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Thyroid hormone status and membrane *n*-3 fatty acid content influence mitochondrial proton leak

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Abstract

Proton leak, as determined by the relationship between respiration rate and membrane potential, was lower in mitochondria from hypothyroid rats compared to euthyroid controls. Moreover, proton leak rates diminished even more when hypothyroid rats were fed a diet containing 5% of the lipid content as *n*-3 fatty acids. Similarly, proton leak was lower in euthyroid rats fed the 5% *n*-3 diet compared to one containing only 1% *n*-3 fatty acids. Lower proton leaks rates were associated with increased inner mitochondrial membrane levels of *n*-3 fatty acids and a decrease in the ratio of *n*-6/*n*-3 fatty acids. This trend was evident in the phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and cardiolipin phospholipid fractions. These results suggest that a significant portion of the effect of thyroid hormone status on proton leak is due to alterations in membrane fatty acid composition, primarily changes in *n*-3 content. Both the hypothyroid state and dietary effects appear to be mediated in part by inhibition of the Δ^6- and Δ^5-desaturase pathways.

Keywords: Mitochondria; Proton leak; *n*-3 fatty acid; Thyroid hormone; Membrane potential

1. Introduction

While our understanding of how mitochondria carry out oxidative phosphorylation is extensive, the mechanisms by which external effectors, such as hormones, regulate energy production are less well defined. In particular, thyroid hormone is known to have multiple physiological effects on most organ systems, and through its influence on mitochondrial energetics contributes to the control of energy expenditure [1–3]. Nevertheless, the sites of action of thyroid hormone and the signaling mechanisms that link thyroid hormone to mitochondrial energy-producing pathways have not been fully elucidated.

In theory at least, the pumping of protons from the mitochondrial matrix creates an electrochemical potential or proton motive force (Δp) that drives protons back through the ATP synthase in a coupled reaction during phosphorylation. Experimentally, however, the coupling of electron transport to phosphorylation is variably incomplete in that significant transport-driven oxygen consumption occurs during non-phosphorylating respiration (state 4) and to a lesser degree during phosphorylation (state 3 respiration). The back flow of protons into the mitochondrial matrix without ADP phosphorylation is termed proton leak and gives rise to uncoupled or inefficient

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metabolism [4]. It is estimated that proton leak comprises approximately 20% of liver mitochondrial oxygen consumption, the remainder being tightly coupled to ATP synthesis [5].

The effect of thyroid hormone on proton leak is clearly evident in the higher rates of state 4 respiration seen in mitochondria isolated from hyperthyroid rats [6]. Moreover, the observation that thyroid state affects proton leak in intact hepatocytes [7] is consistent with the view that the basal rate of cellular oxygen consumption is dependent on relative levels of thyroid hormone and that thyroid hormone activity accounts for species differences in resting metabolic rate [8]. It can also be assumed that thyroid hormone status could be a contributing factor in pathophysiological conditions that involve mitochondrial dysfunction.

Possible explanations for higher leak rates in the hyperthyroid state are either a hormone-induced increase in surface area of the inner membrane [9], or an increase in proton permeability brought about by an alteration in membrane fatty acid composition [5,10]. Conversely, in the hypothyroid state, a decrease in surface area or membrane permeability would account for a lower leak rate. Changes in mitochondrial inner membrane area appear to account for less than half of the effect of a change in thyroid hormone status on proton leak, however, with the remainder of any alteration in proton permeability presumably being due to changes in membrane fatty acids [9]. In particular, Porter et al. [11] and others [12] have suggested a role for *n*-3 fatty acids in the determination of proton permeability of the inner mitochondrial membrane. Despite some observed species differences, however, evidence supporting membrane fatty acids as mediators of the effects of thyroid hormone on proton leak has been largely circumstantial to date.

In this study, we demonstrate that reduced proton leak in mitochondria from hypothyroid rats is associated with an increase in inner membrane phospholipid *n*-3 fatty acid content. Furthermore, we show, for the first time, that physiological levels of dietary *n*-3 fatty acids reduce the proton permeability of the inner mitochondrial membrane in euthyroid animals, and further reduce proton leak in the hypothyroid state.

2. Materials and methods

Male weanling Sprague–Dawley rats (50–55 g) were randomly divided into two groups and fed one of two semi-purified diets, ad libitum. Both diets contained 20% lipid (w/w) with one diet containing 1% of the total lipid as *n*-3 fatty acids and the other diet 5% *n*-3 fatty acids. The 1% *n*-3 diet ensured adequate levels of all essential fatty acids and represents a control diet. After 2 weeks half of each diet group was made hypothyroid by continuous administration of 0.05% propylthiouracil (PTU) in drinking water and was provided with the same diet they had been consuming for a further 28 days. At the end of each experimental period plasma T_4 levels were 58 ± 5.9 and 61 ± 6.1 pmol/l in euthyroid rats fed the low and high *n*-3 diets, respectively, and 6.8 ± 1.3 and 11.1 ± 2.0 pmol/l in hypothyroid rats.

2.1. Diets

Diets were formulated to contain equivalent nutrients per caloric content and were comprised of the following nutrients (per kg); 270 g casein, 200 g starch, 207 g glucose, 50 g non-nutritive cellulose, 10 g vitamin mix, 50.85 g mineral mix, 2.75 g choline, 6.25 g inositol, 2.5 g L-methionine. The lipid content was achieved by the addition of 20% fat (w/w) to the basal mix as a combination of beef tallow, safflower oil, olive oil, linseed oil, or fish oil (28% 20:5(*n*-3); 12% 22:6(*n*-3)) [13]. Diets were adjusted to contain equivalent levels of total sterols.

2.2. Preparation of mitochondria and measurement of proton leak

Liver mitochondria were isolated by differential centrifugation using a buffer consisting of 250 mM sucrose, 3 mM HEPES, pH 7.4, 1 mM EGTA and 0.5 mg/ml BSA. Oxygen consumption was measured at 30°C with a Clark-type oxygen electrode in buffer consisting of 100 mM KCl, 20 mM glucose, 20 mM sucrose, 3 mM HEPES, 2 mM KH_2PO_4, 2 mM $MgCl_2$, 1 mM EGTA, 100 μM acetate, and 5 μM rotenone, pH 7.4. Respiration was initiated by the addition of 5 mM succinate. State 4 respiration was measured in the absence of ADP, but in the presence

of 2 μg/ml oligomycin and 0.4 μg/ml nigericin. Membrane potential ($\Delta\psi$) was measured in parallel from the distribution of tetramethylphenylphosphonium ion ($TPMP^-$) using a TPMP-sensitive electrode and a high impedance pH electrode as reference [14]. $TPMP^-$ binding corrections were calculated as described by Brand [15]. Mitochondrial volume and ΔpH were determined by the distribution of ^3H_2O and [^{14}C]sucrose, and by [^{3}H]acetate and [^{14}C]sucrose, respectively [16]. Titration of respiration and $\Delta\psi$ with serial additions of 250 μM malonate was used to calculate the ratio of $\Delta\psi$ to O_2 consumption, i.e. proton leak. All measurements were performed in triplicate.

2.3. Analysis of inner mitochondrial membrane fatty acid composition

An inner mitochondrial membrane fraction was isolated essentially as described by Schnaitman and Greenawalt [17]. The inner membrane fraction was collected after purification on sucrose density gradients and total lipids extracted with chloroform/methanol (2:1) and then partitioned against 0.1% KCl. All solvents contained 0.05% butylated hydroxytoluene as antioxidant. Individual phospholipid classes were identified 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) [18]. Phospholipid classes were visualized under UV light after staining with 0.05% (w/v) 8-anilino-1-naphthalene sulfonic acid and methyl esters of their constituent fatty acids prepared with fresh 14% boron trifluoride in methanol. 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/min using split injection. Temperature programming from 90 to 220°C allowed separation of all saturated, mono-, di-, and poly-unsaturated fatty acids from C_{12} to C_{24} in chain length. Fatty acids were identified using internal standards and by comparison against authentic standards (Supelco; NHI/NIH FAME standards).

2.4. Materials

6-*n*-Propylthiouracil (PTU) was obtained from Sigma, St. Louis, MO. All other chemicals and biochemicals were of the highest purity available.

2.5. Statistical analysis

The influence of dietary levels of *n*-3 fatty acids and the hypothyroid state on phospholipid fatty acid composition was determined using Neuman–Keuls multiple range test following a 2 × 2 factorial ANOVA. In the first instance, the effect of a high (5%) *n*-3 diet on membrane fatty acid composition was determined in euthyroid rats. The effect of the hypothyroid state on membrane fatty acid composition was determined in hypothyroid animals fed the control (1% *n*-3) diet. Finally, the interaction of *n*-3 fatty acids and the hypothyroid state was determined in euthyroid and hypothyroid animals fed control or high *n*-3 diets.

3. Results

3.1. Effect of n-3 fatty acids and hypothyroid state on proton leak

At high $\Delta\psi$, the apparent proton leak was significantly different between euthyroid and hypothyroid mitochondria and between mitochondria from euthyroid or hypothyroid animals fed different diets (Fig. 1). For any given $\Delta\psi$ respiration rates were in the order; hypothyroid/high *n*-3 diet < hypothyroid/low *n*-3 diet < euthyroid/high *n*-3 diet < euthyroid/low *n*-3 diet. Differences in leak persisted as $\Delta\psi$ was lowered during malonate titration, whereas there were no differences in either membrane ΔpH or matrix volume between preparations.

3.2. Effect of n-3 fatty acids on inner mitochondrial membrane fatty acid composition

The fatty acid composition of the inner mitochondrial membrane was altered by dietary *n*-3 fatty acids and by the hypothyroid state. The most significant changes occurred in the relative distribution of *n*-6 and *n*-3 fatty acid moieties with the extent of these changes being different in each phospholipid class. In the phosphatidylcholine (PC) fraction, 18:2*n*-6 was 28% higher and 20:4*n*-6 45% lower in euthyroid ani-

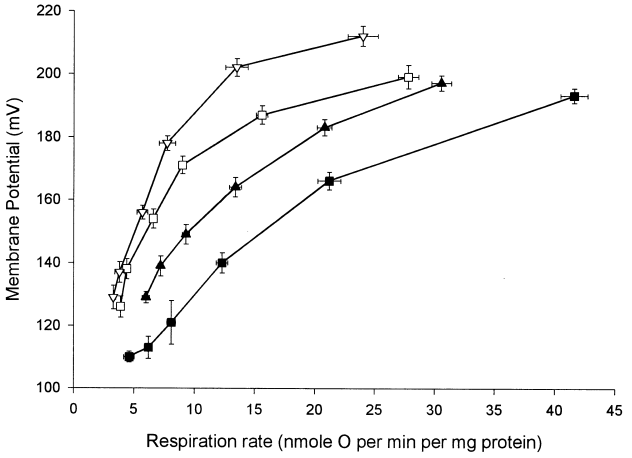

Fig. 1. Effect of hypothyroid state and *n*-3 fatty acids on mitochondrial proton leak. Relationship between respiration rate and $\Delta\psi$. Mitochondria from euthyroid (closed symbols) and hypothyroid rats (open symbols) fed diets containing either 1% (▲, ▽) or 5% (■, □) *n*-3 fatty acids were incubated in 2.5 ml of 100 mM KCl, 20 mM glucose, 20 mM sucrose, 3 mM HEPES, 2 mM KH_2PO_4, 2 mM $MgCl_2$, 1 mM EGTA, 100 μM acetate, and 5 μM rotenone, pH 7.4. The $TPMP^-$ electrode was calibrated with aliquots of Ph_3MePBr to a final concentration of 5 μM and then state 4 respiration initiated by the addition of 5 mM succinate. Respiration was inhibited step-wise with additions of 200 μM malonate. Values are means ± S.E.M. for at least six experiments. Triplicate determinations were made for each animal.

mals fed the high *n*-3 diet compared to the control diet. As a result, the Δ^6-desaturase index (20:4/18:2) was lower (Table 1). At the same time, accumulation of 20:3*n*-6 (450% increase) resulted in a dramatic decrease in the Δ^5-desaturase index (20:4/20:3). The overall effect of feeding the high *n*-3 diet was to lower the PC content of *n*-6 fatty acids and to increase *n*-3 content. As a consequence, the *n*-6/*n*-3 ratio was 78% lower.

The effect of the high *n*-3 diet on phosphatidylethanolamine (PE) was to decrease the content of 20:4*n*-6 (51%) but with no change in 18:2*n*-6. Again, the high *n*-3 diet lowered both the Δ^6- and Δ^5-desaturase indices and the ratio of *n*-6/*n*-3 fatty acids.

The largest single effect of the high *n*-3 diet on the phosphatidylinositol (PI) fraction was a 35% lower content of 20:4*n*-6 in membranes from euthyroid animals. This was reflected in a lower Δ^6-desaturase index (Table 3). A 5-fold increase in 20:3*n*-6 content (0.4 ± 0.1 to 2.2 ± 0.4 mol%) resulted in a significant decrease in the Δ^5-desaturase index as well. Similar to the situation in the PC and PE fractions, a decrease in the total *n*-6 fatty acid content coupled with a nearly 5-fold increase in *n*-3 fatty acids lowered the *n*-6/*n*-3 ratio (Table 3).

The membrane cardiolipin fraction from euthyroid rats fed the high *n*-3 diet was more saturated than from animals fed the low *n*-3 diet, but contained less

Table 1
Summary of phosphatidylcholine fatty acid composition of inner mitochondrial membranes from euthyroid and hypothyroid rats fed diets containing either 1 or 5% (w/w) *n*-3 fatty acids

Fatty acid (w/w%)	Euthyroid			Hypothyroid				
	1% *n*-3 Diet	5% *n*-3 Diet	Diet effect	1% *n*-3 Diet	5% *n*-3 Diet	Diet effect	1% *n*-3/ Hypo effect	5% *n*-3/ Hypo effect
Σ*n*-6	37.4 ± 1.8	30.8 ± 1.3	*	39.2 ± 1.4	29.9 ± 1.0	**	NS	NS
Σ*n*-3	2.2 ± 0.4	8.4 ± 0.5	**	1.6 ± 0.1	11.6 ± 0.5	**	*	**
n-6/*n*-3	17.0 ± 1.8	3.7 ± 0.3	**	24.5 ± 1.4	2.6 ± 0.5	**	**	*
ΣSAT	54.2 ± 2.1	51.6 ± 1.3	NS	51.6 ± 1.0	41.2 ± 1.3	*	NS	**
ΣMONO	2.9 ± 0.2	6.9 ± 0.8	**	3.9 ± 0.3	7.2 ± 0.4	**	*	NS
20:4/18:2	1.9 ± 0.2	0.8 ± 0.2	*	1.4 ± 0.2	0.6 ± 0.1	*	*	NS
20:4/20:3	122.0 ± 2.0	12.1 ± 0.7	**	56.5 ± 1.7	15.4 ± 0.8	**	**	*

Values are means of mol% ± S.E.M. for at least six animals. Σ*n*-6, sum of *n*-6 fatty acyl species; Σ*n*-3, sum of *n*-3 fatty acyl species; ΣSAT, sum of all saturated fatty acyl species; ΣMONO, sum of all monounsaturated fatty acyl species. Δ^6-Desaturase index is determined by the ratio 18:2*n*-6/20:4*n*-6 and is a measure of the enzymatic conversion of 18:2 to 20:4. Δ^5-Desaturase index is determined by the ratio 20:4*n*-6/20:3*n*-6 and is a measure of the enzymatic conversion of 20:4 to 20:3.
* $P < 0.05$.
** $P < 0.01$.

Table 2
Phosphatidylethanolamine fatty acid composition of inner mitochondrial membranes from euthyroid and hypothyroid rats fed diets containing either 1 or 5% (w/w) *n*-3 fatty acids

Fatty acid (w/w%)	Euthyroid			Hypothyroid				
	1% *n*-3 Diet	5% *n*-3 Diet	Diet effect	1% *n*-3 Diet	5% *n*-3 Diet	Diet effect	1% *n*-3/ Hypo effect	5% *n*-3/ Hypo effect
Σ*n*-6	29.2 ± 1.4	18.7 ± 0.9	**	34.8 ± 0.5	18.9 ± 1.1	**	*	NS
Σ*n*-3	3.5 ± 0.3	12.5 ± 0.5	**	3.5 ± 0.2	13.9 ± 0.5	**	NS	NS
n-6/*n*-3	8.3 ± 1.4	1.5 ± 0.9	**	9.9 ± 0.5	1.4 ± 0.6	**	*	NS
ΣSAT	59.9 ± 1.4	62.0 ± 1.0	NS	55.0 ± 2.2	58.8 ± 1.8	NS	NS	NS
ΣMONO	3.2 ± 0.3	4.6 ± 0.2	*	3.2 ± 0.2	5.3 ± 0.3	*	NS	*
20:4/18:2	3.6 ± 0.3	1.6 ± 0.1	*	1.9 ± 0.1	0.8 ± 0.1	*	*	*
20:4/20:3	228.0 ± 2.0	28.0 ± 0.9	**	56.3 ± 1.1	16.6 ± 0.7	**	**	**

Values are means of mol% ± S.E.M. for at least six animals. Σ*n*-6, sum of *n*-6 fatty acyl species; Σ*n*-3, sum of *n*-3 fatty acyl species; ΣSAT, sum of all saturated fatty acyl species; ΣMONO, sum of all monounsaturated fatty acyl species. Δ^6-Desaturase index is determined by the ratio 18:2*n*-6/20:4*n*-6 and is a measure of the enzymatic conversion of 18:2 to 20:4. Δ^5-Desaturase index is determined by the ratio 20:4*n*-6/20:3*n*-6 and is a measure of the enzymatic conversion of 20:4 to 20:3.
* $P < 0.05$.
** $P < 0.01$.

n-6 and mono-unsaturated fatty acids (Table 4). This phospholipid fraction was unique in that there was no significant influence of diet on either the Δ^6- or Δ^5-desaturase indices. Feeding the high *n*-3 diet also had the effect of elevating levels of the major *n*-3 fatty acids, i.e. 20:5*n*-3, 22:5*n*-3 and 22:6*n*-3 (data not shown), with the result the *n*-6/*n*-3 ratio was significantly lower.

3.3. Effect of hypothyroid state on inner mitochondrial membrane fatty acid composition

In the membrane PC fraction, 18:2*n*-6 was 26.5% higher and 20:4*n*-6 7.4% lower in hypothyroid animals compared to euthyroid controls when both were fed the control diet. The Δ^6- and Δ^5-desaturase indices were both lower in hypothyroid animals (Table

Table 3
Summary of phosphatidylinositol fatty acid composition of inner mitochondrial membranes from euthyroid and hypothyroid rats fed diets containing either 1 or 5% (w/w) *n*-3 fatty acids

Fatty acid (w/w%)	Euthyroid			Hypothyroid				
	1% *n*-3 Diet	5% *n*-3 Diet	Diet effect	1% *n*-3 Diet	5% *n*-3 Diet	Diet effect	1% *n*-3/ Hypo effect	5% *n*-3/ Hypo effect
Σ*n*-6	44.1 ± 0.9	33.0 ± 0.6	**	39.7 ± 0.6	38.0 ± 0.5	NS	*	*
Σ*n*-3	0.9 ± 0.1	4.4 ± 0.3	**	1.3 ± 0.2	2.0 ± 0.1	*	*	**
n-6/*n*-3	49.0 ± 1.2	7.5 ± 0.7	**	30.5 ± 0.9	6.1 ± 0.2	*	*	*
ΣSAT	51.2 ± 0.6	53.8 ± 0.7	NS	57.4 ± 0.3	52.1 ± 0.8	NS	*	NS
ΣMONO	1.1 ± 0.1	2.8 ± 0.7	*	1.6 ± 0.3	2.6 ± 0.3	**	*	**
20:4/18:2	20.6 ± 1.2	11.3 ± 1.0	**	5.6 ± 0.4	8.9 ± 0.4	**	**	**
20:4/20:3	104.0 ± 1.8	12.4 ± 0.5	**	26.4 ± 0.9	8.9 ± 0.6	**	*	*

Values are means of mol% ± S.E.M. for at least six animals. Σ*n*-6, sum of *n*-6 fatty acyl species; Σ*n*-3, sum of *n*-3 fatty acyl species; ΣSAT, sum of all saturated fatty acyl species; ΣMONO, sum of all monounsaturated fatty acyl species. Δ^6-Desaturase index is determined by the ratio 18:2*n*-6/20:4*n*-6 and is a measure of the enzymatic conversion of 18:2 to 20:4. Δ^5-Desaturase index is determined by the ratio 20:4*n*-6/20:3*n*-6 and is a measure of the enzymatic conversion of 20:4 to 20:3.
* $P < 0.05$.
** $P < 0.01$.

Table 4
Summary of cardiolipin fatty acid composition of inner mitochondrial membranes from euthyroid and hypothyroid rats fed diets containing either 1 or 5% (w/w) *n*-3 fatty acids

Fatty acid (w/w%)	Euthyroid			Hypothyroid				
	1% *n*-3 Diet	5% *n*-3 Diet	Diet effect	1% *n*-3 Diet	5% *n*-3 Diet	Diet effect	1% *n*-3/ Hypo effect	5% *n*-3/ Hypo effect
Σ*n*-6	66.2 ± 1.4	60.0 ± 2.2	*	69.5 ± 1.4	73.7 ± 2.0	NS	NS	*
Σ*n*-3	1.6 ± 0.1	3.5 ± 0.2	*	2.4 ± 0.2	2.1 ± 0.1	NS	*	*
n-6/*n*-3	41.4 ± 1.4	13.3 ± 2.2	**	28.9 ± 2.0	18.0 ± 1.8	**	**	*
ΣSAT	19.9 ± 2.1	25.2 ± 1.3	**	9.8 ± 0.7	13.6 ± 0.6	**	**	**
ΣMONO	15.2 ± 0.3	9.8 ± 1.0	**	13.7 ± 0.6	7.5 ± 0.6	**	NS	*
20:4/18:2	0.02 ± 0.1	0.05 ± 0.1	NS	0.01 ± 0.1	0.01 ± 0.1	NS	NS	*
20:4/20:3	1.0 ± 0.1	2.6 ± 0.2	**	0.67 ± 0.1	0.64 ± 0.1	NS	NS	**

Values are means of mol% ± S.E.M. for at least six animals. Σ*n*-6, sum of *n*-6 fatty acyl species; Σ*n*-3, sum of *n*-3 fatty acyl species; ΣSAT, sum of all saturated fatty acyl species; ΣMONO, sum of all monounsaturated fatty acyl species. Δ^6-Desaturase index is determined by the ratio 18:2*n*-6/20:4*n*-6 and is a measure of the enzymatic conversion of 18:2 to 20:4. Δ^5-Desaturase index is determined by the ratio 20:4*n*-6/20:3*n*-6 and is a measure of the enzymatic conversion of 20:4 to 20:3.
* $P < 0.05$.
** $P < 0.01$.

1). Total *n*-6 content was not different between the two groups; however, a decrease in total *n*-3 content resulted in an increase in the *n*-6/*n*-3 ratio. The effect of diet exacerbated the influence of the hypothyroid state in that 20:4*n*-6 content was reduced by 52% in membrane PC from hypothyroid rats fed the high *n*-3 diet compared to the low *n*-3 diet. Conversely, levels of the major *n*-3 fatty acids were higher in hypothyroid animals fed the high *n*-3 diet with the result the *n*-6/*n*-3 ratio was several-fold lower (Table 1).

The effect of the hypothyroid state on PE fatty acid composition was to increase 18:2*n*-6 content with no change in 20:4*n*-6. As a result the Δ^6-desaturase index was lower as was the Δ^5-desaturase index (Table 2). Similar to the situation in PC, there was a small, but significant, increase in the *n*-6/*n*-3 ratio. Feeding the high *n*-3 diet to hypothyroid rats reduced the Δ^6- and Δ^5-desaturase indices even further and, in contrast to the control diet, induced a 7-fold decrease in the *n*-6/*n*-3 ratio (Table 2).

In the PI fraction, there was a 185% increase in 18:2*n*-6 in hypothyroid animals fed the control diet and a corresponding 23.8% decrease in 20:4*n*-6. 20:3*n*-6 increased three-fold. As a result, the Δ^6- and Δ^5-desaturase indices were substantially lower (Table 3). The hypothyroid state lowered the total PI content of *n*-6 fatty acids thereby lowering the *n*-6/*n*-3 ratio compared to euthyroid controls. Feeding the high *n*-3 diet did not affect the *n*-6 content of PI but significantly increased levels of *n*-3 fatty acids with the result the *n*-6/*n*-3 ratio decreased even more.

In membrane cardiolipin, the characteristically high 18:2*n*-6 content was not affected by the hypothyroid state nor was the 20:4*n*-6 or 20:3*n*-6 content (Table 4). As a result, the Δ^6- and Δ^5-desaturase indices were not significantly different. Increased levels of 20:5*n*-3(57%) and 22:6*n*-3(43%), however, contributed to a decrease in the *n*-6/*n*-3 ratio in the hypothyroid state. The other significant change in fatty acid composition occurred in the amount of total saturated species that declined by 50% in the hypothyroid state.

4. Discussion

The major findings of this study are that the fatty acid composition of the inner mitochondrial membrane is a determinant of proton leak kinetics, and that the effect of the hypothyroid state on proton leak is mediated in part by alterations in membrane fatty acid composition. The most significant influence on proton leak appears to be inner membrane phospholipid content of *n*-3 fatty acids relative to *n*-6 species. Chronic hypothyroidism induces a decrease

in phospholipid *n*-6/*n*-3 ratio that is correlated with reduced proton leak. In euthyroid rats, enhancing total membrane levels of *n*-3 fatty acids by dietary incorporation of 20:5*n*-3, 22:5*n*-3 and 22:6*n*-3 also lowers the *n*-6/*n*-3 ratio with a concomitant decrease in proton leak. Furthermore, the depressed proton leak rate characteristic of mitochondria from hypothyroid rats is lowered further when these animals are fed a diet high in *n*-3 fatty acids. Of particular interest is the observation that the fatty acid compositions of individual phospholipid classes are affected differently either by the hypothyroid state or by diet. This suggests the possibility of a disproportionate influence of phospholipid classes on proton leak pathways.

Two distinct mechanisms appear to be functioning with respect to the effects of the hypothyroid state and *n*-3 fatty acids on membrane fatty acid composition. The first is an apparent suppression of Δ^6- and Δ^5-desaturase activity in the desaturation/elongation pathways for long-chain *n*-6 and *n*-3 fatty acids. This results in an accumulation of 18:2 as synthesis of longer chain *n*-6 species is blocked, and an overall reduction in total *n*-6 content. While depressed Δ^6- and Δ^5-desaturase activity has previously been reported in the hypothyroid state [10] and in diabetes [19–21], our study is the first to report that this effect can be exacerbated by dietary *n*-3 fatty acids, particularly in the PE and PI fractions. Previous studies have shown that dietary 18:3*n*-3 inhibits the enzymes in the pathway for desaturation and elongation of *n*-6 fatty acids [20]; however, our diets contained equivalent amounts of 18:3. The *n*-3 fatty acids in our high *n*-3 diet were 20:5*n*-3 and 22:6*n*-3 and would not have required the actions of the Δ^6-desaturase pathway. Rather, they would have been directly incorporated into the various phospholipids and, coupled with an inhibition of the Δ^6- and Δ^5-desaturase enzymes, would account for the dramatic decreases in *n*-6/*n*-3 ratios.

Our results showing increased membrane levels of *n*-3 fatty acids are correlated with decreased proton leak are opposite to what has been predicted by others. Comparing proton leak rates in reptiles and mammals, Brand et al. [1] concluded that the four-fold higher proton permeability seen in the rat coincides with higher mitochondrial content of *n*-3 fatty acids. When the total phospholipid fatty acid content of rat and lizard mitochondria are compared, however, trends similar to our observed effects of the hypothyroid state and dietary *n*-3 fatty acids are seen. Of particular note are higher levels of 18:2 and lower levels of 20:4 seen in lizard phospholipids with a concomitantly lower apparent Δ^6-desaturase activity. The same trends, to greater or lesser degree, are seen in all of the phospholipid classes in the present study and all are associated with reduced proton leak.

When rats are fed a polyunsaturated fatty acid-deficient diet non-ohmic mitochondrial proton leak increases in conjunction with alterations in fatty acid composition [22]. Under these conditions, both total phospholipid *n*-6 and *n*-3 fatty acid contents decrease in response to diet, although the ratio of *n*-6/*n*-3 fatty acids increases two-fold compared to controls. This is in contrast to the up to several-fold decrease in the *n*-6/*n*-3 ratio seen in all phospholipid classes in the present study induced by the hypothyroid state and by dietary *n*-3 fatty acids. This suggests that the ratio of these two groups of polyunsaturated fatty acids may be more important than the absolute amounts of either. It also supports the notion that there is an inverse relationship between proton leak and membrane *n*-6/*n*-3 ratios. While proton leak induced by a polyunsaturated fatty acid-deficient diet is associated with an increase in mitochondrial volume [22], increased leak under hyperthyroid conditions appears to be correlated with an increase in the inner membrane surface area [9]. In the present study, as in a previous report [23], inner membrane cardiolipin content was lower in the hypothyroid state indicating a reduced inner membrane surface area, even in the absence of any change in mitochondrial volume. Nevertheless, Brand et al. [9] have concluded that a thyroid hormone-induced increase in surface area/volume ratio only partially accounts for an increase in proton leak, the remainder presumably being the result of alterations in permeability properties.

Several attempts have been made to confirm the role of membrane phospholipids in mitochondrial proton leak using liposomes made from purified or native lipids. While proton permeability in liposomes formed from mitochondrial phospholipids appears to be correlated with long-chain *n*-3 fatty acid content [11], Brooke and coworkers estimate that proton flux across the phospholipid bilayer represents only about

5% of the total proton permeability of intact mitochondria [24]. Perhaps more importantly, they subsequently showed that there is no correlation between liposome proton permeability and the bilayer phospholipid fatty acid composition [25]. It may be more appropriate, therefore, to consider that the protein–lipid interfaces that exist in intact mitochondria are the regions of highest proton permeability [16] and that this is where the effects of fatty acid changes occur. Any protein–lipid interactions would be modulated by the inherent asymmetry of membrane phospholipids and by the distribution of fatty acid moieties. For this reason, our observation that individual membrane phospholipids are affected differently by thyroid state and by dietary manipulation may be of considerable importance. For example, when hypothyroid rats fed the high *n*-3 diet are compared with euthyroid animals fed the control diet there is a 20% decline in *n*-6 fatty acids in membrane PC and a more than 4-fold increase in total *n*-3 content. This is in contrast to cardiolipin, where *n*-6 levels increased and there was only a 31% increase in *n*-3 fatty acids. When we consider that PC makes up nearly 50% of the phospholipid content of rat liver mitochondria, compared to only 13% for cardiolipin [26], it is apparent that changes to PC would potentially have a greater impact on proton permeability. On the other hand, membrane cardiolipin content and its fatty acid composition influences a number of mitochondrial functions. These include effects on cytochrome oxidase activity [27,28] and phosphate [26] and pyruvate transport [23]. These functions are all altered in the hypothyroid rat and their function is correlated with changes in the relative abundance of cardiolipin in the mitochondrion and its fatty acid composition [26,23]. The fact that these cardiolipin-dependent functions all involve effects on membrane proteins that transport protons or other ions suggests that cardiolipin, and possibly the other phospholipids, modulate the activity of these proteins. Taken together, these findings suggest that inner membrane phospholipids may be involved in the formation of proton pores or channels and that the 'leakiness' of these proton pathways is determined largely by the fatty acyl chains of the phospholipids. How *n*-3 fatty acids reduce proton permeability is highly speculative at this time; however, there is evidence that proton leak in liposomes decreases as chain length of substituted fatty acids increases [29]. Our results show a clear shift toward long-chain *n*-3 fatty acid incorporation into membrane phospholipids at the expense of shorter chain *n*-6 fatty acids (i.e. 18:2 and 20:4). The possibility that the physical properties imparted by the location of the double bond in *n*-3 fatty acids modulates proton permeability remains to be determined.

Another explanation for our observed effects of fatty acid composition on proton leak is through interactions with a recently described mitochondrial uncoupling protein (UCP2) [30], or with other inner membrane proteins known to have sequence homology with the brown adipose tissue mitochondrial uncoupling protein, UCP1 [31]. It is not clear how the structure of the lipid boundary could modulate these, or any other protonophoretic proteins; however, the activity of the proton conductance pathway of BAT mitochondrial UCP1 in obese mice has been shown to increase after enhancing inner membrane *n*-6 fatty acid content [32].

The results of this study are consistent with both the hypothyroid state and dietary *n*-3 fatty acids reducing proton leak through alterations in inner mitochondrial membrane *n*-3 fatty acid content. More importantly, they suggest this is a mechanism by which thyroid hormone and diet determine the overall efficiency of mitochondrial energy production. The overall implications are that resting metabolic rate can be determined as much by diet as by thyroid hormone state. The precise nature of the role *n*-3 fatty acids play in proton leak is not clear at present, but most probably occurs at the lipid–protein interface.

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