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Influence of hyper- and hypothyroidism on lipid peroxidation, unsaturation of phospholipids, glutathione system and oxidative damage to nuclear and mitochondrial DNA in mice skeletal muscle

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Abstract

While the biochemical literature on free radical metabolism is extensive, there is little information on the endocrine control of tissue oxidative stress, and in the case of thyroid hormones it is mainly limited to liver tissue and to short-term effects on a few selected biochemical parameters. In this investigation, chronic hypothyroidism and hyperthyroidism were successfully induced in mice, and various oxidative-stress-related parameters were studied in skeletal muscle. *In vivo* and *in vitro* lipid peroxidation significantly increased in hyperthyroidism and did not change in the hypothyroid state. The fatty acid composition of the major phospholipid classes was affected by thyroid hormones, leading to a significant decrease in total fatty acid unsaturation both in hypothyroid and hyperthyroid muscle in phosphatidylcholine and phosphatidylethanolamine fractions. In cardiolipin, however, the double bond content significantly increased as a function of thyroid status, leading to a 2.7 fold increase in the peroxidizability index from euthyroid to hyperthyroid muscle. Cardiolipin content was also directly and significantly related to thyroid state across the three groups. Glutathione system was not modified by thyroid state. The oxidative damage marker 8-oxo-7,8-dihydro-2′-deoxyguanosine did not change in mitochondrial DNA, and decreased in genomic DNA both in hypothyroid and hyperthyroid muscle. The results indicate that chronic alterations in thyroid status specially affect oxidative damage to lipids in skeletal muscle, with a probably stronger effect on mitochondrial membranes, whereas the cytosolic redox potential and DNA are better protected possibly due to homeostatic compensatory reactions on the long-term. (Mol Cell Biochem **221**: 41–48, 2001)

Key words: thyroid hormones, free radicals, polyunsaturated fatty acids, 8-oxo-7,8 dihydro-2′-deoxyguanosine, oxidative stress

Abbreviations: 8-oxodG – 8-oxo-7,8-dihydro-2′-deoxyguanosine; mtDNA – mitochondrial DNA; DBI – double bond index; PI – peroxidizability index; GSH – reduced glutathione; GSSG – oxidized glutathione

Introduction

The endogenous generation of oxygen radicals is known to be involved in the development of many diseases. A main source of oxygen radicals in the cell is mitochondrial respiration, which can be manipulated by thyroid hormones. Since mitochondrial oxygen consumption is among the factors which, theoretically at least, could modify the rate of mitochondrial oxygen radical generation, we hypothesized that experimental manipulation of thyroid hormones could be able

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to change the level of oxidative stress in the muscle, an aerobic tissue. Surprisingly, however, while the biochemical literature on free radical metabolism is extensive, there is little information on the endocrine control of tissue oxidative stress.

Concerning markers of oxidative damage, the small number of previous studies about the effects of thyroid hormones show various limitations. First, the majority of those studies concern liver [1–5] with very scarce information available for skeletal muscle. Second, the majority of the investigations only studied thyroid hormone short-term effects not allowing to ascertain if the tissues have capacity for homeostatic adaptation to thyroid-induced oxidative stress on the long-term. In addition, the majority of the studies measured lipid peroxidation or glutathione [1–5] and this second parameter was studied in liver, not in the muscle. There are also contradictions concerning changes in lipid peroxidation in hypothyroid muscle [6, 7]. On the other hand, endogenous oxygen radical production occurs mainly in cellular membranes and unsaturated fatty acids are the most sensitive macromolecules to lipid peroxidation. But the effects of thyroid hormones on the fatty acid composition and double bond content of the different membrane fractions have been studied again in liver [5, 8, 9] but not in skeletal muscle. Finally, while other oxidatively damaged cellular macromolecules can be more easily substituted by turnover using the information coded in DNA, damage to DNA itself could have more serious long-term consequences specially in postmitotic tissues like muscle. However, although there are reports on increased lipid (see above) and protein [10, 11] oxidative damage after thyroid hormone administration, the possible effect of thyroid hormones on oxidative damage to nuclear and mitochondrial DNA has never been studied.

In order to overcome some of those limitations, in this investigation mice were chronically rendered hypothyroid or hyperthyroid, and *in vivo* and *in vitro* lipid peroxidation, fatty acid composition and the unsaturation degree of the major phospholipid classes, reduced and oxidized glutathione, and the oxidative damage marker 8-oxo-7,8-dihydro-2′-deoxyguanosine in both nuclear and mitochondrial DNA were simultaneously studied in skeletal muscle. Cytochrome oxidase was also measured as a marker of maximum mitochondrial aerobic capacity.

Materials and methods

Animals, treatments and thyroid status

OF1 female mice of 12 weeks of age were obtained from Iffa-Creddo (Lyon, France). They were maintained at 22 ± 2°C, 12:12 (light:dark) cycle, 50 ± 10% relative humidity and fed *ad libitum*. Mice were rendered hypothyroid by administration of 0.05% 6-n-propyl-2-thiouracil in their drinking water for 5 weeks. Hyperthyroidism was induced through treatment with 12 mg of T_4 per liter of drinking water for 5 weeks also. Rectal temperature was monitored throughout the experimental period with a Cole Parmer thermocouple (Chicago, IL, USA). After treatment, the animals were sacrificed by decapitation and a blood sample was obtained. Hindlimb skeletal muscle and sera were stored at –80°C. The serum T_4 concentration was measured by radioimmunoassay (RIA kit, CIS Bio International, Gif-Sur-Yvette, France). Cytochrome c oxidase activity was measured in muscle supernatants following the rate of oxidation of cytochrome c at 550 nm [12].

Lipid analyses

Skeletal muscle lipids were extracted into chloroform: methanol (2:1 v/v) according to the method of Folch *et al*. [13] in the presence of 0.01% butylated hydroxytoluene. The chloroform phase was separated and stored at –80°C until analysis. In preliminary experiments and in order to guaranty total lipid recovery, this procedure was optimised until the absence of a detectable amount of lipids by thin layer chromatography was achieved. The phospholipid classes were separated by preparative thin-layer chromatography on silica gel plates. Development with n-hexane:1,2 dichloroethane:methanol:formic acid (16:14:4:1 v/v) followed by 1,2-dichloroethane solvent system was used. Fractions were made visible by spraying with 0.02% 8-aniline-1-naphthalenesulfonic acid in ethanol. The bands of the plates corresponding to major phospholipids were scrapped, transferred to screw capped tubes and redissolved with transesterification mixture. Lipids were transesterified with 5% methanolic HCl at 75°C for 90 min. The fatty acid methyl esters (FAME) were extracted in n-pentane in the presence of saturated NaCl. After drying under N_2, the FAME were redissolved in CS_2 for gas chromatography and mass spectrometry (GC/MS) analysis. GC separation was performed in a SP2330 capillary column (30m × 0.25 mm × 0.20 mm) in a Hewlett Packard 5890 Series II gas chromatograph, by using helium as carrier gas at constant flow. For detection a Hewlett Packard 5989 A mass spectrometer was used in electron-impact ionization mode. GC/MS conditions were as follows: injector and detector port temperature 220 and 250°C, respectively; column temperature ranging from 100–200°C with an increase of 10°C/min, from 200–240°C at 5°C/min, and a final hold of 12 min, sing a splitless injection mode (inlet P45 kPa and linear velocity 28.5 cm/sec) and an injection volume of 1 µl. Identification of methyl esters was made by retention time and mass spectra comparison with authentic standards (Sigma). Only fatty acids contributing more than 0.25% of total fatty acids are shown in the tables. For % quantification of major phospholipid classes, lipid samples were separated in Silica gel 60A LK6D plates for thin layer chromatography (Whatman, Cli-

fton, NJ, USA), using the solvent system described above. Separated lipid fractions were detected using a 10% cupric sulphate in 8% phosphoric acid solution, followed by charring at 160°C for 20 min, and were quantified by scanning densitometry using a Shimadzu CS-9001PC dual-wavelength flying spot scanner (Shimadzu Europe GmbH, Duisburg, Germany).

Lipid peroxidation

Endogenous *in vivo* skeletal muscle lipid peroxidation was measured by a thiobarbituric acid test specially adapted to tissue samples [14] in the presence of 0.07 mM butylhydroxytoluene, added as an antioxidant to avoid artefactual lipid peroxidation during the assay. Muscle sensitivity to *in vitro* lipid peroxidation was estimated by incubating muscle homogenates with 0.4 mM ascorbate and 0.05 mM $FeSO_4$ for 6 h at 37°C before performing the lipid peroxidation assay. This incubation time ensures that the lipid peroxidation process has reached saturation in this particular sample. Malondialdehyde-bis(dimethylacetal) (Merck, Germany) was used as standard and *in vivo* and *in vitro* lipid peroxidation values were expressed as nanomoles of malondialdehyde per g of tissue.

Glutathione system

Skeletal tissue was homogenized in cold 5% trichloroacetic acid with 0.01 N HCl, and total glutathione was measured by a spectrophotometric recycling assay [15] in the presence of 5-5′-dithiobis (2-nitrobenzoic acid), NADPH and glutathione reductase at 412 nm. Oxidized glutathione (GSSG) was assayed by the same method after derivatization of reduced glutathione (GSH) with 12.5 mM N-ethylmaleimide (NEM) followed by alkaline hydrolysis of NEM [16] with direct control of pH with a micropHmeter and presence of NEM in the homogenization buffer to avoid GSH artefactual oxidation. GSH values were obtained by subtracting GSSG from total glutathione.

Oxidative DNA damage

Genomic DNA was isolated, after SDS treatment of skeletal muscle samples, by chloroform extraction and ethanol precipitation [17]. Mitochondrial DNA (mtDNA) was isolated by the method of Latorre *et al*. [18] with some modifications [19]. The mtDNA preparations were free of nuclear DNA as tested by agarose gel electrophoresis and staining with ethidium bromide. Isolated genomic and mitochondrial DNA were digested to deoxynucleoside level by incubation at 37°C with 5 U of nuclease P1 (in 20 µl of 20 mM sodium acetate, 10 mM $ZnCl_2$, 15% glycerol, pH 4.8) during 30 min and 1 U of alkaline phosphatase (in 20 µl of 1 M Tris-HCl, pH 8.0) for 1 h [17]. The concentrations of 8-oxodG and deoxyguanosine (dG) were measured by HPLC with on line electrochemical and ultraviolet detection respectively. For analyses, the nucleoside mixture was injected into a reverse-phase Beckman Ultrasphere ODS column (5 µm, 4.6 mm × 25 cm), eluted with 2.5% acetonitrile in 50 mM phosphate buffer pH 5. The amount of deoxynucleosides injected in the HPLC was higher than the minimum needed to avoid potential artifacts due to injection of small quantities of deoxynucleosides in the HPLC system [20]. A Waters 510 pump at 1 ml/min was used. 8-oxodG was detected with an ESA Coulochem II electrochemical coulometric detector (ESA Inc., Bedford, MA, USA) with a 5011 analytical cell run in the oxidative mode (225 mV/10 nA), and dG was detected with a Biorad model 1806 UV detector at 254 nm. For quantification peak areas of dG standards and of three level calibration of pure 8-oxodG standards (Sigma) were analyzed during each HPLC run.

Statistical analyses

Comparisons between hypothyroid and euthyroid and between euthyroid and hyperthyroid groups were statistically analyzed with Student's *t*-tests. The 0.05 level was selected as the point of minimal statistical significance in all the analyses.

Results

In order to ascertain the effectiveness of the experimental treatments, three parameters were used. First, rectal temperature was measured weekly during the 5 weeks of treatment. Rectal temperature significantly increased during the experimental time in the hyperthyroid group, decreased in the hypothyroid group, and did not show changes in euthyroid animals (results not shown). On the fifth week rectal temperature was significantly higher in the hyperthyroid (39.12 ± 0.11°C) than in the euthyroid (37.48 ± 0.06°C) group ($p < 0.001$) and was significantly lower in hypothyroid (36.65 ± 0.05°C) than in euthyroid animals ($p < 0.001$). Hyperthyroidism significantly increased ($p < 0.001$) serum T_4 from 58.9 ± 3.5 ng/ml in the euthyroid to 402.8 ± 6.0 ng/ml in the hyperthyroid group, whereas T_4 levels were significantly lower ($p < 0.001$) in hypothyroid (19.5 ± 1.1 ng/ml) than in euthyroid animals. The treatment was also effective at tissue level, since the cytochrome oxidase activity, a marker of mitochondrial aerobic capacity, was significantly increased in

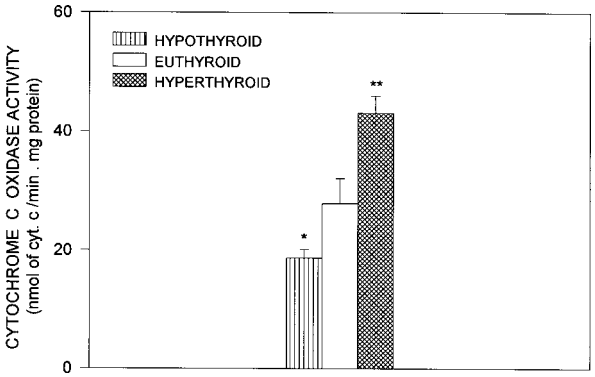

Fig. 1. Cytochrome c oxidase activity of mouse skeletal muscle. Values are means ± S.E.M. from 6–8 animals per group. Asterisks describe significant differences in relation to the euthyroid group: *p < 0.05; **p < 0.01.

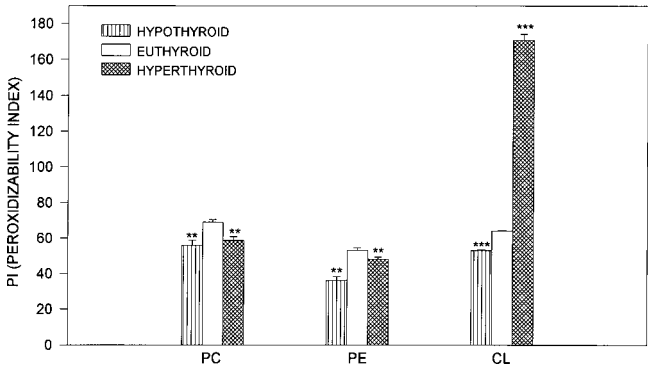

Fig. 2. Peroxidizability Index (PI) of fatty acids in the phosphatidylcholine (PC), phosphatidylethanolamine (PE), and cardiolipin (CL) phospholipid fractions of mouse skeletal muscle. PI = [(%Monoenoic × 0.025) + (%Dienoic × 1) + (%Trienoic × 2) + (%Tetraenoic × 4) + (%Pentaenoic × 6) + (%Hexaenoic × 8)]. Values are means ± S.E.M. from 6 animals per group. Asterisks describe significant differences in relation to the euthyroid group: **p < 0.01; ***p < 0.001.

hyperthyroidism and significantly decreased by the hypothyroid treatment (Fig. 1).

The fatty acid profiles of the three main phospholipid membrane fractions were analyzed. In the phosphatidylcholine fraction 16:0 was significantly increased and 18:2n-6 and 20:4n-6 were significantly decreased both by hyperthyroidism and hypothyroidism (Table 1). As a result of this, the total number of fatty acid double bonds (double bond index, DBI) was decreased (by 16%) at both extremes of thyroid status (Table 1). The peroxidizability index (PI), which takes into account that sensitivity of fatty acids to peroxidation exponentially increases as a function of the number of double bonds per fatty acid molecule, was also decreased both in hypothyroid (by 18%) and in hyperthyroid (by 10%) animals (Fig. 2).

In the phosphatidylethanolamine fraction (Table 2), hypothyroidism increased 18:0 and decreased 18:1n-9, 18:2n-6 and 20:4n-6, whereas hyperthyroidism increased 16:0 and 18:0 and decreased 18:1n-9, and 18:2n-6. In consequence, similarly to what happened in the phosphatidylcholine fraction, the DBI (Table 2) and the PI (Fig. 2) were moderately decreased both by hypothyroidism (by 34 and 32%, respectively) and by hyperthyroidism (by 22 and 9%, respectively).

The phospholipid fraction in which double bonds were most profoundly affected (in the case of hyperthyroidism) was cardiolipin (Table 3). In hypothyroid animals, 16:0, 16:1n-7 and 18:2n-6 increased and 18:3n-3 and 18:4n-3 decreased. The result of this was, like in the other two membrane fractions, a moderate decrease in DBI (by 7%; Table 3) and PI (by 17%; Fig. 2). The effect of hyperthyroidism on cardiolipin, however, was stronger and of opposite sense in relation to those observed in the two other phospholipid fractions. In this case hyperthyroidism decreased 16:0, 16:1n-7, 18:1n-9, 18:2n-6, 18:3n-3, and 18:4n-3, and increased 18:0, 20:4n-6 and 22:6n-3. The strong increase in fatty acids containing a high number of double bonds (20:4n-6 and specially 22:6n-3), which were not detected in euthyroid or hypothy-

Table 1. Fatty acid composition (mol%) of mouse skeletal muscle phosphatidylcholine

	Hypothyroid		Euthyroid		Hyperthyroid
14:0	0.60 ± 0.04		0.72 ± 0.11		0.65 ± 0.05
16:0	47.24 ± 0.65	***	40.66 ± 0.33	***	47.49 ± 0.40
16:1n-7	10.53 ± 0.65		9.16 ± 0.56		7.52 ± 0.77
18:0	8.03 ± 0.70		8.93 ± 0.77		9.90 ± 0.38
18:1n-9	11.45 ± 0.33		12.96 ± 0.93		12.01 ± 0.41
18:2n-6	10.67 ± 0.78	*	13.51 ± 0.65	***	10.06 ± 0.38
20:3n-6	0.58 ± 0.04		0.63 ± 0.02		0.62 ± 0.04
20:4n-6	10.91 ± 0.80	*	13.43 ± 0.49	*	11.74 ± 0.59
DBI	88.68 ± 1.85	***	104.73 ± 1.16	***	88.47 ± 1.48

Values are means ± S.E.M. from 6 animals per group. DBI (Double bond index) = (∑mol% of unsaturated fatty acids × number of double bonds of each unsaturated fatty acid). Asterisks describe significant differences between hypothyroid and euthyroid or between euthyroid and hyperthyroid groups. *p < 0.05; ***p < 0.001.

Table 2. Fatty acid composition (mol%) of mouse skeletal muscle phosphatidylethanolamine

	Hypothyroid		Euthyroid		Hyperthyroid
16:0	14.78 ± 1.11		12.35 ± 0.55	***	16.04 ± 0.47
18:0	56.93 ± 0.63	***	44.07 ± 1.45	*	53.98 ± 3.05
18:1n-9	15.07 ± 0.60	***	20.45 ± 0.78	***	14.69 ± 0.95
18:2n-6	5.72 ± 0.38	***	13.30 ± 1.13	***	4.46 ± 0.41
20:4n-6	7.49 ± 0.55	*	9.82 ± 0.75		10.83 ± 0.52
DBI	56.48 ± 2.96	***	86.34 ± 4.84	*	66.93 ± 6.62

Values are means ± S.E.M. from 6 animals per group. DBI (Double bond index) = (∑mol% of unsaturated fatty acids × number of double bonds of each unsaturated fatty acid). Asterisks describe significant differences between hypothyroid and euthyroid or between euthyroid and hyperthyroid groups. *p < 0.05; ***p < 0.001.

Table 3. Fatty acid composition (mol%) of mouse skeletal muscle cardiolipid

	Hypothyroid		Euthyroid		Hyperthyroid
16:0	22.43 ± 0.54	*	21.08 ± 0.05	***	8.75 ± 0.78
16:1n-7	7.57 ± 0.05	***	6.42 ± 0.05	***	1.43 ± 0.06
18:0	8.28 ± 0.43		8.55 ± 0.08	***	41.79 ± 0.57
18:1n-9	21.55 ± 1.20		23.83 ± 0.02	***	17.07 ± 0.25
18:2n-6	34.53 ± 0.55	***	29.40 ± 0.02	***	7.40 ± 0.07
18:3n-3	2.35 ± 0.07	***	4.44 ± 0.12	***	0.98 ± 0.15
18:4n-3	3.29 ± 0.20	***	6.29 ± 0.11	***	0.00 ± 0.00
20:4n-6	0.00 ± 0.00		0.00 ± 0.00	***	4.91 ± 0.14
22:6n-3	0.00 ± 0.00		0.00 ± 0.00	***	17.67 ± 0.44
DBI	118.39 ± 1.18	***	127.49 ± 0.07	***	161.91 ± 2.35

Values are means ± S.E.M. from 6 animals per group. DBI (Double bond index) = ∑mol% of unsaturated fatty acids × number of double bonds of each unsaturated fatty acid). Asterisks describe significant differences between hypothyroid and euthyroid or between euthyroid and hyperthyroid groups. *p < 0.05; *** p < 0.001.

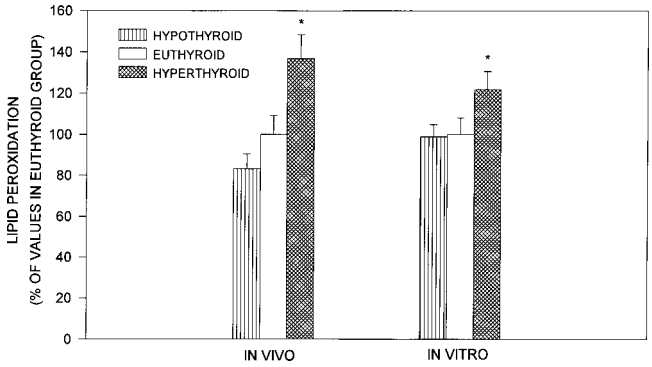

Fig. 3. *In vivo* and *in vitro* lipid peroxidation in mouse skeletal muscle. Values are means ± S.E.M. and are expressed as % of the mean in the euthyroid group. Absolute values in euthyroid animals (nanomoles of malondialdehyde per g of tissue): 68.1 ± 6.3 for *in vivo* and 901 ± 72 for *in vitro* lipid peroxidation. Asterisks describe significant differences in relation to the euthyroid group: *p < 0.05.

roid animals, was responsible for the increase in DBI (by 27%) and for the strong increase in PI (to 266% of the values present in euthyroidism; Fig. 2) in the hyperthyroid group.

The thyroid status also affected phospholipid distribution into the different fractions (Table 4). Hypothyroidism increased sphingomyelin and lysophosphatidylcholine and decreased phosphatidylcholine, cardiolipin and phospholipid/cholesterol ratio, whereas in hyperthyroid animals sphingomyelin, lysophosphatidylcholine and cardiolipin increased and phosphatidylcholine and phosphatidylethanolamine decreased in relation to euthyroid animals. Cardiolipin was the only phospholipid which varied in the same sense from the hypothyroid to the euthyroid and from the euthyroid to the hyperthyroid group: the higher the thyroid status, the higher were the cardiolipin levels.

In vivo lipid peroxidation was significantly increased by hyperthyroidism and was not modified by hypothyroidism (Fig. 3). *In vitro* incubation with ascorbate-iron successfully increased lipid peroxidation values by 12–15 fold in the three groups. Similarly to what happened for *in vivo* values, *in vitro* lipid peroxidation was higher in the hyperthyroid than in the euthyroid group and was not changed by hypothyroidism (Fig. 3).

Neither reduced or oxidized glutathione or GSSG/GSH ratio changed as a function of the thyroid status (Table 5). Although higher absolute levels of GSSG and GSSG/GSH ratio were observed in the hyperthyroid group, these changes did not reach statistical significance.

8-oxodG in genomic DNA was decreased both by hypothyroidism and hyperthyroidism (Table 6). In the case of mtDNA, 8-oxodG levels did not show significant changes as a function of the thyroid status.

Table 4. Phospholipid distribution (mol%) and phospholipid/cholesterol ratio in mouse skeletal muscle

	Hypothyroid		Euthyroid		Hyperthyroid
SM	10.00 ± 0.28	**	6.85 ± 0.02	***	7.74 ± 0.09
LPC	6.66 ± 0.18	**	4.56 ± 0.04	***	5.15 ± 0.06
PC	33.30 ± 0.35	**	35.90 ± 0.29	**	33.97 ± 0.24
PE	39.12 ± 0.69		40.20 ± 0.13	*	39.38 ± 0.17
CL	10.88 ± 0.12	***	12.46 ± 0.04	**	13.72 ± 0.26
P/C	2.80 ± 0.11	**	3.39 ± 0.03		3.11 ± 0.11

Values are means ± S.E.M. from 6 animals per group. SM – Sphingomyelin; LPC – Lysophosphatidylcholine; PC – Phosphatidylcholine; PE – Phosphatidylethanolamine; CL – Cardiolipin; P/C – Phospholipid/cholesterol ratio. Asterisks describe significant differences between hypotheyroid and euthyroid or between euthyroid and hyperthyroid groups. *p < 0.05; **p < 0.01; ***p < 0.001.

Table 5. Reduced (GSH), oxidized (GSSG) and GSSG/GSH ratio in mouse skeletal muscle

	Hypothyroid	Euthyroid	Hyperthyroid
GSH	908 ± 63	916 ± 74	915 ± 52
GSSG	25 ± 3	24 ± 2	28 ± 2
GSSG/GSH	0.027 ± 0.003	0.028 ± 0.004	0.033 ± 0.004

Values are means ± S.E.M. for 8–9 animals per group and are expressed in nanomoles per g of tissue. No significant differences were observed between groups.

Table 6. 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) levels in genomic (gDNA) and mitochondrial (mtDNA) DNA of mouse skeletal muscle

	Hypothyroid		Euthyroid		Hyperthyroid
gDNA	0.72 ± 0.10	*	1.03 ± 0.11	*	0.79 ± 0.06
mtDNA	1.96 ± 0.43		2.25 ± 0.25		2.24 ± 0.36

Values are means ± S.E.M. from 6–10 animals per group and are expressed as 8-oxodG/10^5dG. Asterisks describe significant differences between hypothyroid and euthyroid or between euthyroid and hyperthyroid groups. *p < 0.05.

Discussion

In this investigation, chronic hyperthyroid and hypothyroid states were successfully obtained as deduced from serum levels of T_4, rectal temperatures, and muscle cytochrome oxidase activities, in agreement with previous reports in muscle tissues [21–24]. Thus, oxidative metabolism progressively increases across the three groups from the hypothyroid to the hyperthyroid animals. This is also true for the content of cardiolipin, a mitochondrial phospholipid, which is consistent with an increase in mitochondrial mass of the muscle as a function of thyroid state and agrees with known effects of thyroid hormones on cardiolipin biosynthesis [25].

Our results showing that hyperthyroidism increases lipid peroxidation in mouse skeletal muscle are in agreement with previous findings of increased conjugated dienes and malondialdehyde in cat muscle [26], increased lipid peroxidation in rat muscle [6, 21, 27], and increases in malondialdehyde and hydroperoxides in rat muscle [7] during hyperthyroidism. In the case of hypothyroidism, however, contradictory evidence was available, since some authors found decreases in rat muscle lipid peroxidation [6] whereas other authors did not find changes [7]. This difference seems to be independent of the experimental period used, 15 days in the case of Venditti *et al*. [7], 35 days in that of Pereira *et al*. [6], and 35 days also in our case. Thus, similarly to what happens in rat liver tissue [1, 3, 4, 28] or human urine [29], lipid peroxidation is a reliable indicator of the oxidative stress induced in rodent skeletal muscle by hyperthyroidism, whereas it does not seem to change in the muscle during short- or long-term hypothyroidism.

Lipid peroxidation depends both on the rates of free radical generation, which are thought to be increased *in vivo* by thyroid hormones, and on the unsaturation degree of the fatty acid substrates, high fatty acid unsaturation strongly increasing the rates of lipid peroxidation. This is a reason why fatty acid composition was measured in our study. While the effects of thyroid hormones on fatty acid composition have been studied in other tissues [8, 9, 30], mainly in liver, to our knowledge no data are available for skeletal muscle. In our case the total number of double bonds (DBI) moderately decreased both in hypothyroid and hyperthyroid muscle in the phosphatidylcholine and phosphatidylethanolamine fractions, whereas in the cardiolipin fraction the DBI continuously increased from hypothyroidism to euthyroidism and from euthyroidism to hyperthyroidism, the change found in hyperthyroidism leading almost to a triplication of the peroxidizability index. Those changes can be due to thyroid hormone effects on desaturases [8, 9, 31], phospholipases [32], acyltransferases [33], or fatty acid catabolism.

The increase in DBI found in the cardiolipin fraction of hyperthyroid muscle was mainly due to decreases in 16:0, 16:1n-7, 18:1n-9, 18:2n-6, 18:3n-3 and 18:4n-3 together with increases in the highly unsaturated 20:4n-6 and 22:6n-3. Among these changes, those affecting n-6 and n-3 fatty acids can be explained by the stimulation of delta-6 and delta-5 desaturases by thyroid hormones which has been previously described [8, 9]. The increased desaturase activities will stimulate the conversion of 18:2n-6 into 20:4n-6 in the n-6 biosynthetic pathway and the conversion of 18:3n-3 and 18:4n-3 into 22:6n-3 in the n-3 pathway. Modulation of monolysocardiolipin acyltransferase can also be implicated in the fatty acid changes observed, since recent data show that this activity is increased by hyperthyroidism in rat heart [33]. The final result is a strong increase in the peroxidizability index of cardiolipin in the hyperthyroid muscle because the sensitivity to peroxidation exponentially increases as a function of the number of double bonds per fatty acid molecule. Since cardiolipin is almost exclusively a mitochondrial phospholipid (mostly at the inner membrane; [34]), that change would increase the risk of lipid peroxidation in the hyperthyroid mitochondria *in vivo*. This would be further enhanced by the fact that the majority of oxygen radical generation of healthy tissues occurs in mitochondria (again at the inner membrane). Being very reactive, free radicals can not diffuse away, and they react with molecules placed near their sites of generation. The occurrence of an special impact on muscle mitochondria would also agree with the observation that thyrotoxicosis leads to functional cardiomyopathy but the only histopathological lesions found in the cardiac muscle are alterations in mitochondrial ultrastructure, mainly disrupted cristae [23, 24]. Thus, part of the increase in lipid peroxidation found in hyperthyroid muscle in our investigation can be due to the strong increase in the content of highly unsaturated fatty acids of the cardiolipin fraction together with the increased cardiolipin content. In the case of hypothyroidism, however, other factors in addition to double bond content determine lipid peroxidation since, in spite of the decrease in DBI observed in the three phospholipid fractions, lipid peroxidation was not significantly decreased in hypothyroid muscle.

In our study, no changes were observed in either reduced or oxidized glutathione or GSSG/GSH ratio either in hypothyroid or hyperthyroid animals. Previous studies have shown that short-term (1–3 days) hyperthyroidism decreases liver GSH in rat liver [3, 4] due to an increase in GSH utilization during oxidative stress [2]. While the difference of these liver results with our findings in skeletal muscle could be due in principle to tissue or species differences, it seems to be related to the length of the thyroid treatment, since in a previous long-term study (5 weeks as in the present investigation) neither hypothyroidism or hyperthyroidism changed mouse liver GSH, GSSG or GSSG/GSH [5]. In the short-term studies mentioned above in which liver GSH was decreased by hyperthyroidism, the thyroid hormone treatment had already stimulated GSH synthesis by 82–97% [2]. Thus, the liver reacts to thyroid hormone oxidative stress with an in-

crease in GSH synthesis which, during the first 3 days, is not able to fully compensate for the increased GSH utilization, whereas in the long-term a new steady-state is reached in which increased but balanced synthesis and utilization could probably explain a maintenance of liver and muscle GSH levels of obvious adaptive nature. Further studies on GSH synthesis are needed to clarify this possibility.

The effect of thyroid hormones on oxidative damage to genomic and mitochondrial DNA has not been previously studied. In this investigation, it was found that the oxidative damage marker 8-oxodG decreases in the genomic DNA (99.85% nuclear DNA in rat muscle; [35]) of hypothyroid muscle, in agreement with the concept of a lower oxidative stress in that condition. However, in the hyperthyroid group a decrease in 8-oxodG was also found. This could be due to an overcompensation by induction of 8-oxodG-repairing enzymes, a phenomenon already observed in other models of oxidative stress [36–38]. Concerning mtDNA, contrarily to previous belief, it is now known that mitochondria contain very active enzymatic systems repairing 8–oxodG, at least so active as those in nuclear DNA [39]. The existence of those very active systems is logical since free radical generation is specially high near mtDNA due to the vicinity of the respiratory chain. Adjustments of those repair systems in response to changes in mitochondrial oxygen free radical production induced by thyroid hormones could also help to explain the lack of differences in 8-oxodG damage in mtDNA in the different thyroid states. No studies on the effects of thyroid hormones on 8-oxodG repair are available and are thus needed. On the other hand, a recent short-term (10 days) study from our laboratory [40] in rat heart has shown that, whereas total H_2O_2 generation from the mitochondria to the cytosol is increased by thyroid hormones secondarily to the increase in total mitochondrial density of the tissue, H_2O_2 production per mg of mitochondrial protein is not increased in hyperthyroid heart mitochondria. If the same happens in mouse muscle mitochondria after 5 weeks of treatment, it could also explain the lack of increase in 8-oxodG in mtDNA in hyperthyroidism found here. In the case of hypothyroidism, however, a decrease in mitochondrial oxygen radical generation per mg of mitochondrial protein was found in that study [40]. In any case, in agreement with previous investigations [41, 42], our results indicate that DNA seems to be better protected in vivo from oxidative stress than other macromolecules like lipids (see above) and proteins [10], probably due to its vital function as depositary of information in its gene sequences.

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