Thyroxine regulation of monolysocardiolipin acyltransferase activity in rat heart

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Treatment of rats with thyroxine has been shown to elevate the biosynthesis and content of cardiolipin in the heart [Cao, Cheng, Angel and Hatch (1995) Biochim. Biophys. Acta **1256**, 241–244]. Treatment with thyroxine resulted in a 1.8-fold increase ($P <$ 0.025) in [1-^{14}C]linoleate and a 1.7-fold increase ($P < 0.025$) in [1-^{14}C]oleate incorporated into cardiolipin in perfused hearts, compared with controls. The mechanism for the elevation in incorporation of unsaturated fatty acids into cardiolipin was a 1.6-fold ($P < 0.025$) increase in mitochondrial monolysocardiolipin acyltransferase activity. The results demonstrate that the acylation of cardiac monolysocardiolipin is regulated by thyroid hormone. Thus an elevation in cardiolipin biosynthesis is accompanied by an elevation in monolysocardiolipin acyltransferase activity to maintain the appropriate molecular species composition of cardiolipin in the cardiac mitochondrial membrane. We postulate that monolysocardiolipin acyltransferase might be a rate-limiting enzyme for the molecular remodelling of cardiolipin in the heart.

Key words: cardiolipin, phospholipid metabolism, thyroid hormone

INTRODUCTION

Cardiolipin (CL) is the main polyglycerophospholipid found in mammalian tissues [1,2]. In the heart, CL comprises approx. 15% of the entire phospholipid mass of the organ and resides exclusively within mitochondria [3,4]. CL has been shown to modulate the activity of a number of mitochondrial membrane enzymes involved in the generation of ATP (reviewed in [5]). It is well documented that an alteration in the content and the molecular composition of CL alters oxygen consumption in mitochondria [6,7]. Thus proper maintenance of the biosynthesis and molecular composition of CL in cardiac mitochondria is probably essential for cardiac function.

Cardiac CL biosynthesis occurs exclusively in mitochondria via the cytidine-5′-diphosphate-1,2-diacyl-*sn*-glycerol (CDP-DG) pathway [4]. Phosphatidic acid is converted into CDP-DG by phosphatidic acid:CTP cytidylyltransferase. CDP-DG and *sn*-glycerol 3-phosphate are then converted into phosphatidylglycerol (PtdGro) catalysed by PtdGro phosphate synthase and phosphatase. Finally, the PtdGro formed is condensed with another molecule of CDP-DG to form CL, a reaction catalysed by CL synthase. Once CL is synthesized *de novo* by this pathway it must be remodelled to obtain the appropriate molecular species composition found in the membrane. The species pattern of phosphatidic acid, CDP-DG and PtdGro formed *de novo* were similar enough to imply that the rat liver enzymes of the CL biosynthetic pathway were not specific to molecular species [8]. Remodelling of a phospholipid requires the concerted action of phospholipase A_2 (PLA_2) followed by the reacylation of the resulting lysophospholipid [9]. CL was shown to be readily hydrolysed by mitochondrial PLA_2 [10,11]. The acyltransferases involved in the reacylation of monolysocardiolipin (MLCL) to CL have recently been characterized in the rat heart [12].

Thyroxine (T_4) is the main form of thyroid hormone [13,14]. In thyroid-responsive tissues it is deiodinated to 3,3′,5-tri-iodothyronine (T_3), the biologically active form. T_3 is transported to the nucleus of the cell, where it acts directly through DNA-bound hormone receptors to stimulate or inhibit gene transcription. Although mitochondrial thyroid hormone receptors have been proposed, they have not been proved to exist. The heart is a target organ of thyroid hormone action; the effects of hyperthyroid states on the cardiovascular system are well known. The heart hypertrophies and exhibits increased contractility, cardiac output and oxygen demand in the presence of increased peripheral resistance [15]. At the cellular level, excess T_4 is associated with larger, more numerous mitochondria and, in cardiac myocytes, altered oxidative phosphorylation [16].

Treatment of rats with T_4 was shown to elevate CL synthase activity and resulted in elevated levels of cardiac mitochondrial CL [17,18]. It would be logical to surmise that the activity of the enzymes that remodel CL should be elevated to accompany this increase in CL biosynthesis. In this study we used the extensively studied T_4-treated rat model to determine whether an elevation in cardiac CL biosynthesis was accompanied by an alteration in the activities of the enzymes that remodel CL.

MATERIALS AND METHODS

Adult male Sprague–Dawley rats (150–170 g body weight) were maintained on Purina rat chow and tap water *ad libitum*, in a light- and temperature-controlled room. Treatment of animals conformed to the Guidelines of the Canadian Council on Animal Care. [1-^{14}C]Oleic acid, [1-^{14}C]linoleic acid, [*dipalmitoyl*-1,2-^{14}C]phosphatidylcholine (PtdCho) and [1-^{14}C]oleoyl-CoA were obtained from Mandel Scientific (Missassauga, Ontario,

Abbreviations used: AT, acyltransferase; CDP-DG, cytidine-5′-diphosphate-1,2-diacyl-*sn*-glycerol; CL, cardiolipin; i.p., intraperitoneal; KHB, Krebs–Henseleit buffer; lysoPtdGro, lysophosphatidylglycerol; MLCL, monolysocardiolipin; PLA_2, phospholipase A_2; PtdCho, phosphatidylcholine; PtdGro, phosphatidylglycerol; T_3, 3,3′,5-tri-iodothyronine; T_4, thyroxine.

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Canada). [1-^{14}C]Linoleoyl-CoA was obtained from American Radiolabelled Chemicals (St. Louis, MO, U.S.A.). Fatty acids and acyl-CoAs were obtained from Serdary Research Laboratories (Englewood Cliffs, NJ, U.S.A.). MLCL was obtained from Avanti Polar Lipids (Alabaster, AL, U.S.A.). Ecolite scintillation cocktail was obtained from ICN Biochemicals (Costa Mesa, CA, U.S.A.) and thin-layer plates (silica gel 60, 0.25 mm thickness) were obtained from Fisher Scientific (Winnipeg, Manitoba, Canada). All other biochemicals were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

For heart perfusions, the rat was killed by decapitation; the heart was removed and cannulated via the aorta with a modified syringe needle (18 gauge). The blood in the coronary circulation was removed by injecting the heart with freshly prepared Krebs–Henseleit buffer (KHB) [19] by using a 10 ml syringe. The heart was placed on the perfusion apparatus and perfused for 5 min or until electrical stabilization was achieved. Viability of the heart throughout the perfusion experiments was monitored via electrocardiac analysis. After stabilization, the heart was perfused for 30 min in the Langendorff mode [20] with 12.5 ml of KHB containing 0.1 mM [1-^{14}C]fatty acid (0.4 μCi/ml) bound to BSA in a 1:1 molar ratio [12]. The lipids were extracted and the radioactivity incorporated into PtdGro and CL was determined as described [4].

For assay of enzyme activities, a 10 % (w/v) homogenate was prepared by homogenizing the heart (Polytron, 20 s burst) in 0.25 M sucrose/10 mM Tris/HCl (pH 7.4)/2 mM EDTA, followed by differential centrifugation. The homogenate was centrifuged at 1000 ***g*** for 5 min and the resulting supernatant was centrifuged at 12000 ***g*** for 10 min. The pellet obtained was washed once in homogenizing buffer and then resuspended in 1–2 ml of homogenizing buffer with a tightly fitting Dounce A homogenizer; this suspension was designated the mitochondrial fraction. The supernatant was centrifuged at 100000 ***g*** for 60 min. The pellet obtained was washed once in homogenizing buffer and then resuspended in 1–2 ml of homogenizing buffer with a tightly fitting Dounce A homogenizer; this suspension was designated the microsomal fraction. Mitochondrial MLCL acyltransferase (AT) activity was assayed with exogenous MLCL and [1-^{14}C]linoleoyl-CoA, as described previously [12]. [1-^{14}C]Linoleoyl-CoA was used as substrate because this molecular species is found in the highest concentration in CL. CL generated from the MLCL AT assay was treated with *Naja mocambique mocambique* PLA_2. Radioactivity in the resulting lysophospholipid was determined [12]. Mitochondrial lysophosphatidylglycerol (lysoPtdGro) AT activity was assayed with 1-oleoylglycerophosphoglycerol and [1-^{14}C]oleoyl-CoA, as described previously [21]. These conditions provided the highest lysoPtdGro AT activity for mitochondrial fractions. Mitochondrial PLA_2 was assayed as described, except that [1,2-^{14}C-dipalmitoyl]PtdCho was used as substrate [22]. These conditions provided the lowest specific activity of all commercially available PLA_2 substrates examined. Acyl-CoA synthetase activities were assayed as described [22]. Mitochondrial succinate dehydrogenase activity was assayed as described [23]. Protein was determined as described [24]. The fatty acid composition of cardiac PtdGro and CL was determined as described [12]. The data were analysed with Student's *t* test. The level of significance was defined as $P < 0.025$.

RESULTS

To examine whether an elevation in CL biosynthesis was accompanied by an elevation in the activity of CL-remodelling enzymes, rats were treated with 250 μg of T_4/day per kg for 5 consecutive days; the hearts were then removed and weighed. Heart wet weight from hyperthyroid rats was 27 % higher than that of controls (0.70 ± 0.06 g hyperthyroid compared with 0.55 ± 0.03 g control; mean ± S.D. for eight rat hearts in each group). Mitochondrial PtdGro and CL levels and the biosynthesis of PtdGro and CL were examined. As previously reported by our group [18], the levels of PtdGro and CL were elevated by approx. 35 % and 25 % respectively compared with controls (results not shown). These elevations in PtdGro and CL levels are due to approx. 3-fold increases in PtdGro phosphate synthase and CL synthase activities [17,18]. As a further control, the activity of an inner-mitochondrial membrane marker, succinate dehydrogenase was determined. Cardiac mitochondrial succinate dehydrogenase activity was increased from 29.7 μmol/min per mg of protein in control to 36.6 μmol/min per mg of protein in T_4-treated rats. Thus the elevated heart weight, mitochondrial PtdGro and CL levels and succinate dehydrogenase activities confirmed the hyperthyroid state of the animals.

Table 1 Effect of T_4 on [1-^{14}C]linoleate and [1-^{14}C]oleate incorporation into PtdGro and CL in isolated perfused rat hearts

Rats were treated with T_4 and the hearts were perfused for 30 min with buffer containing 0.08 mM [1-^{14}C]linoleate or 0.1 mM [1-^{14}C]oleate; the radioactivity incorporated into PtdGro and CL was determined. Results are means ± S.D. for four hearts. *$P < 0.025$.

		10^{-5} × Radioactivity incorporated (d.p.m./g of freeze-dried ventricles)	
Addition	Phospholipid	Control	Hyperthyroid
[1-^{14}C]Linoleate	PtdGro	0.07 ± 0.02	0.06 ± 0.02
	CL	0.17 ± 0.03	0.28 ± 0.03*
[1-^{14}C]oleate	PtdGro	0.13 ± 0.05	0.15 ± 0.04
	CL	0.14 ± 0.04	0.25 ± 0.04*

Table 2 Effect of treatment with T_4 on fatty acid composition of cardiac PtdGro and CL

Rats were treated with T_4; the hearts were isolated and the fatty acid compositions of CL and PtdGro were determined. Results are means ± S.D. for four hearts.

	Content (%)			
	CL		PtdGro	
Fatty acid	Control	Hyperthyroid	Control	Hyperthyroid
$C_{16:0}$	1.1 ± 0.1	1.8 ± 0.7	36.8 ± 1.0	36.1 ± 1.6
$C_{18:0}$	1.5 ± 0.3	1.8 ± 1.7	15.5 ± 2.2	14.3 ± 1.4
$C_{18:1}$	11.4 ± 1.8	12.7 ± 2.6	35.9 ± 3.9	39.0 ± 4.7
$C_{18:2}$	82.5 ± 8.6	80.7 ± 5.1	10.1 ± 0.4	8.3 ± 1.7
Others	2.0 ± 0.1	1.4 ± 0.6	1.6 ± 1.5	1.8 ± 1.9

We initially examined the ability of isolated hearts to incorporate unsaturated fatty acids into PtdGro and CL. Rats were treated with 250 μg of T_4/day per kg for 5 consecutive days; the hearts were removed and perfused for 30 min in the Langendorff mode with KHB containing [1-^{14}C]oleate or [1-^{14}C]linoleate and the radioactivity incorporated into PtdGro and CL was determined. As seen in Table 1, treatment with T_4 did not affect [1-^{14}C]oleate or [1-^{14}C]linoleate incorporation into cardiac PtdGro. In contrast, treatment with T_4 resulted in a 1.8-fold increase ($P < 0.025$) in [1-^{14}C]linoleate and a 1.7-fold

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Table 3 Effect of T_4 on mitochondrial MLCL AT and lysoPtdGro AT activities

Rats were treated with T_4; cardiac mitochondrial fractions were prepared and MLCL AT and lysoPtdGro AT activities were determined. Results are means ± S.D. for four hearts. *$P < 0.025$.

	Activity (pmol/min per mg of protein)	
Enzyme	Control	Hyperthyroid
MLCL AT	67 ± 5	106 ± 19*
LysoPtdGro AT	190 ± 22	201 ± 20

Table 4 Effect of T_4 on mitochondrial PLA_2 and mitochondrial and microsomal long-chain acyl-CoA synthetase activities

Rats were treated with T_4; mitochondrial and microsomal fractions were prepared from the hearts. Mitochondrial PLA_2 activity and mitochondrial and microsomal long-chain acyl-CoA synthetase activities were determined as described in the Materials and methods section. Results are means ± S.D. for four hearts.

		Activity (nmol/min per mg of protein)	
Enzyme	Location	Control	T_4
PLA_2	Mitochondria	0.12 ± 0.02	0.11 ± 0.02
Fatty acyl-CoA synthetase	Microsomes	62 ± 2	62 ± 5
	Mitochondria	40 ± 5	33 ± 5

increase ($P < 0.025$) in [1-^{14}C]oleate incorporated into CL compared with controls. Thus treatment with T_4 specifically elevated the incorporation of unsaturated fatty acids into CL, but not into its immediate precursor PtdGro. We next examined the relative percentage fatty acid composition of cardiac PtdGro and CL in T_4-treated rats. As seen in Table 2, CL contained primarily unsaturated fatty acids (mainly linoleate), whereas PtdGro contained a mixture of saturated and unsaturated fatty acids. Treatment with T_4 did not affect the percentage composition of fatty acids in cardiac PtdGro or CL.

We examined the mechanism for the increase in incorporation of radiolabelled unsaturated fatty acids into CL in the hearts of hyperthyroid animals. Hearts from control and T_4-treated rats were removed and mitochondrial fractions were prepared. In T_4-treated rats, cardiac MLCL AT activity was elevated 1.6-fold ($P < 0.025$) in comparison with controls (Table 3). In contrast, lysoPtdGro AT activity was unaltered and thus served as a negative control for a mitochondrial enzyme activity not affected by treatment with T_4. We reported previously that transacylase activity did not contribute to the acylation of MLCL in rat heart [12]. The radioactive CL formed from the MLCL AT assay of cardiac mitochondria prepared from T_4-treated rats was incubated with *N. mocambique mocambique* PLA_2 and the radioactivity in the MLCL product was determined. No radioactivity was recovered in MLCL. Therefore the elevation in cardiac MLCL AT activity observed in T_4-treated animals was not due to transacylase activity.

Mitochondrial PLA_2 and cardiac acyl-CoA synthetase activities were then determined in hearts prepared from T_4-treated rats. As seen in Table 4, mitochondrial PLA_2 activity was unaltered by treatment with T_4. T_4 treatment did not affect mitochondrial or microsomal acyl-CoA synthetase activities. We previously reported that cardiac acyl-CoA hydrolase activities were unaltered in rats treated with T_4 under conditions identical with those in the present study [22]. Therefore the increased incorporation of radiolabelled unsaturated fatty acids into CL was due to an increase in MLCL AT activities that accompanied the elevation in CL biosynthesis and CL levels observed in the hyperthyroid state.

DISCUSSION

The objective of this study was to determine whether an elevation in cardiac CL biosynthesis was accompanied by an elevation in the activity of CL-remodelling enzymes. T_4 administration to rats is known to result in tissue-specific elevations in lipid biosynthesis [25,26]. We used the T_4-treated rat because this model consistently produces an elevation in cardiac CL biosynthesis and content [17,18]. Our results in both the isolated intact perfused rat heart and isolated rat heart mitochondrial fractions clearly indicated that an elevation in cardiac CL biosynthesis was accompanied by an elevation in cardiac MLCL AT activity. To our knowledge this is the first demonstration of the regulation of MLCL AT activity in any organism.

Phospholipid remodelling is defined as the conversion of one molecular species to another. It was previously suggested that phospholipids such as PtdCho are remodelled by deacylation/reacylation reactions at rates of approx. 10–15%/h [27]. The enzymes involved in CL biosynthesis *de novo* were shown to exhibit limited molecular species specificity [8]. CL must therefore be remodelled to obtain its high level of linoleoyl molecular species. A previous study had reported that liver mitochondrial PLA_2 activity was unaltered in hyperthyroid rats [28]. In the present study, cardiac mitochondrial PLA_2 activity was unaltered by treatment with T_4. Because PLA_2 activity exceeded the MLCL AT activity, it is possible that MLCL AT activity is rate-limiting for CL remodelling in the heart. Evidence in support of this was the absence of alterations in the relative fatty acid composition of CL in T_4-treated rats. Therefore the elevation in cardiac mitochondrial MLCL AT activity was probably required for remodelling the increased amount of newly synthesized CL observed in the hearts of T_4-treated animals [18].

Interestingly, treatment with T_4 did not affect lysoPtdGro AT activities or the incorporation of radioactive fatty acids into cardiac PtdGro. We had previously demonstrated that cardiac PtdGro biosynthesis and content were elevated in T_4-treated rats, probably to serve as a substrate for the observed accelerated rate of CL biosynthesis [18]. In addition, perfusion of the heart with [^{14}C]lysoPtdGro bound to albumin resulted in the formation of [^{14}C]PtdGro, but not [^{14}C]CL [21]. These results, taken together with our present observations, suggest that lysoPtdGro AT activity is not regulated by thyroid hormone and that newly remodelled PtdGro enters into a pool that might not be readily available for CL biosynthesis. Thus CL biosynthesis and remodelling are independent of PtdGro remodelling.

It had been postulated that acyl-CoA synthetase and hydrolase activities might be important in the fatty acyl distribution of cardiac PtdCho [29]. In the present study, fatty acyl-CoA synthetase activities were unaltered in cardiac microsomes or mitochondria prepared from T_4-treated rats. Using an identical dose and duration of treatment with T_4 [intraperitoneal (i.p.) 250 μg/day per kg for 5 days] we reported previously that T_4 did not alter cardiac fatty acyl-CoA hydrolase activity [22]. A previous study had shown that T_3 administration (i.p. 500 μg/day per kg for 14 days) resulted in a 10.3% increase in the specific activity of long-chain acyl-CoA synthetase in rat heart homo-

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genates [30]. Administration of T_4 (i.p. 500 μg/day per kg for 7 days) resulted in a 35 % increase in rat liver microsomal acyl-CoA hydrolase activity [31], whereas T_3 administration (i.p. 250 μg/day per kg for 14 days) resulted in a 36–42 % decrease in microsomal acyl-CoA synthetase but no alteration in acyl-CoA hydrolase activities [32]. In addition, treatment of rabbits with T_3 did not affect liver acyl-CoA hydrolase activity [33]. The difference between all of these studies is probably related to both the duration and the level of thyroid hormone administration. T_3-mediated alterations in acyl-CoA synthetase activities in rat lung were shown to be dependent on dose and duration of treatment [34]. In summary, our results demonstrate clearly that rat heart MLCL AT activity is regulated by T_4. We suggest that an elevation in CL biosynthesis is accompanied by an elevation in MLCL AT activity to maintain the appropriate molecular species composition of CL in the cardiac mitochondrial membrane. In addition, we postulate that MLCL AT might be a rate-limiting enzyme for the molecular remodelling of CL.

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