Role of Thyroid Hormone in De Novo Synthesis of Cholinephosphotransferase in Guinea Pig Lung Mitochondria and Microsomes

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ABSTRACT: *It is known that thyroid hormone enhances the biosynthesis of phosphatidylcholine in the lung. The purpose of the present study was to investigate the effects of thyroid hormone on the activity of cholinephosphotransferase, the terminal enzyme in the CDP-choline pathway, in guinea pig lung mitochondria and microsomes. Intramuscular injection of triiodothyronine (T_3, 0.25–4 mg/kg body wt) stimulated the activities of both mitochondrial and microsomal enzymes in a dose-dependent manner. However, the stimulation was much more pronounced in the microsomes than in the mitochondria. The stimulatory effect of T_3 was blocked by the intraperitoneal injection of both actinomycin D and cycloheximide in the microsomes, whereas in the mitochondria, the hormonal effect was blocked only by cycloheximide. Thus, it is suggested that T_3 stimulates not only the nucleocytoplasmic system for the de novo synthesis of the enzyme, but possibly also the regulation of the transport of the synthesized protein into the mitochondria. Furthermore, administration of T_3 produced an increase in the uptake and incorporation of [^{14}C]choline into phospholipids of lung slices in vitro. However, this effect was blocked by intraperitoneal injection of both actinomycin D and cycloheximide. Thus, the change in CPT activity by T_3 in mitochondria is not reflected by enhanced incorporation of choline into phosphatidylcholine.*

INTRODUCTION

Thyroid hormone plays an important role in the maturation and regulation of surfactant phospholipid synthesis of fetal lungs [1, 2]. In vitro stimulation of phosphatidylcholine (PC) synthesis by thyroid hormone, as observed in the fetal rabbit and rat lung [3, 4], is probably due to the

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stimulatory effect of the hormone on the activity of the rate-limiting enzyme for the cytidine diphosphocholine (CDP-choline) pathway, cholinephosphate cytidyltransferase [5]. We recently reported that in the guinea pig lung, thyroid hormone stimulates the activity of glycerophosphate acyltransferase, a regulatory enzyme for PC biosynthesis [6]. It is possible that the stimulation of PC synthesis by thyroid hormone may involve nuclear receptors, de novo RNA, and protein synthesis. We previously reported that cholinephosphotransferase, the terminal enzyme in CDP-choline pathway, is present in the outer mitochondrial and microsomal membrane of lung [7]. Furthermore, a close examination of the developmental pattern of cholinephosphotransferase activity of fetal guinea pig lung [8] and that of the fetal guinea pig plasma thyroid level [9] shows a similar pattern of gestational variation. The purpose of this study was to investigate whether thyroid hormone modulates the activity of cholinephosphotransferase in guinea pig lung, and if so, to determine its mechanistic basis. In this regard, it is important to know if the change in CPT activity by thyroid hormone is reflected by enhanced incorporation of choline into PC.

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MATERIALS AND METHODS

Chemicals

All biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Radioactive cytidine diphosphocholine and choline chloride were purchased from New England Nuclear (Boston, MA). Butanol was purchased from Malinckrodt Inc. (Paris, KY). Universol cocktail was purchased from ICN Radiochemicals (Irvine, CA).

Animals

Adult female guinea pigs (Hartley strain) were obtained from Camm Laboratory Animals (Wayne, NJ). All animals were housed in stainless steel cages and fed ad libitum. The experimental animals were injected intramuscularly for 2 days with a freshly prepared solution of T_3 in 5 mM NaOH, at a daily dose of 0.25 to 4.0 mg/kg body wt. The control animals received the vehicle only. To determine whether the increased cholinephosphotransferase activity observed after the hormonal treatment was associated with the enzyme synthesis, another experiment was carried out in which the guinea pigs were injected intraperitoneally with actinomycin D (200 μg/kg body wt) or cycloheximide (200 μg/kg body wt) daily for 2 days during the hormonal treatment (1 mg/kg body wt). All animals were killed on the third day by decapitation.

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Subcellular Fractionation

The lungs were quickly excised, washed in ice cold saline (0.9%), blotted dry, weighed, and homogenized in 10 volumes of 0.25 M sucrose/1 mM EDTA (pH 7.4) using a Potter Elvehjem homogenizer. After the removal of nuclei and cell debris by centrifugation at 600 *g* for 10 min, mitochondria were sedimented at 10000 *g* for 10 min in a Sorvall SS-34 rotor at 4°C. The 10000 *g* supernatant fluid was centrifuged at 105000 *g* for 1 h to sediment the microsomes. The mitochondrial and microsomal pellets were washed with 0.1 M Tris–HCl/1 mM EDTA (pH 7.4) and suspended in the same buffer. The mitochondrial fraction was further purified by linear sucrose density gradient [7]. Protein concentration was measured by the procedure of Lowry et al. [10].

Enzyme Assays

The authenticity of the mitochondria and microsomes was established by the determination of the activities of three markers: monoamine oxidase, succinic dehydrogenase, and rotenone-sensitive NADPH cytochrome c reductase [7, 11]. Cholinephosphotransferase activity was assayed by measuring the incorporation of radioactivity from cytidine diphospho-[methyl-^{14}C]choline into PC using dioleoylglycerol as a substrate, as described earlier [8].

Incorporation of [^{14}C]Choline into Lung Tissue Slices

Groups of control and treated animals were anesthetized with sodium pentobarbital (100 mg/kg IP) containing 4000 U heparin. The chest cavity was opened and the lungs were perfused by gravity with a prewarmed (37°C) solution of phosphate-buffered saline containing glucose. During the perfusion, the lungs were ventilated manually with air via a cannula inserted into the trachea. The lung lobes were dissected from the hilus and cleaned of extraneous tissue. They were rinsed three times in ice-cold oxygenated Krebs-Ringer bicarbonate buffer supplemented with 10 mM glucose, 1 mM pyruvate, and 21 amino acids [12], and blotted dry and weighed. In a cold room at 4–6°C, lung slices of uniform thickness (0.5 mm) were prepared from all lobes by a Mcilwain tissue chopper. The lung slices from each animal were pooled in 25-mL Erlenmeyer flasks containing 9.5 mL of modified Krebs-Ringer buffer. One microcurie [^{14}C]choline chloride, in a total volume of 0.5 mL of buffer, was added to each flask and mixed by swirling [13]. A 25-μL aliquot was taken from each flask and transferred to scintillation vials containing 10 mL Universol cocktail for determination of preincubation radioactivity. The flasks were incubated at 37°C in a Dubnoff metabolic shaker for 3 h under an atmo-

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sphere of 95% O_2 + 5% CO_2 (v/v). After incubation, another 25-μL aliquot was taken to determine postincubation radioactivity. The contents of the flask were then poured off onto a 20-μm nylon cloth to recover lung slices. The slices were rinsed several times with ice-cold isotonic saline to remove unincorporated [^{14}C]choline from the surfaces.

Tissue Extraction and Analysis of Choline-Containing Lipids and Water-Soluble Intermediates of Phosphatidylcholine

The slices were transferred to a 50-mL plastic centrifuge tube and homogenized in 5 mL isotonic saline by a Brinkman polytron homogenizer. A 0.2-mL aliquot from each sample was frozen at −20°C for measurement of protein. To the remaining homogenate, 15 mL of chloroform/methanol (4:1, v/v) was added for lipid extraction. Three milliliters of water was added to the homogenate to create a two-phase system, which was vortexed for 1 min. The samples were then centrifuged at 3000 rpm for 10 min to establish a clear two-phase system. The organic phase was transferred to a set of 50-mL round-bottom flasks and evaporated to dryness under nitrogen in an Organomation N evaporator. The aqueous phase was transferred to another set of 50-mL round-bottom flasks and the pellets were discarded. The aqueous phase was dried in the same manner as the organic phase.

The organic phase was reconstituted in 1 mL of chloroform/methanol (4:1, v/v), and a 25-μl aliquot was taken to determine the total radioactivity in the extract. One hundred microliters of the reconstituted sample was spotted on a 20 × 20-cm Brinkman silica gel, thin-layer chromatography plate and developed in a tank with a mobile phase of chloroform/methanol/acetic acid/H_2O (50:37.5:3.5:2, by vol) [13]. Lysophosphatidylcholine (LPC), sphingomyelin (SM), and phosphatidylcholine (PC) were visualized by spraying the plate with a saturated solution of fluorescein in methanol and exposing it to ammonia vapor in a developing tank. The plate was illuminated with an ultraviolet lamp to clearly differentiate the bands, which were circled and scraped into scintillation vials and to which 10 mL of Universol ES (ICN Biochemicals) was added for radioactivity assay. To determine the total disaturated PC (DSPC), the lipids were reacted with osmium tetroxide dissolved in carbon tetrachloride [14]; they were separated into individual phospholipid components by TLC and processed as above.

The aqueous phase was reconstituted in 1 mL methanol and a 100-μL aliquot was taken to determine total radioactivity. One hundred microliters of sample was spotted on a 20 × 20-cm Brinkman silica gel TLC plate and developed in a tank with a mobile phase of *n*-butanol/acetic acid/water (5:1.6:4, v/v/v) [13]. Cytidine diphosphocholine (CDPcholine), phosphocholine (PPC), and choline (C) were located using radioactive

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standards on the same TLC plate as the samples. The bands were located by exposing the plate to an iodine chamber. They were then circled and scraped into scintillation vials to which 10 mL of Universol was added to quantitate the radioactivity. All liquid scintillation counting was done by a Beckman (LS-355) scintillation counter.

Statistical Analysis

The data are reported as means ± standard errors of the means (SE). Means of control and treated groups were compared using unpaired Student's t test [15]. The p values less than .05 were considered significant.

RESULTS

Intramuscular injection of T_3 to 24-week-old guinea pigs at a daily dose between 0.25 and 4 mg/kg body wt for 2 days stimulated the activity of both mitochondrial and microsomal cholinephosphotransferase in a dose-dependent manner (Fig. 1). The mitochondrial enzyme activity was increased by 25, 56, 113, 103, 94, and 34% by intramuscular injection of T_3 at a dose of 0.25, 0.5, 1, 2, 3, and 4 mg/kg body wt, respectively. At the same time, the microsomal enzyme activity was increased by 26, 74,

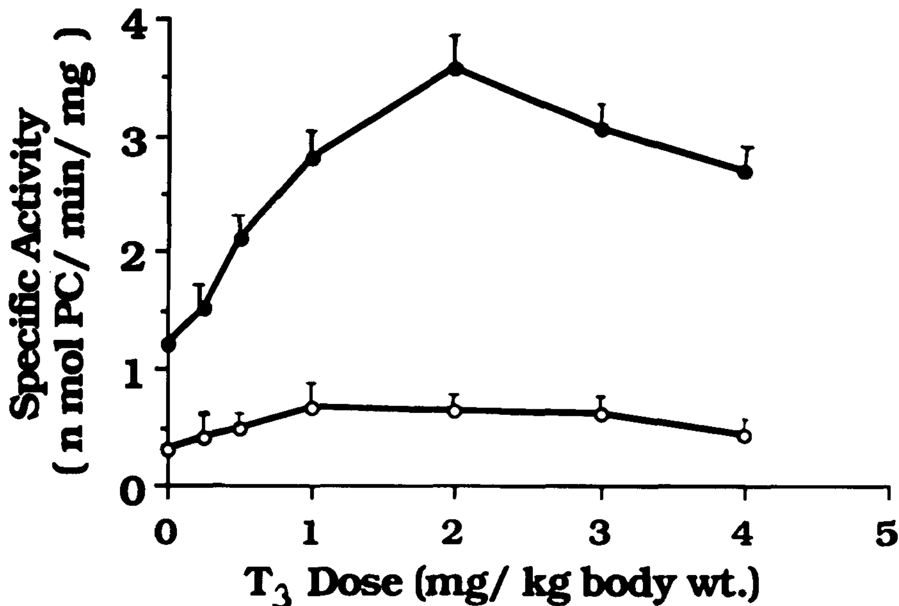

Figure 1 Effect of intramuscular injection of T_3 on the activity of cholinephosphotransferase in guinea pig lung mitochondria and microsomes. Each point on the curves represents a mean of nine samples. The maximum stimulation was achieved in mitochondria at a dose of 1 mg/kg body wt, whereas for microsomes, it was 2 mg/kg body wt. ○, Mitochondria; ●, microsomes. Guinea pigs used in this experiment were 24 wk old.

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132, 196, 154, and 124%, respectively. The optimum stimulation was achieved at 1 and 2 mg/kg, respectively, for the mitochondrial and microsomal enzymes.

There was much more stimulation in the microsomes than in the mitochondria, particularly at higher doses (2–4 mg/kg). Furthermore, after the optimum stimulation was achieved, there was a gradual decline in stimulation of the enzyme activity in both subcellular fractions as the dose of the hormone was increased. Data on the relative distribution of markers in the subcellular fractions revealed that the subcellular fractions were not cross-contaminated (data not shown).

T_3 treatment caused stimulation of mitochondrial and microsomal CPT activity in lungs of animals of different age groups (Table 1). The mitochondrial enzyme activity was stimulated 2.2-, 2.0-, and 1.7-fold, and the microsomal activity was stimulated 1.4-, 2.3-, and 1.9-fold, respectively, in 3-, 24-, and 34-week-old guinea pigs. The stimulatory effect of T_3 was blocked by both actinomycin D and cycloheximide in the microsomal preparation, but only by cycloheximide in the mitochondrial preparation in all cases (Table 1).

We next assessed the uptake of choline into lung slices from control and T_3-treated animals. This was determined by assaying the disappearance of radioactivity from the medium after the incubation of lung slices [13]. In Table 2, the values presented include the [^{14}C]choline-associated radioactivity in the lung slices. T_3 treatment caused 1.57- and 1.9-fold increase in the uptake of choline by lung slices from 3- and 34-week-old guinea pigs, respectively. The stimulatory effect of T_3 was blocked by both actinomycin D and cycloheximide.

Table 3 summarizes the effect of T_3 on the incorporation of [^{14}C]choline into choline-containing lipids LPC, PC, and SM. T_3 produced a significant increase in the incorporation of choline into all choline-containing lipids in both young (3-week-old) and adult (34-week-old) guinea pigs. Both actinomycin D and cycloheximide blocked the stimulatory effects of T_3 for all choline-containing lipids except SM. Actinomycin D did not block the stimulatory effects of T_3 on SM.

Table 4 demonstrates the effect of T_3 on the incorporation of radioactive choline into water-soluble precursors of PC. The greatest accumulation of radioactivity occurred in CDPcholine in all cases. Treatment of T_3 caused an increase in the incorporation of radioactive choline into all intermediates, and this stimulation was blocked by both actinomycin D and cycloheximide.

Table 5 demonstrates the effect of T_3 on the incorporation of radioactive choline into the saturated and unsaturated PC. T_3 treatment caused an increase in the incorporation of radioactivity into both saturated and unsaturated PC, and this effect was counteracted by both actinomycin D and cycloheximide.

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Table 1 Effects of Transcriptional and Translational Inhibitors on T_3-Induced Cholinephosphotransferase Activity in Guinea Pig Lung Mitochondria and Microsomes

	Specific activity (nmol/min mg^{-1} protein)						Total activity (nmol/min g^{-1} tissue)					
	Mitochondria			Microsome			Mitochondria			Microsome		
Group	A	B	C	A	B	C	A	B	C	A	B	C
Control	0.29	0.34	0.42	0.62	1.21	0.48	5.36	6.28	7.72	10.06	19.63	8.78
	(0.01)	(0.03)	(0.02)	(0.06)	(0.08)	(0.04)	(0.25)	(1.42)	(0.25)	(0.35)	(1.93)	(1.15)
T_3	0.65[a]	0.68[a]	0.70[a]	0.87[a]	2.81[a]	0.90[a]	12.83[a]	13.42[a]	13.91[a]	36.33[a]	52.75[a]	26.89[a]
	(0.02)	(0.07)	(0.03)	(0.10)	(0.23)	(0.10)	(0.35)	(2.14)	(0.35)	(0.35)	(4.02)	(2.11)
T_3 + AMD	0.67	0.70	0.64	0.43[b]	1.20[b]	0.44[b]	13.22	13.86	12.72	11.25[b]	22.73[b]	12.10[b]
	(0.04)	(0.04)	(0.05)	(0.02)	(0.10)	(0.06)	(0.10)	(3.62)	(0.85)	(0.25)	(2.11)	(1.03)
T_3 + CHM	0.22[b]	0.39[b]	0.33[b]	0.47[b]	1.52[b]	0.46[b]	7.35[b]	6.55[b]	7.25[b]	13.4[b]	24.99[b]	14.20[b]
	(0.02)	(0.08)	(0.03)	(0.10)	(0.18)	(0.05)	(0.09)	(1.73)	(0.12)	(1.02)	(3.45)	(1.25)

Note. Values are means ± SEM. T_3 (1 mg/kg body wt) was injected intramuscularly daily for 2 days. AMD (actinomycin D) and CHM (cycloheximide) were administered intraperitoneally (200 μg/kg body wt) during the hormonal treatment. Animals were sacrificed after 48 h. A, 3 weeks (n = 4); B, 24 weeks (n = 9); and C, 34 weeks (n = 4).

[a]Significantly different from control.

[b]Significantly different from T_3 alone.

Table 2 Effects of Intramuscular Injection of T_3 on the Uptake of [^{14}C]Choline by Guinea Pig Lung Slices

Group	Uptake of [^{14}C]choline (nmol/g)	
	3 weeks	34 weeks
Control	4.20 ± 0.20	3.13 ± 0.15
T_3	6.31 ± 0.10[a]	5.97 ± 0.12[a]
T_3 + AMD	3.96 ± 0.10	2.99 ± 0.12
T_3 + CHM	3.33 ± 0.16	2.21 ± 0.10

Note. Values are means ± SEM of three experiments. T_3 (1 mg/kg body wt) was injected daily for 2 days. AMD (actinomycin D) and CHM (cycloheximide) were administered intraperitoneally (200 μg/kg body wt) during hormonal treatment. Animals were sacrificed after 48 h.

[a]Significantly different from control.

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DISCUSSION

We established earlier [7] that cholinephosphotransferase exists in the outer membrane of mitochondria. It is generally believed that all of the proteins in the outer mitochondrial membrane are synthesized by the nucleocytoplasmic system, i.e., they are encoded by nuclear genes, translated on cytoplasmic ribosomes, and imported into the mitochondria in subsequent steps [16]. Since actinomycin D has also been widely used as an inhibitor of RNA synthesis in the mitochondria [17], two possibilities may explain why actinomycin D did not block the stimulatory effect of T_3 on the mitochondrial enzyme while blocking the stimulatory effect on the microsomal preparation. One explanation is that thyroid hormone stimulates the transport of the protein translated on cytoplasmic ribosomes into the mitochondria; the other is that it stimulates the transport of some stable preformed mRNA encoded by nuclear genes into the mitochondria.

It is generally believed that cycloheximide does not inhibit protein synthesis in the mitochondria [18–20]. Thus, it is unlikely that cycloheximide will inhibit the translation of some preformed nuclear mRNA that have been transported into the mitochondria. Furthermore, if such a stable preformed mRNA is transported to mitochondria for protein synthesis, it is not clear why this stable mRNA could not be translated outside the mitochondria and transported into the endoplasmic reticulum.

Considering various possibilities, the following hypothesis can be proposed for the subcellular regulation of cholinephosphotransferase by thyroid hormone in guinea pig lung irrespective of age: The mitochondrial and microsomal enzymes are probably produced under the same transcriptional and translational control by thyroid hormone in the cytoplasm and targeted into the respective organelle by subsequent steps, and it is

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Table 3 Effects of Intramuscular Injection of Thyroid Hormone on the Incorporation of [^{14}C]Choline into Choline-Containing Lipids of Guinea Pig Lung Slices

	[^{14}C]Choline incorporation (nmol/g)					
	Phosphatidylcholine		Sphingomyelin		Lysophosphatidylcholine	
Group	3 weeks	34 weeks	3 weeks	34 weeks	3 weeks	34 weeks
Control	2.34 ± 0.21	1.75 ± 0.08	0.14 ± 0.03	0.15 ± 0.02	0.29 ± 0.03	0.43 ± 0.03
T_3	4.19 ± 0.10[a]	3.79 ± 0.26[a]	0.31 ± 0.06[a]	0.28 ± 0.03[a]	0.58 ± 0.11[a]	0.50 ± 0.09[a]
T_3 + ACD	2.40 ± 0.10	1.93 ± 0.11	0.33 ± 0.07	0.31 ± 0.02	0.09 ± 0.001	0.06 ± 0.001
T_3 + CHM	1.77 ± 0.18	1.51 ± 0.13	0.25 ± 0.04	0.16 ± 0.04	0.34 ± 0.04	0.22 ± 0.03

Note. Values are means ± SEM of three experiments. For details see Table 2.

[a]Significantly different from control.

Table 4 Effects of Intramuscular Injection of Thyroid Hormone on the Incorporation of [^{14}C]Choline into Water-Soluble Intermediates of Phosphatidylcholine in Guinea Pig Lung Slices

	[^{14}C]Choline incorporation (pmol/g)					
	CDPcholine		Phosphocholine		Choline	
Group	3 weeks	34 weeks	3 weeks	34 weeks	3 weeks	34 weeks
Control	220 ± 8	190 ± 2	650 ± 10	730 ± 15	20 ± 1	50 ± 2
T_3	300 ± 20[a]	380 ± 8[a]	1110 ± 20[a]	1320 ± 25[a]	90 ± 2[a]	180 ± 20[a]
T_3 + ACD	190 ± 2	120 ± 2	620 ± 2	470 ± 2	90 ± 1	60 ± 1
T_3 + CHM	170 ± 2	140 ± 5	570 ± 5	590 ± 3	30 ± 1	50 ± 5

Note. Values are means ± SEM of three experiments. For details see Table 2.
[a]Significantly different from control.

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likely that the transport of cholinephosphotransferase into mitochondria is also under the control of T_3. The differential regulatory effect of thyroid hormone on the mitochondrial and microsomal cholinephosphotransferase activities further supports our previous findings that the mitochondrial cholinephosphotransferase is not originated from the endoplasmic reticulum [7, 8, 21].

The effect of T_3 on the biosynthesis of PC is shown in Fig. 2. T_3 treatment causes an increase in the uptake of choline from the medium and its incorporation into PC and into the water-soluble intermediates of PC in lung slices. The accumulation of radioactivity in intermediate com-

Table 5 Effects of Intramuscular Injection of Thyroid Hormone on the Incorporation of [^{14}C]Choline into Saturated and Unsaturated Phosphatidylcholine of Guinea Pig Lung Slices

	Phosphatidylcholine (nmol/g)			
	Saturated		Unsaturated	
Group	3 weeks	34 weeks	3 weeks	34 weeks
Control	1.38 ± 0.07	1.15 ± 0.05	0.96 ± 0.03	0.60 ± 0.03
T_3	2.60 ± 0.03[a]	2.18 ± 0.03[a]	1.59 ± 0.06[a]	1.61 ± 0.05[a]
T_3 + ACD	1.11 ± 0.09	1.07 ± 0.01	0.93 ± 0.12	0.86 ± 0.02
T_3 + CHM	1.06 ± 0.07	0.93 ± 0.02	0.71 ± 0.04	0.58 ± 0.02

Note. Values are means ± SEM of three experiments. For details see Table 2.
[a]Significantly different from control.

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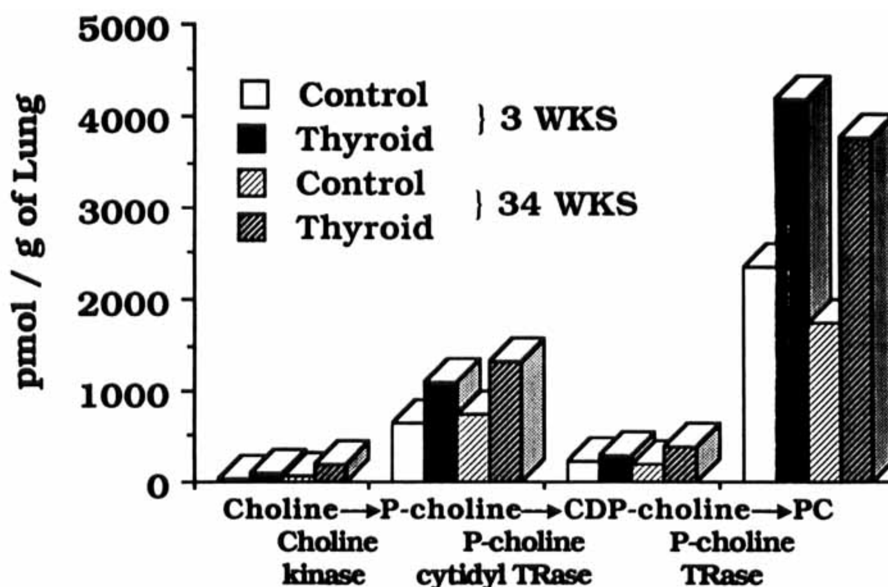

Figure 2 Effect of T_3 on the incorporation of ^{14}C radioactivity into the intermediates and the end products of PC synthesis in guinea pig lung tissue slices. The enzymes involved in the synthesis of PC are listed below the intermediates.

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pounds does not give quantitative information on the changes in the activity of any enzyme in this pathway (choline kinase, phosphocholine cytidyltransferase, and cholinephosphotransferase), but does suggest that each enzyme was stimulated at a different rate.

The stimulatory effects of T_3 on the biosynthesis of PC are counteracted by actinomycin D and cycloheximide (Tables 2 and 3). However, in mitochondria, the stimulatory effect of T_3 on CPT activity is not blocked by actinomycin D (Table 1). Therefore, it is suggested that the stimulation of CPT activity in mitochondria by T_3 is not reflected by increased incorporation of choline into PC. It was reported earlier that rat liver mitochondria can synthesize their own phospholipids in situ, and that the incorporation of radioactive choline and glycerophosphate into phospholipids is inhibited by puromycin and actinomycin C, inhibitors of mitochondrial protein synthesis in vitro, but not by actinomycin D [22].

These studies were done with mitochondria and microsomes derived from whole lung. Since mitochondria and microsomes may originate from many cell types, it is important that we study the effects of T_3 on the cholinephosphotransferase activity in subcellular fractions of a single cell type, such as alveolar type II cells. Our immediate goals are (1) to study the effects of T_3 on the overall rate of PC synthesis in young as well as adult animals, (2) to study the time course for the increased synthesis of cholinephosphotransferase, and (3) to study the effect of the hormonal treatment on the other enzymes of PC synthesis.

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