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Effect of diethylstilboestrol on phosphatidylcholine biosynthesis and choline metabolism in the liver of roosters

Carmen VIGO and Dennis E. VANCE
Department of Biochemistry, University of British Columbia, Vancouver, B.C., V6T 1W5, Canada

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It has been known for 40 years that oestrogens stimulate phospholipid metabolism in roosters. We have investigated *in vivo* the mechanism for this effect. Young roosters were injected daily with 1 mg of diethylstilboestrol for 1–3 days. At 4 h after the last injection, 30 μCi of [*Me*-^{3}H]choline was injected into the portal vein. At periods up to 3 min the livers were freeze-clamped and choline and its metabolites were extracted and resolved by t.l.c. Hormone treatments in the first 2 days resulted in a 2-fold increase in phosphorylation of [*Me*-^{3}H]choline and a decrease in the oxidation of [*Me*-^{3}H]choline to [^{3}H]betaine. The concentrations of phosphocholine in liver were increased 2-fold during the first 2 days concomitant with a 2-fold increase in the rate of phosphatidylcholine biosynthesis. After 3 days of hormone treatment, many of the above effects were reversed and the rate of phosphatidylcholine biosynthesis decreased to approx. 60% of the control value. The results suggest that the initial hormone treatments activate choline kinase within 4 h and, thereby, divert choline form oxidation to betaine. The resulting increased phosphocholine concentrations cause an increase in the activity of CTP:phosphocholine cytidylyltransferase, which results in a doubling of the rate of phosphatidylcholine biosynthesis. After 3 days of hormone treatment, the biosynthesis of phosphatidylcholine is decreased, most likely by an effect on the cytidylyltransferase reaction.

The major pathway for the biosynthesis of phosphatidylcholine was described in the 1950s (Kennedy, 1962). In recent years significant advances have been made in our understanding of the control of this biosynthetic pathway (Porcellati, 1972; Infante, 1977; Vance & Choy, 1979; Rooney, 1979). Most of the evidence suggests that the CTP:phosphocholine cytidylyltransferase catalyses the rate-limiting reaction for phosphatidylcholine biosynthesis (Vance & Choy, 1979). Furthermore, many reports have described interesting regulatory features for this enzyme. In rat liver, the cytidylyltransferase is activated by several phospholipids, of which the most important appears to be lysophosphatidylethanolamine (Choy *et al.*, 1977; Choy & Vance, 1978). The enzyme from rat lung is also activated by phospholipid and in this case phosphatidylglycerol appears to be the most significant (Feldman *et al.*, 1978). Another type of regulation of this enzyme has been described in HeLa cells, where the concentration of CTP in the cytoplasm correlated with the rate of the reaction catalysed by the cytidylyltransferase and the rate of phosphatidylcholine biosynthesis (Vance *et al.*, 1980; Choy *et al.*, 1980). On the other hand theoretical and some experimental work have implicated a regulatory and rate-limiting role for choline kinase (Infante, 1977; Infante & Kinsella, 1978).

Our interest in the control of phosphatidylcholine biosynthesis has focused on the liver. In this organ, the synthesis of phosphatidylcholine is required for the cellular membranes, and is also important for the secretion of plasma lipoproteins and bile. We therefore expected to find several regulatory features in this organ. In our studies with choline-deficient rats we demonstrated a 60% decrease in the activity of the cytidylyltransferase (Schneider & Vance, 1978) and showed that this was not due to a change in the amount of immunoprecipitable enzyme (Choy *et al.*, 1978*a*). More recently, we have demonstrated a 2–3-fold increase in the cytidylyltransferase activity in rats fed a 5% cholesterol/2% cholate diet (P. H. Lim, P. H. Pritchard & D. E. Vance, unpublished work).

Another approach to alter lipid metabolism in the liver would be through hormones that are important for the regulation of growth, differentiation and metabolic activities in most tissues (Chan & O'Malley, 1978). Surprisingly little information is available on how hormones influence phosphatidylchol-

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ine biosynthesis in liver. A search of the literature has shown that a very marked effect occurred when sexually immature chickens were injected with oestrogens or synthetic oestrogens (Entenman *et al.*, 1940; Flock & Bollman, 1942; Taurog *et al.*, 1944). The experiments demonstrated markedly increased concentrations of plasma phospholipids and increased incorporation of ^{32}P into the phospholipids. We thought that these intriguing results of the early 1940s should be re-investigated now that we have a better understanding of phosphatidylcholine biosynthesis. In the present work we confirm these early findings and provide evidence for a mechanism by which phosphatidylcholine biosynthesis is enhanced more than 2-fold in livers from diethylstilboestrol-treated roosters.

Materials and methods

Materials

Diethylstilboestrol, betaine, CTP, CDP-choline, ITP, phosphocholine and choline were from Sigma Chemical Co., St. Louis, MO, U.S.A. [*Me*-^{3}H]-Choline was obtained from Amersham, Oakville, Ont., Canada. All other materials and supplies were obtained from local supply houses.

Animals

Male chickens, 1 day old, were supplied by Western Hatcheries, Abbotsford, B.C., Canada. They were maintained in a light and temperature-controlled room for up to 28 days. They had free access to water and were fed with Purina chick starter every morning. On the day the chicks were killed, they were only supplied with water.

Injection of diethylstilboestrol

Diethylstilboestrol was dissolved in corn oil (20mg/ml) and 1mg was injected intradermally in the legs of the chicks. Control animals received only the corn oil (50μl). The amount of hormone injected was approx. 1.5mg/100g body wt. of chicken. For the experiments reported in the present paper, only chicks that were 7–9 days old were used. Preliminary studies have shown that similar effects of the hormone on choline metabolism were observed in chicks that were 14, 21 and 28 days of age.

Injection of [Me-^{3}H]choline

[*Me*-^{3}H]Choline (30μCi) was dried under N_2 and dissolved in 0.1ml of 0.9% NaCl at a concentration of 17μM. The chickens were lightly anaesthetized with diethyl ether 4h after the last hormone injection. The abdomen was opened and the [*Me*-^{3}H]choline was injected via the portal vein. At intervals up to 3min, part of the liver was rapidly removed and freeze-clamped. The lipids and water-soluble compounds were extracted by the method of Bligh & Dyer (1959).

Determination of [Me-^{3}H]choline in lipids and water-soluble compounds

The organic phase of the extraction was dried under N_2 and dissolved in 250μl of chloroform. An aliquot was spotted on a thin-layer plate (silica gel G), which was developed in chloroform/methanol/water (65:25:4, by vol.). The lipids were detected visually with I_2 vapour, scraped into scintillation vials and the radioactivity was determined. For quantification of the amount of lipid, the silica gel was eluted with 6ml of chloroform/methanol (1:2, v/v) and the lipid phosphorus was determined (Raheja *et al.*, 1973).

The aqueous phase was freeze-dried and dissolved in 0.5ml of water. A portion was applied to a thin-layer plate (silica gel G-25). Carrier compounds (10μg of choline, 50μg of phosphocholine, 30μg of CDP-choline and 20μg of betaine) were also applied to the plate. The compounds were completely resolved by two-dimensional t.l.c. The initial solvent system was methanol/0.6M-NaCl/NH_3 (10:10:1, by vol.) followed by methanol/chloroform/12M-HCl (45:5:2, by vol.). The compounds were detected with I_2 vapour, the silica gel scraped into scintillation vials and the radioactivity determined.

Measurement of phosphocholine

Of the aqueous phase 1ml was treated twice with 0.1g of acid-washed charcoal and the phosphocholine was determined (Choy *et al.*, 1978*b*).

Measurement of nucleotide triphosphates

The method was adopted from the procedures of Elion *et al.* (1977). The internal standard ITP (410nmol) was added to the liver suspension in chloroform/methanol before homogenization. The aqueous phase was freeze-dried and redissolved in 50μl of water. Of this sample 16μl was analysed by high-pressure liquid chromatography on a column (400mm × 2mm) of partisil 10-SAX. The nucleotides were eluted with a linear gradient of 0.1–1.0M-KH_2PO_4, pH3.8. The buffer was pumped through the column at 23ml/h by a Milton Roy pump. The eluate was monitored at 280nm with an Altex detector. The area on the chromatogram for each nucleotide was gravimetrically determined.

Results

Effect of diethylstilboestrol on [Me-^{3}H]choline incorporation into phosphatidylcholine

Diethylstilboestrol had no effect on the weight of the liver per chick on day 1 of the treatment. On day 2 there was a small increase in the liver weight [3.49 ± 0.31g (mean ± S.D.; $n = 4$) for controls and

4.36 ± 0.39 g ($n = 4$) for treated chicks ($P < 0.05$)]. By day 3 the liver had increased in weight by 53% [3.40 ± 0.39 g (mean ± S.D.; $n = 4$) for controls and 5.18 ± 0.13 g ($n = 4$) for treated chicks ($P < 0.001$)] and the liver had a distinctly yellow and fatty appearance. Although the amount of phosphatidylcholine per g of liver did not change during the 3 days of treatment, obviously the total amount of this lipid per liver was increased by day 3.

[*Me*-^{3}H]Choline was injected into the portal vein of the chickens. At various times the liver was rapidly removed, the lipids extracted and resolved by t.l.c. Of the radioactivity 90% was incorporated into phosphatidylcholine, 4% into sphingomyelin and 6% into lysophosphatidylcholine. The synthetic oestrogen did not affect this distribution.

Diethylstilboestrol increased the incorporation of [*Me*-^{3}H]choline into phosphatidylcholine during the first and second day of treatment. The results were similar on both days and so only the data from day 1 are shown in Table 1. A marked decrease in incorporation of labelled choline occurred on day 3 (Table 1). The specific radioactivity in phosphatidylcholine 3 min after injection of [*Me*-^{3}H]choline on day 1 was found to be 76 ± 10 d.p.m./μg (mean ± S.D.) and 190 ± 22 d.p.m./μg ($n = 3$) for control and experimental chicks respectively. On day 3, the ratio was reversed with a specific radioactivity in phosphatidylcholine of 223 ± 23 d.p.m./μg (mean ± S.D.) and 119 ± 22 d.p.m./μg ($n = 3$) in the control and diethylstilboestrol-treated chicks. Approximately half of this decrease in specific radioactivity can be accounted for by the 50% increase in the phosphatidylcholine in the total liver as noted above. As can be seen, the actual specific radioactivities varied from day to day. This may be due to a large number of different factors, which could include the development state of the chicks, the nutritional state and/or variations in the amount of [*Me*-^{3}H]choline taken up by the liver. However, in every case the relative specific radioactivities were higher in the hormone-treated chicks on day 1 and day 2 and lower on day 3.

Effect of diethylstilboestrol on [Me-^{3}H]choline metabolism

The incorporation of labelled choline into betaine and the water-soluble precursors of phosphatidylcholine was also monitored at various times after the injection into the portal vein. The results after day 1 of hormone treatment are shown in Fig. 1. It is clear that the hormone-treated chicks show an increased phosphorylation of choline and decreased oxidation of choline to betaine. Similar results were obtained on day 2 of diethylstilboestrol treatment. In contrast, on day 3 there was no longer a stimulation of the conversion of [*Me*-^{3}H]choline into phosphocholine per g of liver (Fig. 2). However, since the liver weights were increased by 50% on day 3, the total amount of choline phosphorylated was slightly but not significantly elevated in the hormone-treated chicks. It is also evident that the radioactivity associated with CDP-choline (Figs. 1 and 2) was always low and unaffected by diethylstilboestrol. The large amount of radioactivity in the choline pools at 30 s is most likely [*Me*-^{3}H]choline in the blood plasma compartment of the liver tissue.

The results of the labelling studies indicated that diethylstilboestrol treatment stimulated the formation of phosphocholine within 4 h after the first injection. This was confirmed by measurement of the pool sizes of phosphocholine in liver. Table 2 shows that the increased phosphorylation of choline did result in a doubling of the phosphocholine pool by day 2. On day 3 in the diethylstilboestrol-treated chicks, there was a significant decrease compared with day 2 in the phosphocholine pool per g of liver,

Table 1. *Effect of diethylstilboestrol (DES) on the incorporation of [Me-^{3}H]choline into phosphatidylcholine*

Roosters (7 days old) were injected intradermally for 1 or 3 days with 1 mg of diethylstilboestrol in 50 μl of corn oil or with only 50 μl of corn oil. At 4 h after the last injection, the cockerels were lightly anaesthetized with diethyl ether. The abdomen was opened and 30 μCi of [*Me*-^{3}H]choline was injected via the portal vein. At intervals up to 3 min part of the liver was rapidly removed and freeze-clamped. The lipids were extracted by the method of Bligh & Dyer (1959) and resolved by t.l.c. (Vance *et al.*, 1980). Values are means for three determinations ± S.D., except at 0.5 min on day 1, which is the average of two determinations.

Period of treatment (days)	Time after injection (min)	10^{-3} × Radioactivity (d.p.m./g of liver)		10^{-3} × Radioactivity (d.p.m./liver)		Radioactivity (d.p.m./mg of protein)	
		−DES	+DES	−DES	+DES	−DES	+DES
1	0.5	14.2	34.1	48.8	117	114	289
	1.5	30.7 ± 12	105 ± 23	106 ± 41	361 ± 79	246 ± 22	890 ± 17
	3.0	212 ± 28	537 ± 61	729 ± 96	1847 ± 210	1696 ± 152	4550 ± 273
3	0.5	53.7 ± 25.6	33.2 ± 17.9	184 ± 88	171 ± 93	348 ± 83	224 ± 22
	1.5	210 ± 51.1	81.8 ± 33.2	722 ± 176	424 ± 171	1363 ± 327	552 ± 55
	3.0	514 ± 54	192 ± 36	1768 ± 186	995 ± 186	3338 ± 801	1297 ± 130

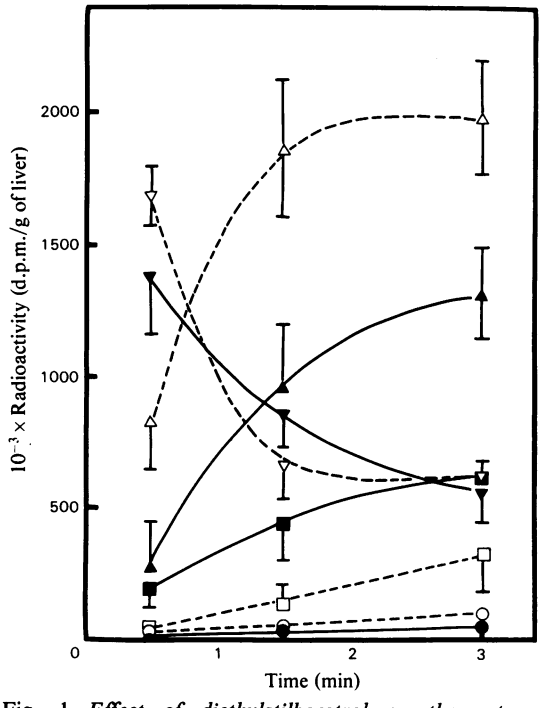

Fig. 1. *Effect of diethylstilboestrol on the rate of [Me-^{3}H]choline metabolism in the liver of roosters after day 1 of treatment*

Open symbols show results for diethylstilboestrol treatment; closed symbols show results for controls. ▲ and △, Phosphocholine; ▼ and ▽, choline; ■ and □, betaine; ● and ○, CDP-choline. Data are means from three to five chickens ± S.D.

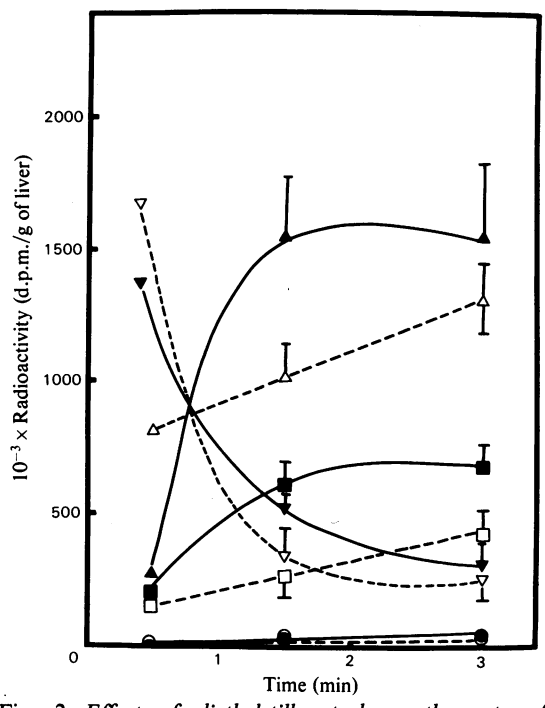

Fig. 2. *Effect of diethylstilboestrol on the rate of [Me-^{3}H]choline metabolism in the liver of roosters after day 3 of treatment*

Open symbols show results for diethylstilboestrol treatment; closed symbols show results for controls. ▲ and △, Phosphocholine; ▼ and ▽, choline; ■ and □, betaine; ● and ○, CDP-choline. Data are means from three chickens ± S.D.

Table 2. *Concentration of phosphocholine in chicken liver*

At 4h after the last hormone injection, the chickens were killed and part of the liver was quickly removed and freeze-clamped. The choline-containing compounds were then extracted, resolved by t.l.c. and phosphocholine concentrations were estimated as described in the Materials and methods section. Results are means ± S.D. for six chickens.

Period of treatment (days)	Treatment	Phosphocholine (nmol/g wet wt.)	
Chicken 1	−Diethylstilboestrol	163 + 63 (6)	$p < 0.05$
	+Diethylstilboestrol	232 + 72 (6)	
2	−Diethylstilboestrol	194 + 72 (6)	$p < 0.005$
	+Diethylstilboestrol	396 + 65 (6)	
3	−Diethylstilboestrol	120 + 48 (6)	$p < 0.025$
	+Diethylstilboestrol	184 + 43 (6)	
Rat	—	1483	

which could not be accounted for by the increase in the liver weight. The concentration of phosphocholine in the control chicken liver is 6–7-fold lower than found in adult rat liver (Choy *et al.*, 1978*b*; Table 1).

From the data in Tables 1 and 2, the rate of synthesis of phosphatidylcholine can be estimated (the difference in d.p.m. in phosphatidylcholine between 1.5 and 3 min divided by the specific radioactivity of phosphocholine) on day 1 to be 37 nmol/min per g of liver for the hormone-treated chicks and 18 nmol/min per g of liver for control chicks. On day 3 the rate of phosphatidylcholine synthesis was estimated to be 18 and 11 nmol/min

per g of liver for control and treated animals respectively. These calculations confirm the impression that one has from Table 1, that phosphatidylcholine biosynthesis is stimulated on day 1 in the hormone-treated roosters, and on day 3 there is a significant decrease in the rate of phosphatidylcholine biosynthesis per g of liver. The rate per liver on day 1 would be twice as high in the hormone-treated compared with the control chicks. On day 3 the rate per liver would be 63 nmol/min in controls and 57 nmol/min in treated chicks.

Nucleotide triphosphate concentrations in rooster livers

Studies with HeLa cells have indicated that the rate of phosphatidylcholine biosynthesis can be altered by a change in the concentration of CTP (Vance *et al.*, 1980; Choy *et al.*, 1980). It was, therefore, of interest to see if the concentrations of CTP were altered in the hormone-treated livers. The results showed no changes in the concentration of CTP [33.9 ± 6.67 nmol/g wet wt. of tissue (mean $\pm$ S.D.)] or the other nucleotide triphosphates. On day 3 the concentration of CTP per liver was increased 1.5-fold by diethylstilboestrol treatment. The concentration per g of tissue presumably reflects more accurately the concentration of CTP in the liver cytoplasm. In either case, the rate of phosphatidylcholine biosynthesis does not seem to correlate with the concentration of CTP in liver.

Discussion

Our studies have demonstrated that diethylstilboestrol treatment will stimulate by 2-fold the synthesis of phosphatidylcholine in the immature rooster liver. Beyond that we have shown that the hormone causes a 2-fold increase in the phosphorylation of choline with a concomitant decrease in the oxidation of choline to betaine. This results in a 2-fold increase in the phosphocholine concentration of chicken liver on the second day of treatment, and a similar increase in the rate of phosphatidylcholine biosynthesis. The results suggest that the hormone activates choline kinase, which diverts more choline into phosphocholine. Because the phosphocholine concentrations are near or below the apparent K_m (0.17 mM) (Choy *et al.*, 1977) for this substrate for cytidylyltransferase, it is likely that the increased phosphocholine concentrations are directly translated into a similar increase in the rate of phosphatidylcholine biosynthesis.

The idea that phosphocholine concentrations could modulate the activity of the cytidylyltransferase and phosphatidylcholine biosynthesis contrasts with the results from rat liver (Choy *et al.*, 1978*b*) and HeLa cells (Vance *et al.*, 1980). In these two systems, the concentration of phosphocholine is in the range 1–2 mM, well above the apparent K_m. Thus fluctuations in the concentration of this substrate would have no effect on the enzyme activity. However, in baby hamster kidney cells the concentrations of phosphocholine are low (0.07 mM) (Choy *et al.*, 1978*b*), and the activity of the cytidylyltransferase in these cells appears to be a function of the concentration of this substrate (Whitehead *et al.*, 1981). Apparently, diverse mechanisms are available for modulating the rate of phosphatidylcholine biosynthesis.

It is noteworthy that the rates of phosphatidylcholine biosynthesis we observed in chicken liver are very similar to those reported for the rat (Sundler *et al.*, 1972) even though the concentrations of phosphocholine in the livers from the two animals differ by 6–7-fold.

It has been argued that the activity of choline kinase could be rate-limiting for phosphatidylcholine biosynthesis (Infante, 1977). On the other hand, most studies support the idea that the rate-limiting reaction is catalysed by the cytidylyltransferase (Vance & Choy, 1979). The results of the present studies support the latter interpretation. If the kinase were rate-limiting, there should be an accumulation of radioactivity in choline. As it disappeared, the radioactivity would quickly pass into phosphatidylcholine via phosphocholine and CDP-choline. As can be seen in Figs. 1 and 2, this is clearly not the case. Rather the radioactivity accumulates in phosphocholine and is only transiently associated with CDP-choline. Secondly, if choline kinase were rate-limiting, there should be no correlation between the concentration of phosphocholine and the rate of phosphatidylcholine biosynthesis. Instead such a correlation was observed on day 1 and day 2. Even though the cytidylyltransferase catalyses the rate-limiting reaction, the rate of this reaction appears to be regulated by the activity of choline kinase.

The results on day 3 of treatment are markedly different from day 1 and day 2 in the treated cockerels. The conversion of [*Me*-^{3}H]choline into phosphocholine has decreased to control values (Fig. 2), although the amount of phosphocholine is still elevated compared with control values. Despite this increased concentration of phosphocholine, phosphatidylcholine biosynthesis per g of liver is decreased to below control values. Thus it appears that by day 3 the activity of the cytidylyltransferase has been altered in some way so that the concentration of phosphocholine is no longer limiting. Further interpretation of the results on day 3 are complicated by the 50% increase in the weight of the livers from the treated chicks.

We have recently assayed under optimal conditions the enzymes involved in phosphatidylcholine biosynthesis in livers from roosters injected with

diethylstilboestrol (Vigo *et al.*, 1981). The results are in good agreement with the data in the present paper. Choline kinase activity is elevated 2-fold on day 1 and remains stimulated on day 2 and day 3. The activity of the cytidylyltransferase is unchanged until day 3 when it is inhibited by 50%.

Obviously, the next study should be directed toward an analysis of the mechanism by which the activity of choline kinase is increased. Secondly, we need to see how the activity of the cytidylyltransferase is inhibited after 3 days of treatment with diethylstilboestrol.

The activity of the third enzyme involved in the synthesis of phosphatidylcholine, CDP-choline: 1,2-diacylglycerol phosphocholinetransferase, has been assayed after a similar treatment of roosters for 5 days (Coleman *et al.*, 1977). They found a small increase in total activity per liver, but a 40% decrease in specific activity. The data in Fig. 2 make it seem unlikely that the activity of the phosphocholinetransferase is limiting the rate of phosphatidylcholine biosynthesis after 3 days of hormone treatment.

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