Choline Deficiency Causes Translocation of CTP:Phosphocholine Cytidylyltransferase from Cytosol to Endoplasmic Reticulum in Rat Liver*

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The choline-deficient rat liver has been chosen as a physiologically relevant model system in which to study the regulation of phosphatidylcholine biosynthesis. When 50-g rats were placed on a choline-deficient diet for 8 days, the activity of CTP:phosphocholine cytidylyltransferase (CT) was increased 2-fold in the microsomes and decreased proportionately in the cytosol. A low titer antibody to CT was obtained from chickens and used to identify the amount of CT protein in cytosol from rat liver. The amount of CT recovered from the choline-deficient cytosol was significantly less than in cytosol from choline-supplemented rats. When hepatocytes were prepared from choline-deficient livers, supplementation of the medium of the cells with choline caused CT to move from the membranes to cytosol within 1–2 h. The activity of another translocatable enzyme of glycerolipid metabolism, phosphatidate phosphohydrolase, was unchanged in cytosol from choline-deficient rat livers, and the microsomal activity of this enzyme was only minimally increased. When the livers were fractionated into endoplasmic reticulum and Golgi, there was a 2-fold increase in the activity on the endoplasmic reticulum from choline-deficient livers but no change in activity associated with Golgi. Thus, the increased association of CT with endoplasmic reticulum in choline-deficient livers appears to be specific to that subcellular fraction, and the subcellular location of other enzymes may not be affected.

A major mechanism for control of PC1 biosynthesis in animal cells involves the regulation of the subcellular location of CT (1). There is general agreement that in cultured animal cells the active form of CT resides on the ER (1–3) and, in liver, the ER and Golgi membranes (1, 3). The cytosolic form of CT is thought to be an inactive reservoir which can be translocated to ER where it becomes activated by interaction with phospholipids on the membrane. Several possible mechanisms which regulate the reversible binding of the enzyme to ER have been identified (1, 4–20). Addition of fatty acids to cultured cells stimulates PC biosynthesis and enhances CT binding to membranes; this process can be readily reversed (4–11). Treatment of cells with phospholipase C causes reversible binding of CT to membranes (12–18), specifically the ER in one study (2). Cyclic AMP analogues inhibit PC synthesis in cultured hepatocytes, and this correlates with a decrease in microsomal CT activity (19). In vitro studies provide evidence that the CT is inactivated by release from membranes by phosphorylation with cAMP-dependent protein kinase, and this can be reversed via the action of a phosphatase (20).

What has been lacking is evidence in intact animals that translocation of CT is a physiologically important control mechanism for PC biosynthesis. Several studies in rats suggest that the translocation of CT may be important. Weinhold et al. (21) showed a correlation between the levels of fatty acids in lung after birth and the CT activity bound to membranes as well as the rate of PC biosynthesis. Lim et al. (22) showed a relationship between PC synthesis and CT activity on microsomes in hypercholesterolemic rats. Developmental studies in rats also showed a correlation between the rate of PC synthesis in liver and the localization of CT to membranes (23). Although these studies are suggestive, the evidence for the mechanisms involved is not compelling.

Because of the situation summarized above, we have been interested in developing a model system that would be physiologically relevant and at the same time allow us to explore mechanisms for regulation of PC synthesis. Over a decade ago we conducted studies on choline-deficient rats which provided some basic information about the PC biosynthetic enzymes in choline-deficient livers (24). At that time we were able to show a 40% decrease in the specific activity of CT in liver cytosol after 2–3 days of choline deficiency (24). Immunoprecipitation experiments showed no change in the amount of cytosolic enzyme for at least 18 days of choline deficiency (25). We were unaware of the importance of microsomal CT at that time, and the CT activity on microsomes was not measured. Because none of the enzymes of PC synthesis had been purified at that time, we were limited in our ability to extend those studies. In the intervening time CT (26), choline kinase (27), and PE methylethyltransferase (28) have been purified to homogeneity and antibodies generated. In addition, the culture of hepatocytes from rats has been well developed (29). Our interest in choline deficiency as a model system was rekindled as a result of these new developments and our

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† The abbreviations used are: PC, phosphatidylcholine; PE, phosphatidylethanolamine; CT, CTP:phosphocholine cytidylyltransferase; ER, endoplasmic reticulum; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylenbis(oxethylaminemirilo)] tetracetate acid.
conjugate were purchased from Organon Teknika Co., West Chester, PA. Male Sprague-Dawley rats, initially weighing 50-55 g, were fed a choline-deficient diet (ICN Biochemicals, Canada) and water ad libitum for 3 days. Control rats were fed with the same diet except that 0.4% (w/w) choline chloride was supplemented. NitroScreen West was purchased from Du Pont-New-England Nuclear. The [methyl-3H]choline chloride (15 Ci/mmol) and UDP-D-[~3H]galactose were from Amersham International, United Kingdom. Phospho[methyl-3H]choline (5-7 mCi/mmol) was synthesized enzymatically from Imethvl-3Hlcholine and ATP with choline kinase as described previously (34). Cell culture medium, Hanks' balanced salt solution, and fetal bovine serum were obtained from GIBCO, and the serum was delipidated and dialyzed as described by Cham and Knowles (35). Primaria culture dishes (60 mm in diameter) were obtained from Sigma. Boron trifluoride (14%) in anhydrous ethyl alcohol was purchased from Zinser Laboratories. 50 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 0.025% (w/v) NaN3 with 0.1% sodium dodecyl sulfate were transferred to Millipore NitroScreen West membrane according to the method of Towbin et al. (38). After transfer, the membrane was incubated with 100 ml of solution T (20 mM Tris-HCl, pH 7.5, 500 mM NaCl) containing 0.1% sodium dodecyl sulfate and incubated with 1:5000 dilution of anti-CT antiserum in 100 ml of the TWEEN solution (1:5000 dilution) for 2 h. The membrane was washed before and incubated with the second probe (rabbit anti-chicken IgG conjugated with peroxidase) in 100 ml...
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do in the Tween solution (1:1000 dilution) for 2 h. The membrane was rinsed with 150 ml of 20 mM Tris-HCl, pH 7.4, 0.1 mM NaCl, and 2 mM CaCl₂ two times and incubated with a solution containing 90 ml of the same buffer plus 54 ml of H₂O₂ and 54 ml of 4-chloro-1-naphtol dissolved in 18 ml of methanol to develop color.

Enzyme Assays—CT activity was determined essentially as described by Weinhold et al. (39) except that phospho[methyl-³H]choline was used as a substrate, and the reaction was stopped by immersion of the assay tubes into boiling water for 1 min. [methyl-³H]CDP-choline was separated from phosphocholine on plastic-backed Silica Gel 60 thin layer chromatography plates (10 × 20 cm) developed in methanol, 0.6% NaCl, 28% NH₄OH (50:50:5, v/v). Lactate dehydrogenase activity (40) and NADPH:cytochrome c reductase activity (41) were assayed according to established procedures. UDP-galactose:N-acetylgalactosamine galactosyltransferase was determined according to Ernster et al. (42).

Chemical Analysis—Phospholipids were quantitated by phosphorus assay as described previously (43). Protein was determined according to Lowry et al. (44) using bovine serum albumin as a standard. Lipids were fractionated and analyzed as described previously (30).

RESULTS

Concentration of Lipids in Livers from Choline-deficient and Choline-supplemented Rats—Animals were maintained on choline-deficient or choline-supplemented diets for 3 days. The livers were excised, perfused with a balanced salt solution, extirpated, and freeze-clamped in liquid nitrogen. Two characteristic features of choline deficiency (45) were produced: a decrease in the PC/PE ratio (choline-supplemented, 1.95; choline-deficient, 1.27) and an accumulation of triacylglycerol (Table I). We also observed a 1.8-fold increase in the concentration of diacylglycerol (p < 0.05), a 1.2-fold increase in PE concentration (p < 0.02), and a slight elevation (p < 0.01) in the unesterified fatty acid levels (Table I). The concentrations of phosphatidylinositol, phosphatidylethanolamine, lysophosphatidylcholine, and sphingomyelin were unchanged by the choline deficiency (data not shown).

Evidence for Translocation of CT Protein—The above data demonstrated that we had achieved a classical choline deficiency in the rats. The activity of CT in cytosol and microsomes prepared from choline-deficient and choline-supplemented livers was measured (Table II). The results confirmed the decrease in cytosolic activity reported previously (24) and demonstrated an increase in the CT activity bound to microsomes. The recovery of microsomes from the homogenate was estimated by assay of NADPH:cytochrome c reductase (29.3 ± 7.1% from choline-deficient rats and 34.7 ± 8.0% from choline-supplemented rats, n = 3). When the CT activity associated with microsomes was corrected for recovery of microsomes, the CT activity (nmol/min/g of liver) on microsomes was 121 for choline-deficient and 58 for choline-supplemented rats.

The increase in microsomal cytidylyltransferase activity in many different experimental systems has always been assumed to be due to a movement of protein from cytosol to membranes rather than an activation of the enzyme. However, in no instance has this been demonstrated. With the availability of pure enzyme (26), we have attempted to produce antibody to CT so that the amount of enzyme in cytosol and microsomes could be titrated. The production of such an antibody in rabbits has been unsuccessful, but we have obtained a low titer antibody in chickens. This polyclonal antibody was not potent enough to detect CT in cytosol or microsomes. However, when CT was purified 25-fold through the octyl glucoside extraction step (26), the protein reacted with antibody in an immunoblot analysis. The recoveries of CT activity in octyl glucoside extracts were 75.2 ± 1.6 for choline-deficient and 74.7 ± 2.4 for choline-supplemented livers. In four separate experiments the amount of CT protein was reduced in the cytosol of the choline-deficient compared with the choline-supplemented rat livers (Fig. 1) in agreement with the reduction of cytosolic CT activity (Table III). Unfortunately, attempts to solubilize microsome-associated CT for immunoblot studies were unsuccessful. Thus, although we have been unable to prove that the increase in CT activity in the microsomes was due to translocation of protein from the cytosol, data presented are consistent with this conclusion.

Reversibility of Increased CT Binding to Microsomes—In order to demonstrate that the translocation of CT to microsomes in choline-deficient rats was reversible, we prepared cultured hepatocytes from these animals. When these cells were maintained in a choline- and methionine-deficient medium, we observed that the increased amount of CT activity on microsomes and a decreased activity in cytosol were preserved. Supplementation of these cells with choline caused a decrease in the enzyme activity on the microsomes and an increase in the cytosol (Fig. 2). The translocation of enzyme was apparent within 1 h and reached the distribution of enzyme found in cells from choline-fed rats within 24 h.

### Table I

<table>
<thead>
<tr>
<th>Diet</th>
<th>PC</th>
<th>PE</th>
<th>TG</th>
<th>1.2-DG</th>
<th>Fatty acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmol/g liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD</td>
<td>11.7 ± 0.5</td>
<td>9.2 ± 0.9</td>
<td>27.6 ± 10.0</td>
<td>0.64 ± 0.16</td>
<td>0.14 ± 0.01</td>
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<tr>
<td></td>
<td>p &lt; 0.01</td>
<td>p &lt; 0.02</td>
<td>p &gt; 0.02</td>
<td>p &lt; 0.05</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>CS</td>
<td>14.5 ± 1.4</td>
<td>7.4 ± 0.4</td>
<td>9.2 ± 2.4</td>
<td>0.25 ± 0.12</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.01</td>
<td>p &lt; 0.02</td>
<td>p &gt; 0.02</td>
<td>p &lt; 0.05</td>
<td>p &lt; 0.01</td>
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</tbody>
</table>

### Table II

<table>
<thead>
<tr>
<th>Distribution of cytidylyltransferase activity in the livers from choline deficient (CD) and choline-supplemented (CS) rats</th>
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</thead>
<tbody>
<tr>
<td>Liver</td>
</tr>
<tr>
<td>Cytidylyltransferase activity</td>
</tr>
<tr>
<td>Postmitochondrial supernatant</td>
</tr>
<tr>
<td>CD</td>
</tr>
<tr>
<td>CS</td>
</tr>
<tr>
<td>p &lt; 0.01</td>
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<tr>
<td>Cytosol</td>
</tr>
<tr>
<td>CD</td>
</tr>
<tr>
<td>CS</td>
</tr>
<tr>
<td>p &lt; 0.0005</td>
</tr>
<tr>
<td>Microsomes</td>
</tr>
<tr>
<td>CD</td>
</tr>
<tr>
<td>CS</td>
</tr>
<tr>
<td>p &lt; 0.0005</td>
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</tbody>
</table>

Rats were fed with a choline-deficient or choline-supplemented diet for 3 days. Samples of postmitochondrial supernatant, cytosol, and microsomes were prepared from the livers, and CT activity was assayed as described under "Experimental Procedures." The data are presented as mean ± S.D. (n = 3). The significance of difference was determined by t test.
incorporation (Fig. 6 in Ref. 30) or with [3H]glycerol as a precursor (data not shown).

The effect of choline on distribution of CT activity was further examined by digitonin permeabilization experiments. Addition of digitonin disrupts cell membranes and releases cytosolic proteins from cells (37). A nearly linear release of CT by the cells was observed during an 8-min incubation with digitonin (Fig. 3). The rate of release of cytosolic CT was increased in the cells supplemented with choline compared with cells without choline, and the maximal rate was achieved 2–4 h after choline addition. These data are in agreement with the cell fractionation experiments in which the cytosolic activity reached a plateau 2–4 h after the addition of choline (Fig. 2). The release of lactate dehydrogenase from the hepatocytes was not affected by the addition of choline (Fig. 3, inset).

Choline Deficiency Minimally Alters the Location of Phosphatidate Phosphohydrolase in Rat Liver—We were curious how specific the choline deficiency effect was for enzyme translocation. Phosphatidate phosphohydrolase is an important enzyme in glycerolipid biosynthesis and like CT is translocated from cytosol to membranes by fatty acids and other compounds (47). This enzyme activity was, therefore, measured in choline-deficient and choline-supplemented rat liver fractions. There was no effect on the cytosolic activity and a slightly significant increase in microsomal activity (Fig. 4).

Since the magnitude of the effect was small compared with the change in CT distribution in choline-deficient livers, we conclude that the choline-deficient mediated translocation of CT appears to be specific.

Choline Deficiency Stimulates CT Binding to the ER but Not the Golgi—Since Golgi from rat liver contains significant amounts of CT activity (3), we tested to see if choline deficiency caused an increased binding of CT to both Golgi and ER membranes. Surprisingly, the translocation was very specific for the ER with no increase in CT activity in the Golgi (Fig. 5). It was possible that choline deficiency might have altered the properties of the membranes such that the procedure used for isolation of Golgi and ER might be altered. We therefore compared the activity of an ER and a Golgi marker enzyme recovered from choline-deficient and choline-supplemented livers. The results (Table III) show that the enrichment of these two marker enzymes and recovery of ER and Golgi were similar in choline-deficient and choline-supplemented livers. Thus, choline deficiency directs the CT to the ER which is the major site of PC synthesis in liver (3) but not to the Golgi membranes.

**DISCUSSION**

The results of this study provide direct evidence for the translocation theory of CT for regulation of PC biosynthesis in a physiologically relevant animal model. Young rats fed a choline-deficient diet develop fatty livers (i.e. accumulate triacylglycerol) presumably as a consequence of reduced PC biosynthesis and decreased very low density lipoprotein secre-
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FIG. 3. Digitonin-mediated release of cytidylyltransferase activity by cultured hepatocytes supplemented with choline. Hepatocytes were isolated from a choline-deficient rat liver and cultured in a choline- and methionine-free medium + 17% delipidated fetal calf serum. After 4 h the medium was replaced with serum-free medium + 100 μM choline. The cells were incubated in the absence (x) or presence of choline for 1 (A), 2 (B), and 4 h (C). The culture medium was removed, and the cells were washed with ice-cold phosphate-buffered saline once and incubated with 0.8 ml of digitonin solution. Aliquots (60 μl) of the digitonin-released enzyme were collected after 2-, 4- and 8-min incubations and used for CT assay. Each point is the average of two independent experiments which do not differ by greater than 20%. Cytidylyltransferase activities released from the cells cultured in the absence of choline for 1, 2, and 4 h were identical. The unit of enzyme activity is nanomoles of CDP-choline formed per min. Inset, the release of lactate dehydrogenase (LDH) activity from the hepatocytes + choline after incubation with digitonin for 4 min. Activity of lactate dehydrogenase is assayed by measuring the decrease in the absorbance of NADH (ΔA/min).

The experiments in choline-deficient and choline-supplemented livers provide the first direct evidence that the translocation of CT activity is due to a movement of the mass of the enzyme. This is not an unexpected result. Nevertheless in previous studies it was not possible to exclude an activation of CT already on membranes or an inactivation of CT present in the cytosol. In previous experiments using chicken antiserum prepared against partially purified CT, we were unable to detect a change in the amount of immunotitratable CT in choline-deficient liver cytosol even though there was a decrease of 40% in the cytosolic CT activity (25). However, in
the present studies, the antigen for immunization was almost pure, and the immunoblots were performed after the enzyme had been denatured and separated by electrophoresis. The result that the mass of CT in the choline-deficient cytosol was decreased was clearly demonstrated and highly reproducible. Immunoblotting will still have to be applied to other independent experiments. CD, choline-deficient; CS, choline-supplemented.

Since fatty acids cause both CT and phosphatidate phosphohydrolase activities to translocate from cytosol to microsomes, we expected that the phosphatidase might also be increased in the microsomes of choline-deficient livers. However, the choline-deficient state had a minimal effect on the distribution of phosphatidate phosphohydrolase; thus, there is good reason to believe that the translocation of CT is a highly specific phenomenon. We have also observed a specific effect of choline deficiency on very low density lipoprotein secretion with no effect on the secretion of other proteins or lipoproteins from rat hepatocytes (30). Such specificity observed in these experiments suggests that the choline deficiency model is a particularly useful system for study of PC biosynthesis and function without many additional complications.

The major question that arises out of this investigation is what is the mechanism by which CT is reversibly translocated in choline-deficient animals? Possible mechanisms have been explored in the companion paper (49) in which we present evidence that the translocation to ER in choline-deficient livers is due to a decrease in the amount of PC in the ER.

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REFERENCES

31.旄, F., Record, M., Robert, E., and Sandi Ungarian for technical assistance. We wish to thank Drs. Ashley Martin and David Brindley for assays of phosphatidic acid phosphohydrolase and Dr. Jean Vance for helpful discussions.

TABLE III
Recovery of marker enzymes for Golgi and endoplasmic reticulum from the liver

Preparations of subfractions of liver, protein analysis, and enzyme assays were performed as described under "Experimental Procedures." For the enzyme assays, the two ER fractions (ER I and ER II) (36) were pooled. The specific activity of NADPH:cytochrome c reductase (ER marker) is expressed as nanomoles of NADP produced per min per mg of protein. The units for specific activity of galactosyltransferase (Golgi marker) are nanomoles of galactose transferred per h per mg of protein. The values are means ± S.D. for four independent experiments. CD, choline-deficient; CS, choline-supplemented.

<table>
<thead>
<tr>
<th></th>
<th>Microsomal protein</th>
<th>NADPH:cytochrome c reductase</th>
<th>Galactosyltransferase</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>mg/g liver</td>
<td>nmol/min/mg</td>
<td>nmol/h/mg</td>
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<tr>
<td>Homogenate</td>
<td></td>
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<tr>
<td>CD</td>
<td>3.8 ± 2.9 (4)</td>
<td>10.7 ± 13.0 (3)</td>
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</tr>
<tr>
<td>CS</td>
<td>4.3 ± 2.3 (4)</td>
<td>12.7 ± 11.1 (4)</td>
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<tr>
<td>Golgi</td>
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<tr>
<td>CD</td>
<td>0.37 ± 0.10 (8)</td>
<td>1.45 ± 7.7 (4)</td>
<td>840 ± 115 (4)</td>
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<tr>
<td>CS</td>
<td>0.37 ± 0.13 (8)</td>
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<td>786 ± 84 (4)</td>
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<td>ER</td>
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<tr>
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Choline deficiency causes translocation of CTP:phosphocholine cytidylyltransferase from cytosol to endoplasmic reticulum in rat liver.
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