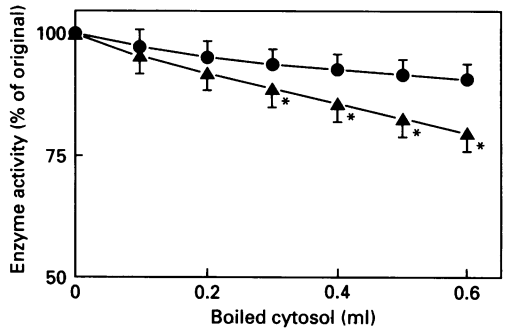

Figure 2 Inhibition of cholinephosphotransferase activity by heat-treated liver cytosol of the fasted hamster

Liver cytosol described in Figure 1 was incubated at 100 °C for 10 min. The heat-treated cytosol was centrifuged to remove the precipitated proteins, and 0.1–0.6 ml of supernatant was added to the enzyme assay mixture. Enzyme activity without any addition is expressed as 100%. The symbols used are the same as in Figure 1. Each point is the mean of three to five separate determinations and the vertical bar is the S.D. *Value obtained from the 24 h-fasted hamster is significantly different ($P < 0.05$) from the control.

animals, a 54% inhibition of enzyme activity was produced whereas the same amount of cytosol from the fasted animals produced a 66% inhibition (Figure 1). The difference in enzyme inhibition was abolished by extensive dialysis of the cytosols from both animal groups. When 0.6 ml of dialysed cytosols from the control or fasted animals was added to the assay mixture, a 45% inhibition of enzyme activity was observed. It is clear from this study that the cholinephosphotransferase activity was inhibited by two components in the cytosol. There was no quantitative difference in the non-dialysable component between the two animal groups. The dialysable component probably consisted of low-M_r entity(s) which was produced in greater quantity by the liver during fasting.

The nature of the dialysable cholinephosphotransferase inhibitor in the cytosol was further investigated. Liver cytosols from the control and the 24 h-fasted animals were incubated at 100 °C for 10 min and the precipitated proteins were removed by centrifugation. Enzyme activity was assayed in the presence of 0.1–0.6 ml of supernatant obtained from the heat-treated cytosol. As depicted in Figure 2, inhibition of enzyme activities was detected in both samples, but a higher degree of inhibition was displayed by the heat-treated cytosol from the fasted animals. When 0.6 ml of the heat-treated cytosol from the control animals was added to the assay mixture, 10% inhibition of enzyme activity was produced, whereas the same amount of heat-treated cytosol from the fasted animals elicited 20.5% inhibition.

Isolation and identification of the cholinephosphotransferase inhibitor

Our next objective was to isolate the inhibitor(s) in the heat-treated cytosol and to study its inhibitory effect on the enzyme. The cytosol (10 ml) was incubated at 100 °C for 10 min and centrifuged at 5000 *g* for 30 min. The supernatant was filtered through an Amicon Centriflo cone with a molecular cut-off at

25000-M_r. The filtered sample was applied to an f.p.l.c. Mono Q HR 5/5 column equilibrated with 10 mM Tris/HCl (pH 9.5). After sample application, the column was washed with a linear pH gradient from 9.5 to 7.5. As depicted in Figure 3, the fractions containing the inhibitory activity were eluted from the column at pH 8.5. The fractions were pooled and the volume reduced by freeze-drying. The freeze-dried sample was dissolved in 1 ml of water and 10 μl was applied to a cellulose t.l.c. The plate was developed in a solvent system containing butanol/formic acid/water (15:3:2, by vol.). After chromatography, the sample fractions and standards on the thin-layer plate were visualized by ninhydrin spray (Figure 4). The sample was resolved into one major band and four minor bands. The major band had an R_F value of 0.08 which was identical with the R_F value of argininosuccinate. In a separate experiment, the chromatogram containing the sample was divided into 0.5 cm fractions ranging from the origin to the solvent front. The cellulose gel of each fraction was removed from the plate and the content of each fraction was eluted with 0.5 ml of water. A portion of the eluate (0.2 ml) from each fraction was analysed for its ability to inhibit cholinephosphotransferase activity. Only the fractions corresponding to the major band were found to exhibit inhibitory activity toward cholinephosphotransferase.

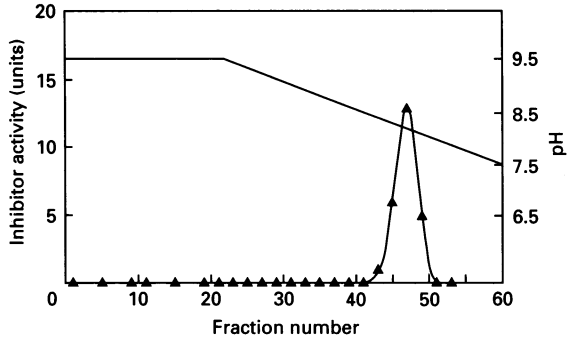

Figure 3 Purification of cholinephosphotransferase inhibitor by Mono Q anion-exchange chromatography

Heat-treated hamster cytosol (5 ml) was centrifuged and the supernatant applied to a Mono Q HR 5/5 column equilibrated with 10 mM Tris/HCl at pH 9.5. The column was washed with 10 ml of the equilibrating buffer followed by a linear pH gradient (pH 9.5–7.5) of 10 mM Tris/HCl (——). Fractions (1 ml) were collected and a portion (0.5 ml) was added to the cholinephosphotransferase reaction mixture. The percentage inhibition was calculated from the difference between the enzyme activity in the control and that in the presence of the eluate (▲).

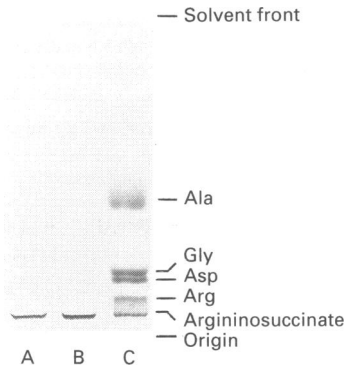

Figure 4 Separation and identification of cholinephosphotransferase inhibitor by t.l.c.

The active fractions from the Mono Q column were pooled and a sample was applied to a cellulose t.l.c. plate. The plate was developed in the solvent containing butanol/formic acid/water (15:3:2, by vol.). The locations of the sample and other standards were visualized by a ninhydrin (2%) spray reagent. Lane A, 10 μl of sample; lane B, 10 μl of sample + 5 μg of argininosuccinate; lane C, a mixture of argininosuccinate, Arg, Asp, Gly and Ala (5 μg each).

Characterization of the cholinephosphotransferase inhibitor

The purity and chemical nature of the inhibitor isolated by t.l.c. was evaluated by an amino acid analyser equipped with a high-resolution Ultrapac 8 resin cation-exchange column. The column was calibrated with standard amino acids and argininosuccinate. Argininosuccinate has been shown to exist in free acid and anhydride forms (Westall, 1960; Portoles and Rubio, 1986). The anhydrides are formed from the free form by heat-treatment. The different forms of argininosuccinate cannot be separated by t.l.c. but these forms are readily resolved by cation-exchange chromatography. The sample was resolved into four separate fractions on an amino acid analyser (Figure 5) and the identities of the peaks were established by using authentic standards: peak 1 (argininosuccinic acid), peak 2 (anhydride II), peak 3 (anhydride I) and peak 4 (ammonia). The analysis revealed that the sample contained only argininosuccinate and its anhydride forms.

Effect of argininosuccinate on phosphatidylcholine-biosynthetic enzymes

The pool sizes of argininosuccinate in the livers of the control and fasted hamsters were determined. Similarly, the amount of argininosuccinate in the liver cytosols of the two animal groups were estimated and the results are depicted in Table 4. A 2-fold increase in the level of argininosuccinate was found in the liver and the cytosol of the 24 h-fasted animals. Fasting for 48 h only produced small increases in argininosuccinate contents in the tissue and cytosol over the values obtained from the 24 h-fasted animals. The direct effect of argininosuccinate on the enzymes in the CDP-choline pathway was investigated. Argininosuccinate (0.1–0.5 mM) was added to the enzyme assay mixture, and the enzyme activity obtained was compared with the control (no argininosuccinate). Argininosuccinate did not cause any inhibition of the activities of choline kinase or cytidylyltransferase (Table 5) As predicted, it inhibited cholinephosphotransferase activity in a dose-dependent manner. In the presence of 0.08 mM argininosuccinate, 5% of the enzyme activity was inhibited, whereas 0.18 mM caused the inhibition of 15% of the enzyme

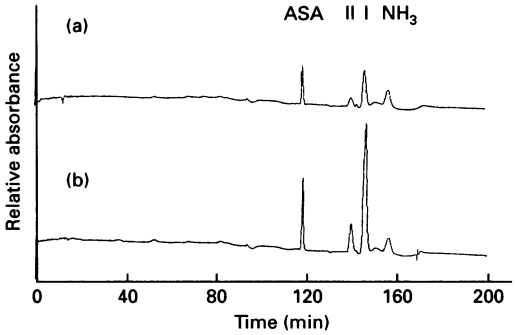

Figure 5 Amino acid analysis of isolated cholinephosphotransferase inhibitor from hamster liver cytosol and argininosuccinate

The sample isolated by t.l.c. was analysed by an automatic amino acid analyser. (**a**) Argininosuccinate; (**b**) sample isolated from hamster liver cytosol. A series of elution buffers was used in the following order: (1) 0.2 M lithium citrate, pH 2.80 (0–17 min); (2) 0.3 M lithium citrate, pH 3.0 (17–65 min); (3) 0.6 M lithium citrate, pH 3.02 (65–93 min); (4) 1 M lithium citrate, pH 3.45 (93–141 min); (5) 1.65 M lithium citrate, pH 3.55 (141–204 min). ASA represents the free argininosuccinate and I and II are the anhydride forms of argininosuccinate.

Table 4 Pool size of argininosuccinate in the hamster liver during fasting

Livers were perfused with Krebs–Henseleit buffer for 40 min. The average weights of the control, 24 h-fasted and 48 h-fasted hamster livers after perfusion were 5.34, 4.68 and 4.21 g respectively. In each study, part of the liver was removed and extracted with chloroform/methanol (1:1, v/v), and chloroform and water were added to the extract to cause phase separation. The upper phase was collected and used for argininosuccinate determination. The remainder of the liver was homogenized in 0.25 M sucrose/10 mM Tris/HCl (pH 7.4). Liver cytosol was prepared from the homogenate by differential centrifugation. The argininosuccinate contents in the liver cytosols were determined. Each value represents the mean ± S.D. of at least three separate experiments. *$P < 0.05$ when compared with the control value.

	Control	24 h-fasted	48 h-fasted
Argininosuccinate			
Liver (μmol/g wet wt.)	0.50 ± 0.13	1.09 ± 0.18*	1.12 ± 0.32*
Liver cytosol (mM)	0.13 ± 0.04	0.29 ± 0.04*	0.31 ± 0.05*

Table 5 Effect of argininosuccinate on phosphatidylcholine-biosynthetic enzymes in hamster liver

The activities of choline kinase, CTP:phosphocholine cytidylyltransferase and cholinephosphotransferase were determined in the absence (control) or presence of argininosuccinate. The cytosolic CTP:phosphocholine cytidylyltransferase was assayed in the presence of 1 mg/ml total lipid extracted from the liver. The results are depicted as means ± S.D. from three separate experiments. *$P < 0.05$ when compared with the control value.

	Specific activity (nmol/min per mg of protein)			
		+ Argininosuccinate		
Enzymes	Control	0.08 mM	0.18 mM	0.5 mM
Choline kinase	0.68 ± 0.05	0.67 ± 0.02	0.67 ± 0.01	0.69 ± 0.02
Cytidylyltransferase				
Microsomal	0.33 ± 0.03	0.33 ± 0.02	0.31 ± 0.04	0.30 ± 0.03
Cytosolic	1.14 ± 0.11	1.09 ± 0.04	1.13 ± 0.04	1.07 ± 0.01
Cholinephosphotransferase	0.78 ± 0.01	0.74 ± 0.02*	0.67 ± 0.02*	0.53 ± 0.02*

activity (Table 5). A similar pattern of inhibition was obtained with heat-treated argininosuccinate (containing the free and anhydride forms). As a positive control, the effects of other metabolites of the urea cycle including arginine, ornithine and citrulline were examined for their inhibitory effect on the enzymes of the CDP-choline pathway. None of these metabolites (0.1–1.0 mM) caused any significant change in the enzyme activities. The mechanism of inhibition of the cholinephosphotransferase activity by argininosuccinate was examined by kinetic studies. Enzyme activities were assayed in the presence of argininosuccinate (0.5 and 1.0 mM) at different concentrations of CDP-choline or 1,2-diacylglycerol. The Lineweaver–Burk plot with various concentrations of CDP-choline displayed a classical non-competitive inhibition pattern in the presence of argininosuccinate (results not shown). When enzyme activities were determined with different concentrations of diacylglycerol, a mixed type of inhibition was observed (results not shown).

DISCUSSION

In this study, we confirmed the previous finding that phosphatidylcholine biosynthesis is reduced in the liver of the fasting animal (Park et al., 1972; Groener et al., 1979; Tijburg et al., 1988). Our analysis revealed that after 48 h of fasting, the reduction in phosphatidylcholine biosynthesis was caused by changes in several factors, including a diminishing rate of choline uptake, reductions in the pool sizes of ATP, CTP and choline-containing metabolites and alterations in activities of the enzymes in the CDP-choline pathway. The reduction in the high-energy nucleotide level in the liver during fasting has been documented (Leelavathi and Guynn, 1977). We have shown in the hypoxic heart (Hatch and Choy, 1990) and during the development of cardiomyopathies (Choy, 1982) that the impairment of phosphatidylcholine biosynthesis was a direct result of severe reductions in high-energy nucleotides levels. Since the reductions in the ATP and CTP levels (33–37% of control values) in the liver of the 48 h-fasted animal were similar to that observed in the hypoxic heart and during the development of cardiomyopathy, such a reduction would result in decreases in phosphocholine and CDP-choline formation. In addition, the conversion of CDP-choline into phosphatidylcholine was further attenuated by the enhanced production of argininosuccinate (see below). Taken together, these factors would probably cause the 52% reduction in phosphatidylcholine biosynthesis in the liver of the 48 h-fasted animal. The activation of the cytidylyltransferase by translocation may be regarded as a compensatory mechanism to maintain a minimal rate of phosphatidylcholine biosynthesis under diminishing ATP and CTP levels (Hatch and Choy, 1990).

A 25% reduction in phosphatidylcholine biosynthesis was also detected in the liver of the 24 h-fasted animal. The reduction in ATP and CTP levels (61–66% of control values) was considerably less severe than that found in the 48 h-fasted animal. It was shown in a previous study (Choy, 1982) that a 30% decrease in the high-energy nucleotide levels was not serious enough to cause a significant change in the formation of phosphocholine or CDP-choline from their precursors. The fact that the labelling of choline-containing metabolites was not reduced in the liver of the 24 h-fasted hamster lends support to this notion. The accumulation of radioactivity in the CDP-choline fraction suggests that its conversion into phosphatidylcholine was impaired. In view of the fact that the level of diacylglycerol was reduced in the liver of the 24 h-fasted animal, the contribution of the diminished level of diacylglycerol to the attenuation of phosphatidylcholine formation was evaluated. There was no change in cholinephosphotransferase activities in the microsomal fraction in the presence of exogenous diacylglycerol. This is not surprising since an excess of exogenous diacylglycerol was employed for the assay. However, it was surprising to find that enzyme activity was not significantly reduced when assayed without exogenous diacylglycerol. One difficulty in correlating the change in diacylglycerol content with phosphatidylcholine biosynthesis is the existence of more than one pool of diacylglycerol in the cell (Rustow and Kunze, 1987), and it is not clear which pool is available for the reaction catalysed by cholinephosphotransferase. Since the K_m of the enzyme for diacylglycerol is 0.46 mM in hamster liver (O and Choy, 1990), changes in the diacylglycerol pool should have some direct effects on the rate of phosphatidylcholine biosynthesis. In addition, the 24% reduction in cholinephosphotransferase activity observed in the postmichondrial fraction of the 24 h-fasted animal should be another major factor for producing the 25% decrease in phosphatidylcholine biosynthesis.

Cholinephosphotransferase has been shown to be located in the microsomal fraction and the enzyme displays limited selectivity for diacylglycerol species (Arthur and Choy, 1984). However, the role of the enzyme in the regulation of phosphatidylcholine biosynthesis remains largely undefined. In the past several years, considerable progress has been made in the study of the enzyme in *Saccharomyces cerevisiae* (Hjelmstad and Bell, 1987, 1990). In mammalian tissues, the enzyme activity

is modulated by lysophosphatidylcholine (Parthasarathy and Baumann, 1979) and calcium concentrations (Taniguchi et al., 1986), but the direct involvement of these inhibitors in the regulation of phosphatidylcholine biosynthesis has not been demonstrated. Recently, the effect of the phosphatidylcholine transfer protein on cholinephosphotransferase activity has been examined (Khan and Helmkamp, 1990). In this study, we are the first to demonstrate the role of argininosuccinate as an endogenous inhibitor of cholinephosphotransferase in the liver. Kinetic studies on the nature of inhibition suggested that the enzyme activity was not inhibited at the substrate level. Evidence to demonstrate that argininosuccinate is the major inhibitor of cholinephosphotransferase in the liver cytosol of the 24 h-fasted animal was obtained from quantitative analysis of the data. In the presence of 0.6 ml of cytosol, a 12% difference in the inhibition of enzyme activity was observed between the fasted animal and the control (Figure 1). In the presence of 0.6 ml of boiled cytosol, a 10.5% difference in the inhibition of enzyme activity was detected between these two animal groups (Figure 2). On the basis of the cytosolic argininosuccinate contents of the 24 h-fasted and control animals, the difference in the inhibition of enzyme activities observed in Figures 1 and 2 could be duplicated by placing the appropriate endogenous amounts of argininosuccinate in the assay mixtures.

In the liver of the fasting animal, the enhancement of amino acid metabolism for gluconeogenesis causes activation of enzymes of the urea cycle (Schimke, 1962; Sakami and Harrington, 1963; Snodgrass, 1981). The increase in argininosuccinate level after 24 h of fasting produces a 10% inhibition of enzyme activity when compared with the control. Fasting for longer periods did not continue to produce substantial increases in argininosuccinate levels. It appears that a maximum of 12–15% inhibition of cholinephosphotransferase can be produced by changes in endogenous argininosuccinate levels during fasting. Under normal conditions, the 12–15% inhibition of enzyme activity may not play a significant role in the regulation of phosphatidylcholine biosynthesis, but the importance of such an inhibition may become more prominent when diacylglycerol is in short supply. The modulation of cholinephosphotransferase activity by a key metabolite of the urea cycle serves as an attractive model to demonstrate the occurrence of co-ordination between phospholipid and protein metabolism during gluconeogenesis. We postulate that the inhibition of enzyme activity by endogenous argininosuccinate, coupled with the decrease in diacylglycerol content, should provide the organ with an immediate but limited mechanism for the transient modulation of phosphatidylcholine biosynthesis during short-term fasting.

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