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Hypothyroid state and membrane fatty acid composition influence cardiac mitochondrial pyruvate oxidation

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Abstract

Pyruvate oxidation was measured in cardiac mitochondria from euthyroid and hypothyroid rats fed diets enriched with either $\omega-6$ or $\omega-3$ fatty acids. Both State 4 and State 3 rates of pyruvate-dependent respiration were markedly reduced in hypothyroid mitochondria, regardless of diet consumed, compared to euthyroid controls. Respiratory control ratios and ADP/O ratios were the same under all treatments. While there was no significant effect of diet on respiration in euthyroid mitochondria, pyruvate oxidation was 28% higher in hypothyroid mitochondria from animals fed the $\omega-3$ diet compared to those fed the $\omega-6$ diet. Depressed respiration in the hypothyroid state was correlated with 18% more phosphatidylcholine in the inner mitochondrial membrane whereas phosphatidylethanolamine was 17% lower and cardiolipin 32% lower compared to controls. The total phospholipid fatty acid composition was not affected by the hypothyroid state. However, enhanced respiration in hypothyroid animals fed the $\omega-3$ diet was associated with a 3-fold increase in monounsaturated fatty acids in the cardiolipin fraction, and a 12-fold increase in $\omega-3$ fatty acids, primarily 22:5($\omega-3$) and 22:6($\omega-3$). The data suggest that membrane levels of cardiolipin and its $\omega-3$ fatty acid content modulate pyruvate transport in hypothyroid mitochondria.

Keywords: Hypothyroid state; Mitochondrion; Pyruvate oxidation; Fatty acid composition; (Heart)

1. Introduction

Mitochondria isolated from hypothyroid rats show depressed respiration which is associated with, among other functions, altered transport of substrates across the inner membrane. In particular, the activity of the pyruvate translocator is decreased in the hypothyroid state as determined by the kinetics of transport and pyruvate-supported oxygen consumption [1,2]. At the same time there are several reports that mitochondrial membrane lipid composition is altered in the hypothyroid state, suggesting a link between the function of the pyruvate carrier and its lipid environment. In cardiac mitochondria, changes in membrane lipid composition include the relative distribution of the major phospholipids, and their fatty acid pattern, with cardiolipin in particular being seen to decrease significantly in the hypothyroid state [2].

While a role for the lipid environment in the regulation of pyruvate transport is an attractive hypothesis, to date the evidence supporting this contention is equivocal. Under some experimental conditions no apparent differences in phospholipid distribution were evident in different thyroid hormone states [3], or changes in phospholipid content were not accompanied by altered fatty acid composition [4]. Moreover, complete analyses of individual phospholipid classes associated with the inner mitochondrial membrane site of the pyruvate carrier are lacking. Recently there has been considerable interest in the physiological importance of the $\omega-6$ and $\omega-3$ families of fatty acids, with at least one report suggesting that the metabolism of $\omega-3$ fatty acids is inhibited in hypothyroid mitochondria [5].

This study provides a rigorous analysis of the fatty acid composition of cardiac inner mitochondrial membranes from euthyroid and hypothyroid rats that had been fed diets high in either $\omega-6$ or $\omega-3$ fatty acids. Using this approach we were able to determine to what extent the relative distribution of $\omega-6$ and $\omega-3$ fatty acids is influenced by the hypothyroid state and at the same time

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examine how enriching the inner membrane with $\omega-6$ or $\omega-3$ fatty acids affected pyruvate transport.

2. Materials and methods

2.1. Animals and diets

Male weanling Sprague-Dawley rats (50–55 g) were randomly divided into two groups and fed one of two semi-purified diets enriched in either $\omega-6$ or $\omega-3$ fatty acids, ad libitum. After 2 weeks half of each diet group was then made hypothyroid by continuous administration of 0.05% 6-n-propyl-2-thiouracil (PTU) in drinking water. Animals continued to be fed the same diet formulations for a further 4 weeks at which time plasma T_4 levels were 60 ± 6.1 and 65 ± 5.8 pmol/l in euthyroid animals fed the $\omega-6$ and $\omega-3$ diets, respectively, and 7.0 ± 1.5 and 13.20 ± 1.7 pmol/l in hypothyroid animals fed the $\omega-6$ and $\omega-3$ diets, respectively.

All diets were formulated to contain equivalent nutrients per caloric content for the non-fat components based on the following nutrients (per kg); 270 g casein, 200 g starch, 207 g glucose, 50 g non-nutritive cellulose, 10 g vitamin mix [1], 50.85 g mineral mix [2], 2.75 g choline, 6.25 g inositol, 2.5 g L-methionine. The lipid content of the diets was achieved by addition of 20% fat (w/w) to the basal mix as combinations of beef tallow, safflower oil, olive oil, or fish oil (28% 20:5($\omega-3$); 12% 22:6($\omega-3$)) to achieve the desired fatty acid composition. Diets were adjusted to contain equivalent amounts of total sterols and sufficient linolenic acid (18:3($\omega-3$)) to provide adequate levels of $\omega-3$ essential fatty acids. The fatty acid composition of the diets is presented in Table 1.

Table 1
Fatty acid composition of diets

Fatty Acid	Diet (% w/w)	
	$\omega-6$	$\omega-3$
14:0	1.5	4.6
16:0	14.5	15.8
16:1	–	5.6
17:0	0.6	1.7
18:0	18.9	12.8
18:1($\omega-7$)	11.1	26.6
18:1($\omega-9$)	0.6	2.3
18:2($\omega-6$)	51.8	12.7
18:3($\omega-3$)	1.4	2.6
20:5($\omega-3$)	–	11.0
22:6($\omega-3$)	–	4.3
$\Sigma(\omega-6)$	51.8	12.7
$\Sigma(\omega-3)$	1.4	17.9
$(\omega-6)/(\omega-3)$	37.0	0.7

2.2. Measurement of pyruvate oxidation

Immediately after decapitation hearts were perfused with 10 ml ice-cold isolation medium consisting of 220 mM mannitol, 70 mM sucrose, 2 mM EGTA and 5 mM Mops (pH 7.4), to which 0.4 mg/ml nagarase (Protease, Sigma) was added. Cardiac mitochondria were isolated in the same medium after homogenization of the ventricles as described in [6]. Homogenates were then centrifuged at $600 \times g_{max}$ for 5 min and the supernatant fluids filtered through four layers of cheesecloth. Supernatant fluids were then centrifuged for 15 min at $10\,000 \times g_{max}$. The resultant mitochondrial pellet was washed three times with fresh, nagarase-free medium. Rates of oxygen consumption were measured polarographically at 30° C with a Clark-type oxygen electrode in 100 mM sucrose, 50 mM KCl, 1 mM $MgCl_2$, 0.5 mM EGTA and 20 mM Tris-HCl, pH 7.2. State 4 respiration was initiated by the addition of 0.5 mM pyruvate followed 1 min later by the addition of 2 mM ADP to determine State 3 rates. For the determination of the respiratory control ratio and ADP/O values, 2.5 mM pyruvate and 2.5 mM malate were used as substrates in the presence of 2 mM ADP [2]. Inhibition of pyruvate respiration was determined by titration with 4.0–50 μM α-cyano-4-hydroxycinnamate [7]. Protein concentration was determined using a modification of the Lowry method as described by Markwell et al. [8].

2.3. Lipid analysis

A portion of each heart homogenate was used to isolate an inner mitochondrial membrane fraction, essentially as described by Schnaitman and Greenawalt [9], except that 0.4 mg/ml nagarase remained in the isolation medium and the concentration of digitonin was increased to 0.5 mg/mg protein. The inner membrane fraction was collected after purification on sucrose density gradients [10]. Membranes were then extracted with chloroform/methanol (2:1) and partitioned against 0.1% KCl. All solvents contained 0.05% butylated hydroxytoluene as antioxidant. Individual phospholipid classes were separated on HPK high-performance silica gel plates (Whatman, Clifton, NJ) using the solvent system chloroform/methanol/2-propanol/0.25% KCl/triethylamine (30:9:25:6:18, v/v) [11]. Phospholipid classes were visualized under UV light after staining with 1% (w/v) 8-anilino-1-naphthalene sulphonic acid and quantified by measuring phosphorus content [12]. Fatty acid methyl esters were prepared by heating a portion of each phospholipid spot at 100° C in freshly prepared 14%

[1] AOAC vitamin mix (Teklad test diets, Madison WI) provided the following per kg of complete diet: 20000 IU vitamin A; 2000 IU vitamin D; 100 mg vitamin E; 5 mg menadione; 5 mg thiamin-HCl; 8 mg riboflavin; 40 mg pyroxidine-HCl; 40 mg niacin; 40 mg pantothenic acid; 2000 mg choline; 100 mg myothiositol; 100 mg *p*-aminobenzoic acid; 0.4 mg biotin; 2 mg folic acid; and 30 mg vitamin B-12.

[2] Berrhart Tomarelli mineral mix (General Biochemicals, Chagrin Falls, OH).

boron trifluoride in methanol for 90 min. Fatty acid species were separated by GLC using a fused silica capillary column (BP-20, 25 m × 0.25 mm I.D.; SGE). Helium was used as carrier gas at a flow rate of 1.8 ml per min using split injection. Temperature programming from 90–220° C allowed separation of all saturated, mono-, di-, and polyunsaturated fatty acids from C_{12} to C_{24} in chain length. Fatty acids were identified using internal standards and comparing against authentic standards (Supelco; NHI/NIH FAME standards) with Beckman 'System Gold' data acquisition software.

2.4. Statistical analysis

The effects of individual diet treatments were compared by Student's Neuman-Keuls multiple range test after a significant effect of treatment was shown by analysis of variance.

3. Results

The data show that alterations in pyruvate-supported oxygen consumption are correlated with thyroid state and the fatty acid composition of the inner mitochondrial membrane. Rates of pyruvate oxidation were significantly lower in mitochondria from hypothyroid animals, regardless of diet, compared to their euthyroid controls (Table 2). State 3 and State 4 rates of oxygen consumption were 40% and 50% lower, respectively, in hypothyroid animals fed the $\omega-3$ and $\omega-6$ diets. However, respiration rates were 28% higher in mitochondria from hypothyroid animals fed the $\omega-3$ diet compared to hypothyroid animals fed the $\omega-6$ diet. Despite the differences in rate between the experimental groups, neither the respiratory control ratios nor ADP/O ratios were affected by thyroid status or diet (Table 2).

Inhibition of pyruvate oxidation by α-cyano-4-hydroxycinnamate resulted in linear Dixon plots (least-squares linear regression of 1/respiratory rate vs. inhibitor concentration) for all of the experimental groups indicating that as inhibitor was added, there was a progressive decrease in pyruvate transport that became rate-limiting for respiration (Fig. 1). Extrapolating the linear portion of the respiration curves to zero rate from the mean values for six separate experiments the apparent K_i for α-cyano-4-hydroxycinnamate for euthyroid mitochondria from $\omega-6$- and $\omega-3$-fed rats was 29.3 ± 2.3 μM and 27.2 ± 2.0 μM, respectively, and for hypothyroid mitochondria 36.3 ± 3.1 μM and 28.8 ± 2.2 μM. Under the conditions used V_{max} was affected by thyroid state and diet, but there was no significant difference in K_i.

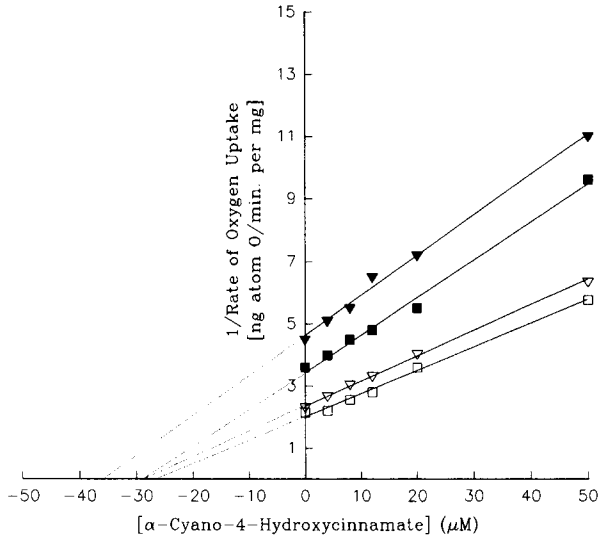

Fig. 1. Dixon plots of the inhibition of pyruvate oxidation by α-cyano-4-hydroxycinnamate in cardiac mitochondria from euthyroid and hypothyroid rats. Pyruvate oxidation was measured as described in Materials and methods. Data were fitted to the equation for non-competitive inhibition using least-squares regression. Data are shown for euthyroid (□, ▽) and hypothyroid (■, ▼) mitochondria from animals fed diets high in $\omega-6$ (▽, ▼) or $\omega-3$ (□, ■) fatty acids.

Table 2
Pyruvate-dependant oxygen consumption in cardiac mitochondria from euthyroid and hypothyroid rats fed diets enriched in either $\omega-6$ or $\omega-3$ fatty acids

	Pyruvate-dependent oxygen consumption (ng atom O/mg per min)			
	$\omega-6$ diet		$\omega-3$ diet	
	euthyroid	hypothyroid	euthyroid	hypothyroid
State 4	33 ± 1.6	18 ± 1.0 [a]	38 ± 1.8	23 ± 0.9 [a,b]
State 3	432 ± 32	215 ± 21 [a]	467 ± 28	276 ± 19 [a,b]
RCR	12.8 ± 0.8	11.8 ± 1.0	12.1 ± 0.9	12.4 ± 0.7
ADP/O	2.78 ± 0.3	2.76 ± 0.2	2.70 ± 0.2	2.80 ± 0.4

Pyruvate-dependent oxygen consumption (ng atom O/mg per min) was measured in mitochondria as described in Materials and methods at 30° C and pH 7.2. Addition of 0.5 mM pyruvate (State 4) to the reaction chamber was followed 1 min later by the addition of 2 mM ADP (State 3). Respiratory control ratios (RCR) and the ADP/O ratio were measured using 2.5 mM pyruvate and 2.5 mM malate. Values represent the means ± S.E. of six experiments.
[a] Significantly different from euthyroid animals fed the same diet.
[b] significantly different from hypothyroid animals fed the $\omega-6$ diet. $P < 0.01$.

Table 3
Phospholipid class distribution in cardiac inner mitochondrial membranes from euthyroid and hypothyroid rats fed diets enriched in $\omega-6$ or $\omega-3$ fatty acids

Phospholipid	Phospholipid class distribution (mol%)			
	$\omega-6$ diet		$\omega-3$ diet	
	euthyroid	hypothyroid	euthyroid	hypothyroid
Phosphatidylcholine	41.6 ± 1.9	48.9 ± 1.8 [a]	40.0 ± 2.1	51.1 ± 2.2 [a]
Phosphatidylethanolamine	34.6 ± 1.9	30.4 ± 1.6 [a]	35.7 ± 1.7	31.4 ± 1.2 [a]
Cardiolipin	19.0 ± 1.0	12.8 ± 0.9 [a]	18.4 ± 1.1	11.9 ± 0.9 [a]
Phosphatidylinositol	1.5 ± 0.2	2.7 ± 0.5 [a]	1.6 ± 0.4	2.5 ± 0.3 [a]
Phosphatidylserine	2.4 ± 0.6	3.7 ± 0.4 [a]	2.9 ± 0.5	3.8 ± 0.5

Each value represents the means ± S.E. for six animals.
[a] Significantly different from euthyroid animals at $P < 0.01$.

3.1. Lipid analysis

The relative distribution of phospholipid classes in the inner mitochondrial membrane was affected by the hypothyroid state but not by diet (Table 3). Cardiolipin and phosphatidylethanolamine (PE) were lower in hypothyroid mitochondria whereas phosphatidylcholine (PC), phosphatidylinositol and phosphatidylserine were all higher. The fatty acid composition of individual phospholipids was profoundly altered by the hypothyroid state with the extent of these changes being determined by the type of fatty acids consumed. In the phosphatidylcholine fraction from rats fed the $\omega-6$ diet, 18:2($\omega-6$) was 69% higher and 20:4($\omega-6$) 13% lower in hypothyroid animals compared to controls (Table 4). The ratio 20:4/18:2 was decreased by half in hypothyroid animals indicating an apparent decrement in Δ^6- and/or Δ^5-desaturase activity. Total $\omega-3$ content was lower in hypothyroid animals resulting

Table 4
Fatty acid composition of cardiac inner mitochondrial membrane phosphatidylcholine from euthyroid and hypothyroid rats fed diets enriched in $\omega-6$ or $\omega-3$ fatty acids

Fatty acid	Fatty acid composition (mol%)			
	$\omega-6$ diet		$\omega-3$ diet	
	euthyroid	hypothyroid	euthyroid	hypothyroid
16:0	14.6 ± 0.7	15.5 ± 0.4 [b**]	17.7 ± 0.6 [a**]	18.6 ± 0.2
16:1	tr.	tr. [b*]	0.2 ± 0.1 [a*]	0.3 ± 0.1
18:0	33.4 ± 0.9 [c**]	30.2 ± 0.3 [b**]	32.3 ± 0.8	28.5 ± 0.4 [d**]
18:1($\omega-7$)	1.7 ± 0.1	1.9 ± 0.1 [b**]	2.7 ± 0.1 [a**]	2.9 ± 0.2
18:1($\omega-9$)	tr.	tr. [b**]	3.0 ± 0.1 [a**]	3.9 ± 0.2
18:2($\omega-6$)	10.5 ± 0.6 [c**]	17.7 ± 0.4 [b**]	6.8 ± 0.7 [a*]	10.1 ± 0.1 [d**]
20:3($\omega-6$)	tr.	tr.	0.1 ± 0.1	0.2 ± 0.1
20:4($\omega-6$)	30.5 ± 0.8 [c*]	26.5 ± 0.3 [b**]	20.3 ± 0.8 [a**]	15.3 ± 1.5 [d**]
20:3($\omega-3$)	tr.	tr.	tr.	tr.
20:5($\omega-3$)	tr.	tr. [b**]	2.4 ± 0.1 [a**]	3.7 ± 0.2 [d**]
22:4($\omega-6$)	1.1 ± 0.1 [c**]	0.6 ± 0.1 [b**]	tr. [a*]	tr.
22:5($\omega-6$)	1.6 ± 0.2 [c*]	0.7 ± 0.1 [b**]	tr. [a**]	tr.
22:5($\omega-3$)	0.6 ± 0.2	0.6 ± 0.1 [b**]	1.8 ± 0.2 [a**]	1.9 ± 0.1
22:6($\omega-3$)	1.7 ± 0.2 [c*]	1.1 ± 0.1 [b**]	9.7 ± 0.2 [a**]	10.1 ± 0.7
$\Sigma(\omega-6)$	43.7	45.5	27.3	25.6
$\Sigma(\omega-3)$	2.3	1.7	13.9	15.7
ΣPUFA	46.0	47.2	41.2	41.3
ΣSAT	48.0	45.7	50.0	47.1
ΣMONO	1.7	1.9	2.7	2.9
$\omega-6/\omega-3$	19.0	26.8	2.0	1.6
20:4/18:2	2.9	1.5	3.0	1.5

[a] Significant difference between euthyroid animals fed $\omega-6$ or $\omega-3$ diet.
[b] Significant difference between hypothyroid animals fed $\omega-6$ or $\omega-3$ diet.
[c] Significant difference between euthyroid and hypothyroid animals fed $\omega-6$ diet.
[d] Significant difference between euthyroid and hypothyroid animals fed $\omega-3$ diet.
* $P < 0.05$; ** $P < 0.01$. $\Sigma(\omega-6)$, sum of $\omega-6$ fatty acid species; $\Sigma(\omega-3)$, sum of $\omega-3$ fatty acid species; ΣPUFA, sum of polyunsaturated fatty acid species; ΣSAT, sum of saturated fatty acid species; ΣMONO, sum of monounsaturated fatty acid species; $(\omega-6)/(\omega-3)$, ratio of sums of $\omega-6$ and $\omega-3$ fatty acyl species, respectively; 20:4/18:2, ratio of 20:4 and 18:2 fatty acids, respectively; tr, trace.

in an increase in the $(\omega-6)/(\omega-3)$ ratio. Significantly lower levels of 22:4($\omega-6$) and 22:5($\omega-6$) were seen in hypothyroid animals.

Feeding the $\omega-3$ diet had two major effects on the fatty acid profile of PC. Firstly, the content of 18:2($\omega-6$) was 43% lower in hypothyroid animals compared to hypothyroid animals fed the $\omega-6$ diet and 20:4($\omega-6$) was 42% lower. However, the change in the 20:4/18:2 ratio was the same between control and hypothyroid animals as it was in $\omega-6$-fed animals. This indicates that while the activities of the Δ^6- and/or Δ^5-desaturase enzymes involved in PC synthesis were depressed by the hypothyroid state, they were not influenced by diet. The other significant effect of the $\omega-3$ diet was to increase the total $\omega-3$ content of PC several-fold with the result that the $(\omega-6)/(\omega-3)$ ratio was lowered dramatically. The incorporation of $\omega-3$ fatty acids into PC was at the expense of the longer chain $\omega-6$ fatty acids, 22:4($\omega-6$) and 22:5($\omega-6$), which were only found in trace amounts in hypothyroid and control animals. Despite the fact that the two diets contained nearly equivalent amounts of unsaturated fatty acids, the total levels of unsaturates were lower in hypothyroid animals fed the $\omega-3$ diet (Table 4). This was partially offset by increased levels of monounsaturates, primarily 18:1($\omega-7$) and 18:1($\omega-9$).

In the phosphatidylethanolamine fraction 18:2($\omega-6$) was 82% higher in hypothyroid animals fed the $\omega-6$ diet compared to euthyroid controls (Table 5). However, unlike the situation in PC, 20:4($\omega-6$) also increased (32%). Nevertheless the 20:4/18:2 ratio was lower in hypothyroid animals indicating lower Δ^6- and/or Δ^5-desaturase activity in the synthesis of PE. Both 22:4($\omega-6$) and 22:5($\omega-6$) were significantly lower in hypothyroid animals. While 22:5($\omega-3$) was higher, 22:6($\omega-3$) was lower in hypothyroid animals compared to controls with the result that, again similar to PC, the $(\omega-6)/(\omega-3)$ ratio was elevated in hypothyroid animals. The effect of the $\omega-3$ diet was to lower 18:2($\omega-6$) (54%) and 20:4($\omega-6$) (62%) in PE from hypothyroid animals compared to controls. However, the change in the ratio of these fatty acids was the same as seen in $\omega-6$-fed animals, i.e., the apparent Δ^6- and/or Δ^5-desaturase activity was lowered to the same extent. Both 22:4($\omega-6$) and 22:5($\omega-6$) all but disappeared from PE in animals fed the $\omega-3$ diet. While there was a 3.5-fold increase in the total $\omega-3$ fatty acid content of hypothyroid PE compared to animals fed

Table 5
Fatty acid composition of cardiac inner mitochondrial membrane phosphatidylethanolamine from euthyroid and hypothyroid rats fed diets enriched in either $\omega-6$ or $\omega-3$ fatty acids

Fatty acid	Fatty acid composition (mol%)			
	$\omega-6$ diet		$\omega-3$ diet	
	euthyroid	hypothyroid	euthyroid	hypothyroid
16:0	7.9 ± 0.5	8.7 ± 0.6	8.8 ± 0.5	9.0 ± 0.3
16:1	0.4 ± 0.1 ^{c**}	0.6 ± 0.1	0.3 ± 0.1	0.6 ± 0.1 ^{d**}
18:0	35.3 ± 0.7 ^{c**}	27.6 ± 0.7 ^{b**}	38.9 ± 0.4 ^{a**}	33.6 ± 0.3 ^{d**}
18:1($\omega-7$)	1.6 ± 0.1	1.7 ± 0.1 ^{b**}	3.1 ± 0.1 ^{a**}	3.2 ± 0.1
18:1($\omega-9$)	1.0 ± 0.1 ^{c**}	1.5 ± 0.1 ^{b**}	1.2 ± 0.1 ^{a*}	1.3 ± 0.1 ^{d**}
18:2($\omega-6$)	7.2 ± 0.6 ^{c**}	13.1 ± 0.7 ^{b**}	3.5 ± 0.3 ^{a**}	6.0 ± 0.6 ^{d**}
20:3($\omega-6$)	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
20:4($\omega-6$)	21.5 ± 0.7 ^{c**}	28.3 ± 0.8 ^{b**}	9.0 ± 0.2 ^{a**}	10.8 ± 0.3
20:3($\omega-3$)	tr.	tr.	0.1 ± 0.1	0.1 ± 0.1 ^{d**}
20:5($\omega-3$)	tr.	tr. ^{b**}	2.3 ± 0.1 ^{a**}	3.4 ± 0.2 ^{d**}
22:4($\omega-6$)	2.7 ± 0.3 ^{c*}	2.0 ± 0.1 ^{b**}	0.1 ± 0.1 ^{a**}	0.1 ± 0.1
22:5($\omega-6$)	10.1 ± 1.0	8.5 ± 0.6 ^{b**}	0.1 ± 0.1 ^{a**}	0.2 ± 0.1
22:5($\omega-3$)	1.1 ± 0.1 ^{c*}	1.6 ± 0.1 ^{b**}	1.8 ± 0.1 ^{a**}	2.4 ± 0.1 ^{d**}
22:6($\omega-3$)	9.7 ± 0.2	8.5 ± 0.6 ^{b**}	29.9 ± 0.3 ^{a**}	29.1 ± 1.1
$\Sigma(\omega-6)$	43.6	51.9	12.8	17.2
$\Sigma(\omega-3)$	10.8	10.1	34.0	35.0
ΣPUFA	54.4	61.0	46.8	52.2
ΣSAT	43.2	36.3	47.7	42.6
ΣMONO	3.0	3.8	4.6	4.9
$(\omega-6)/(\omega-3)$	4.0	5.1	0.4	0.5
20:4/18:2	3.0	2.2	2.6	1.8

[a] Significant difference between euthyroid animals fed $\omega-6$ or $\omega-3$ diet.
[b] Significant difference between hypothyroid animals fed $\omega-6$ or $\omega-3$ diet.
[c] Significant difference between euthyroid and hypothyroid animals fed $\omega-6$ diet.
[d] Significant difference between euthyroid and hypothyroid animals fed $\omega-3$ diet.
* $P<0.05$; ** $P<0.01$. $\Sigma(\omega-6)$, sum of $\omega-6$ fatty acid species; $\Sigma(\omega-3)$, sum of $\omega-3$ fatty acid species; ΣPUFA, sum of polyunsaturated fatty acid species; ΣSAT, sum of saturated fatty acid species; ΣMONO, sum of monounsaturated fatty acid species; $(\omega-6)/(\omega-3)$, ratio of sums of $\omega-6$ and $\omega-3$ fatty acyl species, respectively; 20:4/18:2, ratio of 20:4 and 18:2 fatty acids, respectively; tr, trace.

Table 6
Fatty acid composition (mol%) of cardiac inner mitochondrial cardiolipin from euthyroid and hypothyroid rats fed diets enriched in either $\omega-6$ or $\omega-3$ fatty acids

Fatty acid	Fatty acid composition (mol%)			
	$\omega-6$ diet		$\omega-3$ diet	
	euthyroid	hypothyroid	euthyroid	hypothyroid
16:0	6.0 ± 0.2	6.2 ± 0.7	$9.0 \pm 0.5^{a**}$	$6.5 \pm 0.5^{d**}$
16:1	0.2 ± 0.1	$0.3 \pm 0.1^{b**}$	$1.9 \pm 0.1^{a**}$	$1.4 \pm 0.1^{d**}$
18:0	$2.5 \pm 0.2^{c**}$	4.2 ± 0.7	$5.5 \pm 0.8^{a**}$	$3.6 \pm 0.4^{d*}$
18:1($\omega-7$)	$0.6 \pm 0.1^{c**}$	$1.8 \pm 0.1^{b**}$	$4.6 \pm 1.0^{a**}$	$3.0 \pm 0.5^{d*}$
18:1($\omega-9$)	4.9 ± 0.4	$0.7 \pm 0.3^{b**}$	$8.9 \pm 0.9^{a**}$	$3.7 \pm 0.3^{d**}$
18:2($\omega-6$)	78.8 ± 1.1	$78.2 \pm 2.1^{b*}$	$58.9 \pm 3.2^{a**}$	$70.4 \pm 1.7^{d**}$
20:3($\omega-6$)	$0.9 \pm 0.1^{c**}$	$0.3 \pm 0.1^{b**}$	$0.6 \pm 0.1^{a*}$	0.8 ± 0.1
20:4($\omega-6$)	1.6 ± 0.2	1.9 ± 0.2	2.2 ± 0.4	$1.4 \pm 0.2^{d*}$
20:3($\omega-3$)	tr.	tr.	tr.	tr.
20:5($\omega-3$)	tr.	tr.^{b**}	$1.4 \pm 0.1^{a**}$	$1.0 \pm 0.1^{d*}$
22:4($\omega-6$)	tr.	tr.	tr.	tr.
22:5($\omega-6$)	tr.	tr.	tr.	tr.
22:5($\omega-3$)	0.1 ± 0.1	$0.1 \pm 0.1^{b*}$	$0.5 \pm 0.1^{a**}$	0.4 ± 0.1
22:6($\omega-3$)	0.5 ± 0.1	$0.3 \pm 0.1^{b**}$	$4.1 \pm 0.7^{a**}$	3.3 ± 0.6
$\Sigma(\omega-6)$	81.3	80.4	61.7	72.6
$\Sigma(\omega-3)$	0.6	0.4	6.0	4.7
ΣPUFA	81.9	80.8	67.7	77.3
ΣSAT	8.5	10.4	14.5	10.1
ΣMONO	5.7	2.8	15.4	8.1
$(\omega-6)/(\omega-3)$	135.5	201.0	10.2	15.4
20:4/18:2	0.02	0.02	0.04	0.02

[a] Significant difference between euthyroid animals fed $\omega-6$ or $\omega-3$ diet.
[b] significant difference between hypothyroid animals fed $\omega-6$ or $\omega-3$ diet.
[c] significant difference between euthyroid and hypothyroid animals fed $\omega-6$ diet.
[d] Significant difference between euthyroid and hypothyroid animals fed $\omega-3$ diet.
* $P<0.05$; ** $P<0.01$. $\Sigma(\omega-6)$, sum of $\omega-6$ fatty acid species; $\Sigma(\omega-3)$, sum of $\omega-3$ fatty acyl species; ΣPUFA, sum of polyunsaturated fatty acid species; ΣSAT, sum of saturated fatty acid species; ΣMONO, sum of monounsaturated fatty acyl species; $(\omega-6)/(\omega-3)$, ratio of sums of $\omega-6$ and $\omega-3$ fatty acyl species, respectively; 20:4/18:2, ratio of 20:4 and 18:2 fatty acids, respectively; tr, trace.

the $\omega-6$ diet, there was no significant difference from controls.

Cardiolipin was the least affected by the hypothyroid state in $\omega-6$-fed animals. Levels of 18:2($\omega-6$) and 20:4($\omega-6$) were not different from controls and therefore there was no apparent change in Δ^6- and/or Δ^5-desaturase activity (Table 6). However, in animals fed the $\omega-3$ diet, 18:2($\omega-6$) was higher in hypothyroid animals compared to controls but both were significantly lower than respective hypothyroid and control animals fed the $\omega-6$ diet. The 20:4/18:2 ratio in hypothyroid animals was half the value for controls indicating lower Δ^6- and/or Δ^5-desaturase activity. While the $\omega-3$ diet resulted in enhanced levels of 22:5($\omega-3$) and 22:6($\omega-3$) in cardiolipin from hypothyroid and control animals, the $(\omega-6)/(\omega-3)$ ratio was still 50% higher in the former. Total monounsaturates were reduced 47% in hypothyroid animals fed the $\omega-3$ diet with 16:1($\omega-7$), 18:1($\omega-7$) and 18:1($\omega-9$) all significantly lower. This change is particularly noteworthy in that the hypothyroid state did not have a significant effect on monounsaturates in PC (Table 4) or PE (Table 5).

4. Discussion

The results presented in this study confirm that mitochondrial respiration is depressed in the hypothyroid heart, in part due to inhibition of pyruvate transport. The differences in pyruvate-dependent oxidation rates between euthyroid and hypothyroid animals consuming the same diet were nearly identical to those reported by others, i.e., they were about 40% lower in the hypothyroid state [1,2,4]. The efficiency of respiratory-chain activity does not appear to have been altered by the hypothyroid state or diet as respiratory control ratios and the ADP/O ratio in hypothyroid animals were not different from controls. The lack of effect of hypothyroidism on these two parameters [2,4,13] and on the H^+/O ratio [13] has been documented by others and supports the contention that the major effects of the hypothyroid state on pyruvate transport are probably on the turnover rate of the pyruvate carrier. The possibility that differences in respiration rate were due to the number of pyruvate carrier molecules can be discounted as evidence has been presented that shows binding of α-cyanocinnamate, a marker for the pyruvate carrier, is not influ-

enced by thyroid status [1,14]. Regardless of thyroid state or diet, titration of respiration with α-cyano-4-hydroxycinnamate produced linear Dixon plots with no significant difference in the apparent K_i for inhibition, the range of K_i for all of the treatment groups being similar to that reported for pyruvate metabolism in cardiac mitochondria by others [15,16]. The concentration of pyruvate used was within physiological range [17,18], the linear Dixon plots confirming that pyruvate transport was not rate limiting.

Recent reports that the effects of thyroid hormone on pyruvate transport are correlated with changes in membrane lipids have focused primarily on total phospholipid fatty acid composition and a purported crucial role for cardiolipin. In heart mitochondria from hypothyroid rats, Paradies and Ruggerio [2] found that cardiolipin levels were lower than in euthyroid animals and that the cardiolipin content of 18:2($\omega-6$) and monounsaturated fatty acids was less. While the total unsaturation of the mitochondrial membrane remained unchanged, an increase in the 20:4/18:2 ratio in the hypothyroid animals suggests that the metabolism of $\omega-6$ fatty acids was enhanced. However, while the same authors reported an increase in mitochondrial cardiolipin from hyperthyroid rats [14,19], the fatty acid profile of cardiolipin was virtually identical to control animals [19]. Furthermore, the overall 20:4/18:2 ratio was also elevated in hyperthyroid animals to the same degree seen in hypothyroid animals, and the unsaturation index was again unaffected. Others have failed to find any differences in the relative distribution of phospholipids in hypo- and hyperthyroid rat heart mitochondria compared to controls, even though respiration was substantially different [3]. It is difficult, therefore, to conclude that there is any consistent alteration in the total unsaturation of fatty acids that would modify the fluidity of the bulk phase of membrane lipids and thus contribute to the depressed pyruvate transport seen in the hypothyroid state. This contention is further supported by examination of the distribution of fatty acid species in the present study. Taking into consideration the fatty acid profiles of PC, PE and cardiolipin together, the $\Sigma(\omega-6)$ and $\Sigma(\omega-3)$ fatty acids were not different between euthyroid and hypothyroid animals fed the $\omega-6$ diet. Similarly, there was no difference in the total $\omega-6$ and $\omega-3$ fatty acids between the euthyroid and hypothyroid states in animals fed the $\omega-3$ diet. However, the overall $(\omega-6)/(\omega-3)$ ratio was 8-fold higher in $\omega-6$-fed animals. It is clear therefore that hypothyroidism did not alter the total content and distribution of fatty acids but diet did. The significant increase in pyruvate oxidation in hypothyroid animals fed the $\omega-3$ diet could be due to the enrichment of the inner membrane with $\omega-3$ fatty acids or alternatively, to the 2-fold higher content of monounsaturated fatty acids. While no increase in heart mitochondrial respiration was seen in healthy rats after increasing membrane $\omega-3$ levels through diet [20], succinate was used as substrate and therefore the activity of the pyruvate carrier would not have been rate-limiting for respiration. Nevertheless, even when pyruvate was used in this study there was no significant effect on respiration when $\omega-3$ fatty acids were elevated in all phospholipid classes in euthyroid animals fed the $\omega-3$ diet. An alternative explanation is that the nature of the fatty acyl chains of specific phospholipids, such as cardiolipin, become more important in the hypothyroid state. Cardiolipin is known to be required for full cytochrome-*c* oxidase activity [21,22], for the isolation of active phosphate carrier [23], and for the isolation of viable pyruvate carrier [24]. Moreover, both fatty acid synthetase [25] and cardiolipin synthetase [26] appear to be thyroid hormone dependent. A significant reduction in cytochrome-*c* oxidase activity in hypothyroid mitochondria has been correlated with lower levels of cardiolipin, although the fatty acid composition of cardiolipin was not different from normal mitochondria [4]. On the other hand, altering cardiolipin fatty acid composition through dietary manipulation has been shown to affect cytochrome-*c* oxidase activity in rat liver [27] and canine heart [28]. The marked effect of the hypothyroid state on the amount and the fatty acid profile of cardiolipin in the present study is a strong argument for the dependency of the pyruvate carrier on this specific phospholipid.

Lowering of the 20:4/18:2 ratio appears to be a general response to the hypothyroid state with accumulation of 18:2($\omega-6$) and a corresponding decreased synthesis of 20:4($\omega-6$) indicating inhibition of Δ^6- and Δ^5-desaturase activity [29,30]. The inner mitochondrial membrane appears to respond to the lack of thyroid hormone in a similar manner in that the 20:4/18:2 ratio was lower in the PC and PE fractions in hypothyroid mitochondria. The lack of a similar response in cardiolipin may reflect the fact that this phospholipid is synthesized in the inner membrane and is normally very high in 18:2($\omega-6$). The increased levels of $\omega-3$ fatty acids in animals fed the $\omega-3$ diet were probably achieved because the diet contained high levels of 20:5($\omega-3$) and 22:6($\omega-3$). Normally the fatty acids 18:2($\omega-6$) and 18:3($\omega-3$) compete for the enzymes of desaturation and elongation to longer chains, but in this case the 20:5($\omega-3$) and 22:6ω were preformed and were not dependent of the functioning of the biosynthetic pathway. However, the fact that membrane levels of 20:5($\omega-3$) were lower than 22:6($\omega-3$) in hypothyroid mitochondria, despite their higher concentration in the diet, suggests there may have been inhibition of Δ^4-desaturase or the acyltransferases [5].

Any discussion of respiration in hypothyroid mitochondria must include the contribution proton permeability makes to the overall rate of oxygen consumption. Those reactions of oxidative phosphorylation that consume rather than produce protonmotive force (Δp), and therefore contribute to proton 'leak' across the inner membrane, are known to be depressed in hypothyroid mitochondria and enhanced in hyperthyroid mitochondria [31]. It is possible that the alterations in fatty acid composition induced by

our diet treatments may have affected the proton permeability of the inner membrane. Brand and co-workers have proposed that part of the permeability characteristics of the mitochondrial membrane are determined by its fatty acid composition [32].

In conclusion, depressed pyruvate oxidation in mitochondria from hypothyroid rats was correlated with alterations in the relative distribution of inner membrane phospholipids, but not to the overall fatty acid composition. Improved respiration in hypothyroid animals occurred when membrane levels of $\omega-3$ fatty acids were enhanced through dietary treatment. In view of this, we propose that depressed pyruvate transport in the hypothyroid state is primarily due to the direct effects of thyroid hormone on the pyruvate carrier, and only in part as a consequence of a change in membrane fatty acid composition. While the fatty acid distribution in PC, PE and cardiolipin were all affected, it is most probable that cardiolipin is involved in the regulation of pyruvate transport as suggested by others [2] and that increasing its content of $\omega-3$ fatty acids enhances transport in the hypothyroid state.

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