Retinoylation Reactions are Inversely Related to the Cardiolipin Level in Testes Mitochondria from Hypothyroid Rats

Valentina Senatore · Erika Cione · Antonio Gnoni · Giuseppe Genchi

Abstract The effect of hypothyroidism, induced by 6-n-propyl-2-thiouracil (PTU) administration to rats, on the retinoylation reaction and oxidative status was investigated in rat-testes mitochondria. In hypothyroid mitochondria, when compared to euthyroid controls, we found a noticeable increase in the amount of all-*trans*-retinoic acid (atRA) bound to mitochondrial proteins by an acylation process (34.2 ± 1.9 pmoles atRA/mg protein/360 min and 22.2 ± 1.7 pmoles atRA/mg protein/360 min, respectively). This increase, which was time- and temperature-dependent, was accompanied by a strong reduction in the cardiolipin (CL) amount in the mitochondrial membranes of hypothyroid ($2.6\pm0.2\%$) as compared to euthyroid rats ($4.5\pm0.5\%$) Conversely, a decreased retinoylation reaction was observed when CL liposomes were added to mitochondria or mitoplasts from both euthyroid and hypothyroid rats, thus confirming a role of CL in the retinoylation process. In mitochondria from the latter animals an increase of the level of oxidized CL occurred. The ATP level, which was reduced in hypothyroid mitochondria (27.3 ± 4.1 pmoles ATP/mg protein versus 67.1 ± 8.3 pmoles ATP/mg protein of euthyroid animals), was surprisingly increased in mitochondria by the retinoylation reaction in the presence of 100 nM atRA (481.5 ± 19.3 pmoles ATP/mg protein of hypothyroid animals versus 84.7 ± 7.7 pmoles ATP/mg protein of euthyroid animals). Overall, in hypothyroid rat-testes mitochondria the increase in retinoylation activity correlates with a significant depletion of the CL level, due to a peroxidation of this lipid. In addition, an enhanced production of reactive oxygen species was observed.

Keywords Cardiolipin · Hypothyroidism · Mitochondria · Retinoic acid · Retinoylation reaction

Abbreviations

AAPH	2'azobis(2-amidopropane) dihydrochloride
atRA	all-*trans*-retinoic acid
BHT	butylated hydroxytoluene
CDNB	1-chloro-2,4-dinitrobenzene
CL	cardiolipin
GST	glutathione S-transferase
MDA	malondialdehyde
PBS	phosphate buffered saline
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive species
TCA	trichloroacetic acid
PTU	6-n-propyl-2-thiouracil

V. Senatore · E. Cione · G. Genchi
Department of Pharmaco-Biology, Laboratory of Biochemistry,
University of Calabria,
Rende, Cosenza 87036, Italy

A. Gnoni
Department of Medical Biochemistry, Biology and Physics,
University of Bari,
Bari 70124, Italy

G. Genchi (✉)
Dipartimento Farmaco-Biologico, Università della Calabria,
Rende, Cosenza 87036, Italy
e-mail: genchi@unical.it

Introduction

Retinoic acid is an essential physiological regulator of cellular differentiation, immune response, normal growth, vision and reproduction. Retinoids, bound to proteins in the extracellular fluids, are taken up by cell receptors, then transferred into the cytoplasm and subsequently bound to

intracellular proteins (Blomhoff et al. 1991). Retinoids play an important role in the maintenance and regulation of testicular function (Chaudhary et al. 1989), and part of the steroid biosynthesis, stimulated also by all-*trans*-retinoic acid (atRA) (Chaudhary et al. 1989), is performed in testes mitochondria.

Retinoylation is an acylation reaction by which retinoic acid, a metabolite of retinol (vitamin A), acts on cells. The activity of atRA in a variety of cell types is mediated by the nuclear retinoic acid receptors, RAR and RXR (Kastner et al. 1995; Wolf 2000). AtRA is incorporated into proteins of cells in culture (Breitman and Takahashi 1996; Takahashi and Breitman 1989, 1990, 1994; Tournier et al. 1996) and into proteins of rat tissues, both in vivo (Myhre et al. 1996) and in vitro (Renstrom and DeLuca 1989; Myhre et al. 1998; Genchi and Olson 2001; Cione and Genchi 2004). The retinoylation reaction involves the intermediate formation of retinoyl-CoA (Wada et al. 2001) and subsequent transfer and covalent binding of the retinoyl moiety to protein(s) (Renstrom and DeLuca 1989). The covalent linkage between atRA and protein(s) is a thioester bond (Myhre et al. 1996; Renstrom and DeLuca 1989; Myhre et al. 1998; Genchi and Olson 2001). A high amount of bound atRA to proteins was found in mitochondria of rat testes (Genchi and Olson 2001), where the retinoylation reaction has been well characterized (Cione and Genchi 2004). Recently, we showed that the retinoylation reaction in rat-testes mitochondria is strongly influenced by the membrane phospholipid composition, in turn modulated by different dietary fatty acid patterns (Cione et al. 2007).

Cardiolipin (CL) is a complex phosphoglyceride present in the mitochondrial inner membrane. CL is necessary for optimal function of the mitochondrial electron transport chain (ETC) (Pfeiffer et al. 2003) and mitochondrial carriers involved in energy metabolism (Claypool 2009). Chinese hamster ovary cells lacking a functional phosphatidylglycerophosphate synthase, which synthesizes a direct precursor of CL, display reduced levels of CL, altered mitochondrial morphology, reduced ATP concentrations, diminished oxygen consumption and defective ETC activity (Ohtsuka et al. 1993). Thyroid hormones have a profound influence on normal development, differentiation and metabolism. Mitochondria are considered possible subcellular loci of thyroid hormone action in view of their crucial role in energy metabolism (Goglia et al. 1999, 2002). Hypothyroidism, induced by 6-n-propyl-2-thiouracil (PTU) treatment, decreases the activities of CL synthase and acyl CoA-dependent monolysocardiolipin (MLCL) acyltransferase in mammalian heart mitochondria (Taylor et al. 2002), producing a marked loss of CL content and CL-dependent protein functions. Liver and heart mitochondria from hypothyroid rats show a lower activity of some mitochondrial carriers as well as of proteins involved in the

ETC (Paradies and Ruggiero 1991; Paradies et al. 1993; Giudetti et al. 2006; Siculella et al. 2006). These phenomena have been in part correlated to changes in phospholipid content, with particular attention to the decrease of CL in the mitochondrial inner membranes (Pehowich 1995; Paradies et al. 1996; Paradies et al. 1997).

There is growing evidence supporting the role of reactive oxygen species (ROS) in the pathogeneses of thyroid disorders. Increased generation of ROS, particularly in hypothyroidism, has been reported both in humans (Erdamar et al. 2008; Torun et al. 2009) and rats (Cano-Europa et al. 2008).

Recently, Zamoner et al. (2008) showed that PTU-induced congenital hypothyroidism depletes antioxidant defenses in rat testes and supports increased mitochondrial ROS generation, which contributes to biochemical changes in hypothyroid testes.

However, studies on the effect of the animal thyroid state on the retinoylation reaction in testes mitochondria are lacking.

This study presents the first evidence showing an effect of the thyroid state on the retinoylation reaction in rat-testes mitochondria. We found that, compared to euthyroid rats, the incorporation of ^{3}H-atRA into mitochondrial proteins was higher in hypothyroid mitochondria. These changes were associated with a parallel decrease in mitochondrial CL content while there was a significant increase in the level of peroxidized CL, possibly because of enhanced reactive oxygen species production.

Materials and methods

Chemicals

[11-12 ^{3}H] All-*trans*-retinoic acid (^{3}H-atRA) (50 Ci/mmol) was purchased from PerkinElmer Boston, USA. Cardiolipin (CL), 6-n-propyl-2-thiouracil, digitonin, CoASH, ATP, butylated hydroxytoluene (BHT), glutathione S-transferase (GST), 2,2'azobis(2-amidopropane) dihydrochloride (AAPH), all-*trans*-retinoic acid (atRA), 1-chloro-2, 4-dinitrobenzene (CDNB) and thiobarbituric acid (TBA) were purchased from Sigma Aldrich (Milan, Italy). All other chemicals were of analytical reagent grade.

Animal treatment

Male Wistar rats (230–250 g) were used throughout this study. They were housed in cages in a temperature (22±1 °C) and light (light on 8:00–20:00) controlled room and randomly assigned to one of two different groups. The first one is the euthyroid control and the second one was made hypothyroid by continuous administration of PTU

(0.1% w/v, in drinking tap water) for 3-4 weeks (Siculella et al. 2006). Both groups had free access to food that was a commercial mash (Morini S.p.A., Milan, Italy) and water. The experimental design was in accordance with local and national guidelines covering animal experiments.

Isolation of mitochondria and mitoplasts

Rat-testes mitochondria were isolated by differential centrifugation as described by Genchi and Olson (2001). Mitochondria were suspended in a medium containing 250 mM sucrose, 10 mM Tris/HCl, pH 7.4, 1 mM EDTA at a concentration of 15–18 mg protein/ml. This mitochondrial suspension was either immediately used or frozen at -70°C; in both cases the binding activity was the same. The purity of the mitochondrial preparation was checked by assaying marker enzymes for lysosomes, peroxisomes and plasma membranes.

Mitoplasts, which lack the mitochondrial outer membrane, were prepared from the mitochondrial fraction of testes by the procedure of Greenawalt (1974). In brief, mitochondrial pellets were suspended in the isolation medium containing 70 mM sucrose, 220 mM D-mannitol, defatted BSA (0.5 mg/ml), 2 mM HEPES, pH 7.4 and digitonin. The stock solution of digitonin (1.2%) was prepared dissolving it in the same hot (almost boiling) medium to give a desirable ratio of 0.12 mg digitonin/mg mitochondrial protein. After 15 min under gentle agitation, the preparation was diluted with 3 volumes of the same medium and centrifuged in an Eppendorf centrifuge at 13,000 rpm for 20 min. The mitoplasts were suspended in the isolation medium, sedimented and resuspended again in the same medium.

The protein concentration of mitochondria and mitoplasts was determined as described by Genchi and Olson (2001).

Cardiolipin liposome preparation and fusion with membranes of mitochondria or mitoplasts

CL liposomes were prepared by sonicating the desired amount of CL in 250 mM sucrose, 10 mM Tris/HCl, pH 7.4, 1 mM EDTA, with the microtip probe of a Branson sonifier (mod. 250).

The fusion of liposomes with the membranes of mitochondria or mitoplasts was carried out essentially as described by Hackenbrock and Chazotte (1986) and Paradies et al. (1997). Briefly, freshly sonicated liposomes were added to 0.5 mg of mitochondria or mitoplasts at 30°C under constant stirring in order to have a CL concentration in a final range of 0.25–4 mg/ml. After 30 min of incubation, mitochondria or mitoplasts were centrifuged and the pellet was resuspended in the retinoylation reaction incubation buffer (10 mM ATP, 150 µM CoASH, 27 mM $MgCl_2$, 50 mM sucrose, 100 mM Tris, pH 7.4).

## Incorporation of radioactive ³H-atRA

³H-atRA was dissolved in ethanol and diluted in the retinoylation buffer such that the final concentration of ethanol was no higher than 0.5%. The retinoylation reaction was carried out for 90 min or for the times indicated in figures by adding 2.5–3 µl ³H-atRA (300,000–350,000 cpm) to the preparations (0.5 mg protein) in order to have 100 nM ³H-atRA (final concentration) in the incubation buffer in a total volume of 0.5 mL at 37°C. The reaction was stopped by adding TCA at a final concentration of 5%. The mixture was centrifuged in an Eppendorf centrifuge at 13,000 rpm for 10 min, and the sediment was extracted seven times with 1 mL $CHCl_3/CH_3OH$ (2:1), containing 0.005% BHT to remove from membranes of mitochondria and mitoplasts the amount of not specifically bound ³H-atRA (Genchi and Olson 2001). The pellet was solubilized in 1% SDS, 40 mM Tris, 2 mM EDTA, pH 7.5, at 50°C and counted in a Tri-Carb 2100TR liquid scintillation counter (Packard) and the counting efficiency was about 73–75%. All manipulations involving atRA or ³H-atRA were performed under yellow light.

For the time-course determination the reaction was stopped with TCA at times indicated in Fig. 1. Arrhenius plot analysis samples were incubated for 90 min at different temperatures in a range of 5–40°C.

Phospholipid and fatty acid analysis

Total lipids were extracted as in Giudetti et al. (2006) from freshly prepared mitochondria (10 mg protein) of euthyroid and hypothyroid rat testes before the retinoylation reaction assay. The extracts were dried under N_2 flow and resuspended in 100 µL of $CHCl_3$. Phospholipids were
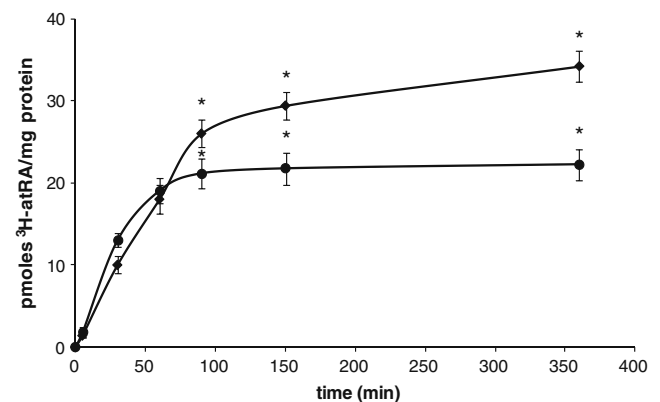
Fig. 1 Time-dependent incorporation of ³H-atRA into proteins of testes mitochondria from euthyroid (●) and hypothyroid (♦) rats. Mitochondria were incubated with 100 nM³H-atRA at 37°C at the indicated times. The data are the means±SD of five independent experiments. *$P<0.01$

separated by HPLC as described by Giudetti et al. (2006) by using a Beckman System Gold chromatograph equipped with an ultrasil-Si column (4.6×250 mm; Chemtek Analytica, Bologna, Italy). The chromatographic system was programmed for gradient elution by using two mobile phases: solvent A, hexane:2-propanol (6:8; v/v), and solvent B, hexane:2-propanol:water (6:8:1.4; v/v/v). The percentage of solvent B in solvent A was increased in 15 min from 0% to 100%. Flow rate was 2 mL/min, and detection was at a wavelength of 206 nm. Single phospholipids were identified with known standards and quantitatively assayed as in Giudetti et al. (2006). To analyze fatty acids, rat-testes mitochondria were saponified with ethanolic KOH for 2 h at 90°C. Fatty acids were extracted as described by Caputi Jambrenghi et al. (2007), and their corresponding methyl esters were prepared by *trans*-esterification with methanolic boron trifluoride (17% BF_3) at 65°C for 30 min. Fatty acid methyl esters (FAMEs) were then analyzed by gas-liquid chromatography. Helium was used as carrier at a flow rate of 1 mL/min. FAMEs were separated on a 30 m×0.32 cm HP5 (Hewlett Packard) capillary column. The injector and detector temperatures were maintained at 250°C. The column was operated isothermally at 150°C for 4 min and then programmed to 250°C at 4°C/min. Peak identification was performed by using known standards, and the relative amounts were automatically determined by peak integration.

Preparation and analysis of peroxidized cardiolipin

Bovine heart cardiolipin was autoxidized overnight in a thin film at 37°C (Paradies et al. 2000). Peroxidized cardiolipin was identified by the normal phase HPLC method described above, with UV detection at 235 nm, indicative of conjugated dienes (Paradies et al. 2000).

Malondialdehyde formation

The lipid peroxidation level was determined through the formation of thiobarbituric acid reactive species (TBARS) following the method of Esterbauer and Cheeseman (1990). After the retinoylation reaction, mitochondria (0.5 mg proteins) were precipitated and resuspended in 940 μL PBS (1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 136.9 mM NaCl, pH 7.2), mixed with 50 μL of 0.5 M 2,2'azobis(2-amidopropane) dihydrochloride (AAPH) and incubated for the indicated times at 37°C. After incubation, 500 μL of a solution containing 15% TCA, 0.4% TBA, 0.2 M HCl and 0.2% BHT was added to the samples and incubated at 80°C for 15 min. The malondialdehyde (MDA) production, expressed as nmoles produced/mg protein, was followed spectrophotometrical-ly at 533 nm.

Mitochondrial ATP determination

The content of ATP in euthyroid and hypothyroid mitochondria after the retinoylation reaction in the presence or in the absence of 100 nM atRA was determined using a commercial ATP kit (Molecular Probes) according to manufacturer instructions based on bioluminescence.

Statistical analyses

Statistical differences were determined by one-way analysis of variance (ANOVA) followed by Dunnett's method, and the results were expressed as mean±SD for the number of experiments indicated in every case. Three replicates were used per point. Differences were considered statistically significant for $P<0.001$, $P<0.01$, and $P<0.05$.

Results

The effectiveness of treatment with PTU in inducing a severe hypothyroid state was confirmed by assaying the rat-serum level of free triiodothyronine (FT_3) and free tetraiodiothyronine (FT_4). In hypothyroid rat serum the FT_3 level decreased by 62% (1.32±0.35 pmoles/L versus 3.42± 0.61 pmoles/L of euthyroid rats, $n=5$) and the FT_4 level by about 90% (0.21±0.05 μg/dL versus 2.93±0.33 μg/dL of euthyroid, $n=5$). Figure 1 shows the time-dependent incorporation of 100 nM of ³H-atRA (about 330,000– 350,000 cpm) into mitochondrial proteins of euthyroid and hypothyroid rat testes. The incorporation of ³H-atRA into mitochondrial proteins of euthyroid rat testes, after the first step of linear increase, reached a plateau at 90 min (21.7± 1.9 pmoles/mg protein), maintaining about the same value until 360 min (22.2±1.7 pmoles/mg protein). The level of ³H-retinoylated mitochondrial proteins of hypothyroid rat testes increased linearly in the first 90 min (26.0± 1.7 pmoles/mg protein) and then slightly further until 360 min (34.2±1.9 pmoles/mg protein). At the latter incubation time there was an increase of 54% of the retinoylation level in hypothyroid rat-testes mitochondria when compared to euthyroid ones.

In testes mitochondria from the two groups of rats, different activation energies were found by the temperature-dependence of the retinoylation reaction. The activation energies (in the range of 5–40°C) were 47.8±6.2 kJ/mol for euthyroid mitochondria (in agreement with Cione and Genchi 2004) and 30.7±4.4 kJ/mol for hypothyroid mitochondria.

Next, the phospholipid and fatty acid composition of testes-mitochondrial membranes from euthyroid and hypothyroid rats were investigated. The total amount of

mitochondrial phospholipids was not significantly changed in mitochondrial preparations of either of these groups of rats (Table 1). However, the majority of individual phospholipid classes showed no significant differences in the percent composition of the two groups of rats, except for cardiolipin, which was strongly decreased (42%) in testes mitochondria from hypothyroid rats.

Therefore, it can be assumed that the changes in the CL level observed in testes mitochondria from hypothyroid rats could be involved in the observed increase in the retinoylation reaction. To prove this more directly, the effect of exogenously added cardiolipin on the retinoylation reaction was investigated. To this end we enriched the membranes of mitochondria and mitoplasts with this phospholipid by using CL liposome preparations to evaluate the retinoylation reaction. Since the mitochondrial inner membrane is physiologically the mitochondrial membrane with the higher CL content and the retinoylation reaction has been found to be of interest also in mitoplasts (Genchi and Olson 2001), we have taken in consideration whether the inhibition could be ascribed to CL enrichment of both mitochondrial outer and inner membranes. In mitochondria and in mitoplasts of euthyroid and hypothyroid rats, the amount of retinoylated proteins decreased with increasing the CL concentration in a range of 0.25–4 mg/mg protein (Fig. 2a-b). At the higher CL concentration of 4 mg/mg protein, it is possible to see a marked inhibition of the retinoylation reaction in proteins of mitochondria (Fig. 2a, 70.6±2.8% for euthyroid and 55.7±1.8% for hypothyroid rats) and in proteins of mitoplasts (Fig. 2b, 63.7±3.0% for euthyroid versus 48.0±1.7% for hypothyroid rats), respectively.

It has been demonstrated that in rat-testes mitochondria PTU-induced hypothyroidism decreased enzymatic and not enzymatic antioxidant defences (Zamoner et al. 2008) and

Table 1 Phospholipid composition (mol%) of testes mitochondria from euthyroid and hypothyroid rats

Phospholipids	Euthyroid	Hypothyroid
Cardiolipin	4.5±0.5	2.6±0.2[a]
Phosphatidylethanolamine	33.2±2.1	32.0±4.8
Phosphatidylinositol	6.5±0.9	5.6±0.8
Phosphatidylserine	4.3±0.9	4.2±0.7
Phosphatidylcholine	53.1±4.5	53.6±6.8
Total phospholipid-Pi (µg Pi/mg protein)	10.1±1.6	11.3±1.8

Phospholipids were extracted from testes mitochondria of euthyroid and hypothyroid rats. Single phospholipids were separated by HPLC, quantified by determining inorganic phosphate, and expressed as a percentage of total phospholipids. The data are the means±SD of five independent experiments

[a] $P<0.001$
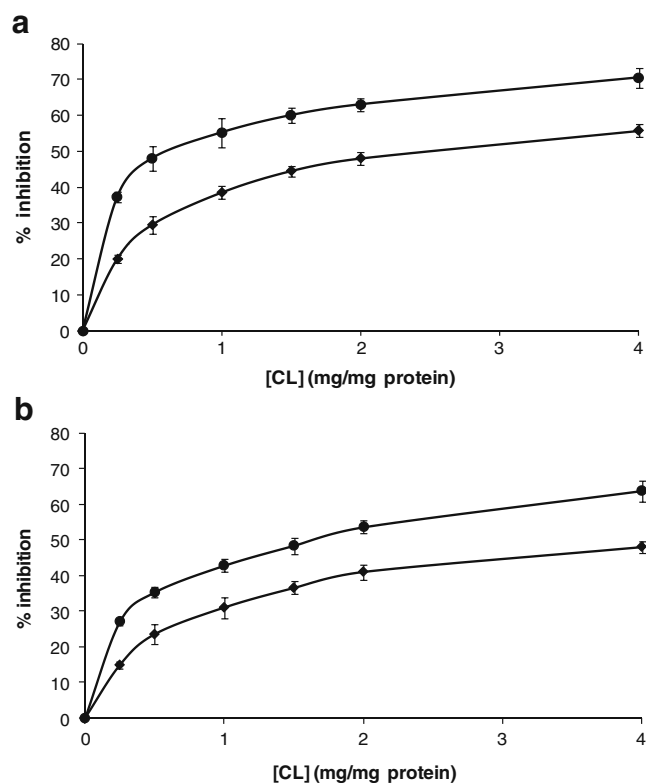
Fig. 2 Effect of increased cardiolipin amounts on incorporation of ^{3}H-atRA into proteins of testes mitochondria from euthyroid (●) and hypothyroid (♦) rats. **a** CL-fused mitochondria (0.5 mg of protein) and **b** CL-fused mitoplasts (0.5 mg of proteins) were incubated with 100 nM ^{3}H-atRA at 37°C for 90 min with the indicated amount of CL (0.25–4 mg/mg protein). The data are the means±SD of three independent experiments. Overall *$P<0.05$

supported an increased mitochondrial ROS generation. To investigate whether the decrease in CL content, observed in mitochondria from hypothyroid rats, could be due to ROS-induced CL peroxidation, the amount of peroxidized CL was assayed. As indicated in Fig. 3, an increase in the level of peroxidized CL was observed in testes mitochondria from hypothyroid rats as compared to euthyroid controls.

It is well known that the membrane fatty acid composition can be an important factor influencing the activities of membrane-associated enzymes. No significant changes of the fatty acid pattern of testes mitochondria between euthyroid and hypothyroid rats were observed in most of the individual fatty acids and in the sum of saturated and unsaturated fatty acids, in their ratio as well as in the unsaturation index (Table 2). However, in hypothyroid mitochondria, a significant decrease in the levels of only some minor fatty acids, i.e. C20:5, n-3 and C22:5, n-3 was observed.

To get a better understanding of the mechanism of retinoylation reactions, euthyroid and hypothyroid mitochondria were incubated with 100 nM atRA and lipid peroxidation
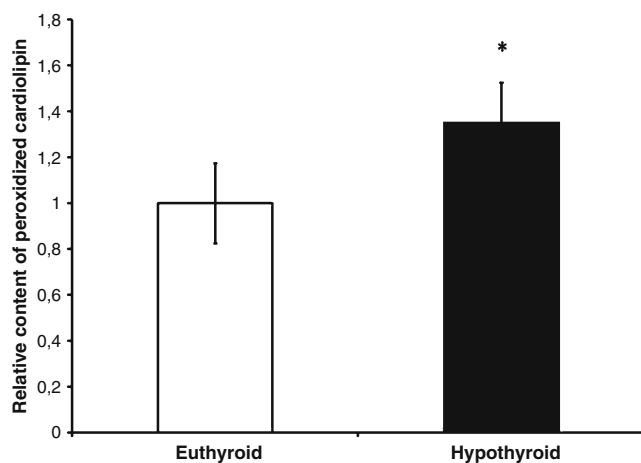
Fig. 3 Relative cardiolipin peroxidation in rat-testes mitochondria from euthyroid and hypothyroid rats. Mitochondrial content of peroxidized cardiolipin, expressed as peak area (at 235 nm) per mg of phospholipids, was assayed by HPLC. Peak area of the euthyroid control is assumed as the unit. The values are the means±SD of five independent experiments. *$P<0.05$ vs control

was determined by TBARS formation. In Fig. 4a (euthyroid mitochondria) and 4b (hypothyroid mitochondria), it is shown that the production of MDA is time-dependent either in the absence or in the presence of 100 nM atRA. In

Table 2 Fatty acid composition (mol%) of mitochondrial membrane phospholipids from rat testes

Fatty acid	Euthyroid	Hypotyroid
14:0	0.5±0.2	0.6±0.3
16:0	38.0±4.2	41.1±3.8
16:1	1.6±1.4	2.1±1.9
18:0	8.6±1.0	8.2±2.3
18:1	12.8±0.9	12.2±0.2
18:2 n6	6.1±1.1	4.9±0.9
18:3 n3	0.2±0.1	0.3±0.1
20:4 n6	15.0±2.9	13.4±2.2
20:5 n3	0.7±0.5	0.1±0.1[a]
22:5 n3	1.6±0.3	1.0±0.1[a]
22:6 n3	14.2±2.7	13.3±2.5
∑Saturated	47.1±3.5	49.9±4.0
∑Unsaturated	52.2±3.6	47.3±5.2
∑Saturated/∑Unsaturated	0.90±0.2	1.05±0.3
Unsaturation index	183.9±28.6	163.9±22.9

Unsaturation index, ∑ mol% of each fatty acid x number of double bonds of the same fatty acid. The data are the means±SD of four independent experiments. Fatty acids were extracted from mitochondrial membrane phospholipids after saponification. Following derivatization with methanolic BF₃, fatty acid methylesters were separated by gas-liquid chromatography and identified by using known standards

∑ saturated = sum of the saturated fatty acids; ∑ unsaturated = sum of the unsaturated fatty acids

[a] $P<0.05$
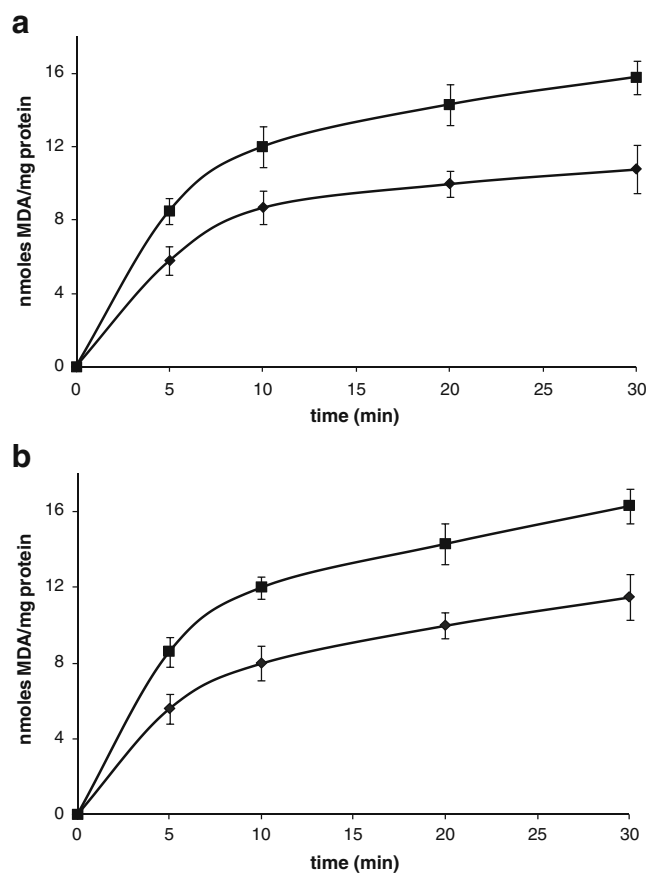
Fig. 4 Determination of TBARS in euthyroid mitochondria **a** and in PTU mitochondria **b** incubated with (■) or without 100 nM atRA (♦) at the indicated times. Lipid peroxidation was evaluated by TBARS assay. The data represent means±SD of three independent experiments

hypothyroid mitochondria the level of MDA is slightly but not significantly higher than the euthyroid mitochondria. As CL is associated with the mitochondrial energy transducing membrane apparatus (Pfeiffer et al. 2003), we evaluated the ATP content in the two groups of rats and we found about a 60% reduction of the ATP level in hypothyroid compared to euthyroid mitochondria (27.3±4.1 pmoles ATP formed/mg protein versus 67.1±8.3 pmoles ATP formed/mg protein, respectively) (Table 3). In the presence of 100 nM atRA a noticeable increase in the ATP content in testes mitochondria of the two groups of rats was observed. Interestingly, ATP increased to a much higher level in hypothyroid mitochondria as compared to euthyroid ones (481± 19.3 pmoles ATP formed/mg protein versus 84.7±7.7 pmoles ATP formed/mg protein, respectively).

Discussion

Retinoylation, a process first studied by Takahashi and Breitman (1989), is an acylation reaction occurring on

Table 3 Content of ATP (pmol/mg protein) in euthyroid and hypothyroid rat testes mitochondria in the absence or in the presence of atRA

	pmoles ATP/mg protein	
	No addition	100nM atRA
Euthyroid mitochondria	67.1±8.3	84.7±7.7
Hypothyroid mitochondria	27.3±4.1	481.5±19.3[a]

The data are the means±SD of three independent experiments

[a] $P<0.01$

proteins by a thioester bond. We previously showed that the retinoylation process is active in testes mitochondria (Genchi and Olson 2001; Cione and Genchi 2004) as well as in normal TM-3 (Cione et al. 2005a) and tumoral MLTC-1 testes-cell lines (Cione et al. 2005b). Recent work indicated that retinoylation of α-actinin by atRA may play a significant role in atRA-induced differentiation in HL60 cells, including the promotion of cytomorphologic changes (Kubo et al. 2008). This is consistent with the fact that retinoylation involves the presence of sulfhydryl functional groups, so it could be strictly dependent on the mitochondrial oxidative status.

Thyroid hormones (THs) are essential for normal postnatal growth and development. TH receptors are highly expressed in neonatal Sertoli cells, and testes may be important TH targets (Jannini et al. 1999). Alterations in thyroid activity are frequently associated with changes in male reproductive functions, since hypothyroidism is associated with a marked delay in sexual maturation and development (Holsberger and Cooke 2005). However, the mechanisms by which the THs are able to control testes metabolism are still uncertain.

Recently, Cione et al. (2007) have shown that retinoylation in rat-testes mitochondria can be influenced by changes in membrane phospholipid pattern. We found higher atRA incorporation when the mitochondrial CL content is lower. It has been reported in rat-testes mitochondria that during spermatogenesis and ageing processes the activities of some enzymes of respiratory chain complexes are greatly influenced by the membrane lipid composition, which in turn affects protein-lipid interactions (Vazquez-Memije et al. 2005). In the present work, we show for the first time that the amount of CL found in mitochondrial membranes of hypothyroid-rat testes was lower than that found in euthyroid control rats (Table 1). This result appears particularly interesting in the light of previous studies showing enhancement of mitochondrial cardiolipin biosynthesis in rat liver of hyperthyroid animals (Paradies et al. 1997). It is important to emphasize that in mitochondria from PTU-treated rats we observed a higher retinoylation activity (34.2 ±1.9 pmoles atRA/mg protein/360 min) as compared to

control rats (22.2±1.7 pmoles atRA/mg protein/360 min). These results suggest that the retinoylation process could be greatly influenced by the mitochondrial CL level. This notion is supported by the reduction in incorporation of atRA when the exogenously added CL was increased in mitochondria or mitoplasts membranes through fusion with CL liposomes (Fig. 2a and b). Notably, changes in CL content have been reported to affect the transport of phosphate in rat-liver mitochondria of hypothyroid rats (Paradies and Ruggiero 1991). Mitochondria are the major source of ROS and constitute a major target of cumulative oxidative stress. It has been shown that in bovine-heart submitochondrial particles the CL content decreases as a consequence of oxidative damage by ROS (Paradies et al. 2000). Indeed, CL molecules are especially susceptible to peroxidative attack by ROS, because of their high level of unsaturated fatty acids. Accordingly, results here reported show, when compared to euthyroid animals, a significant loss in the CL content in hypothyroid rat-testes mitochondria, associated with an increase in peroxidized CL. Zamoner et al. (2008), inducing hypothyroidism by experimental conditions very similar to those reported in the present study, demonstrated an increased ROS generation in hypothyroid rat-testes mitochondria, highlighting an interrelationship between hypothyroidism and oxidative stress. The latter was ascribed to poor ROS scavenging due to decreased enzymatic and non-enzymatic antioxidant defenses.

Of interest, in our experiments we found that ATP content, which was reduced in hypothyroid rat-testes mitochondria as compared to euthyroid ones, was greatly stimulated by addition of atRA to the former organelles (see Table 3). Rapaport et al. (1982) showed in 3T3 cells an increase of the ATP pool in the presence of atRA. Moreover, our results add further support to the very recent findings of Acin-Perez et al. (2010), who found that retinoic acid at μM concentration was able of up-regulating ATP synthase activity in isolated mouse-liver mitochondria. Note that reduced respiration rate (Zamoner et al. 2008) and lower ATP content (Palmero et al. 1994) have been documented in testes mitochondria of hypothyroid rats. Therefore, it can be hypothesized that the increased ATP level we observed in hypothyroid testes mitochondria following atRA addition could play a role in mitochondrial energy homeostasis. However, further studies are needed to clarify this point.

Overall, the present study shows that retinoylation reactions are noticeably stimulated in rat-testes mitochondria from hypothyroid rats as compared to euthyroid ones. The molecular basis of this increase seems to be, at least in part, a decreased amount of mitochondrial CL, occurring in parallel with an increased level of CL oxidized form. The latter finding suggests a role of ROS in this context. Therefore, these results may contribute in elucidating the

mechanisms underlying mitochondrial dysfunction observed in testes mitochondria of hypothyroid animals.

References

Acin-Perez R, Hoyos B, Zhao F, Vinogradov V, Fischman DA, Harris RA, Leitges M, Wongsiriroj N, Blaner WS, Manfredi G, Hammerling U (2010) FASEB J 24:627–636

Blomhoff R, Green MH, Green JB, Berg T, Norum KR (1991) Physiol Rev 71:951–990

Breitman TR, Takahashi N (1996) Biochem Soc Trans 24:723–727

Cano-Europa E, Pérez-Severiano F, Vergara P, Ortiz-Butrón R, Ríos C, Segovia J, Pacheco-Rosado J (2008) Metab Brain Dis 23:275–287

Caputi Jambrenghi A, Paglialonga G, Gnoni A, Zanotti F, Giannico F, Vonghia G, Gnoni GV (2007) Comp Biochem Physiol part B Biochem Mol Biol 147:498–503

Chaudhary LR, Hutson JC, Stocco DM (1989) Biochem Biophys Res Commun 158:400–406

Cione E, Genchi G (2004) J Bioenerg Biomembr 36:211–217

Cione E, Tucci P, Chimento A, Pezzi V, Genchi G (2005a) J Bioenerg Biomembr 37:43–48

Cione E, Tucci P, Senatore V, Ioele G, Genchi G (2005b) Mol Cell Biochem 276:55–60

Cione E, Senatore V, Tucci P, Giudetti AM, Genchi F, Gnoni GV, Genchi G (2007) J Bioenerg Biomembr 39:203–209

Claypool SM (2009) Biochim Biophys Acta 1788:2059–2968

Erdamar H, Demirci H, Yaman H, Erbil MK, Yakar T, Sancak B, Elbeg S, Biberoğlu G, Yetkin I (2008) Clin Chem Lab Med 46:1004–1010

Esterbauer H, Cheeseman KH (1990) Methods Enzymol 186:407–421

Genchi G, Olson JA (2001) Biochim Biophys Acta 1530:146–154

Giudetti AM, Leo M, Siculella L, Gnoni G (2006) Biochim Biophys Acta 1761:484–491

Goglia F, Moreno M, Lanni A (1999) FEBS Lett 452:115–120

Goglia F, Silvestri E, Lanni A (2002) Biosci Rep 22:17–32

Greenawalt JW (1974) Methods Enzymol 31:310–323

Hackenbrock CR, Chazotte B (1986) Methods Enzymol 125:35–45

Holsberger DR, Cooke PS (2005) Cell Tissue Res 322:133–140

Jannini EA, Carosa E, Rucci N, Screponi E, D'Armiento M (1999) J Endocrinol Invest 22:843–848

Kastner P, Mark M, Chambon P (1995) Cell 83:859–869

Kubo Y, Ohba T, Takahashi N (2008) J Biochem 144:349–355

Myhre AM, Takahashi N, Blomhoff R, Breitman TR, Norum KR (1996) J Lipid Res 37:1971–1977

Myhre AM, Hagen E, Blomhoff R, Norum KR (1998) J Nutr Biochem 9:705–711

Ohtsuka T, Nishijima M, Suzuki K, Akamatsu Y (1993) J Biol Chem 268:22914–22919

Palmero S, Trucchi P, Prati M, Fugassa E, Lanni A, Goglia F (1994) Eur J Endocrinol 130:308–312

Paradies G, Ruggiero FM (1991) Biochim Biophys Acta 1070:180–186

Paradies G, Ruggiero FM, Dinoi P, Petrosillo G, Quagliarello E (1993) Arch Biochem Biophys 307:91–95

Paradies G, Ruggiero FM, Petrosillo G, Quagliariello E (1996) FEBS Lett 397:260–262

Paradies G, Ruggiero FM, Petrosillo G, Quagliariello E (1997) FEBS Lett 406:136–138

Paradies G, Petrosillo G, Pistolese M (2000) M Ruggiero. FEBS Lett 466:323–326

Pehowich DJ (1995) Biochim Biophys Acta 1235:231–238

Pfeiffer K, Gohil V, Stuart RA, Hunte C, Brandt U, Greenberg ML, Schägger H (2003) J Biol Chem 278:52873–52880

Rapaport E, Schroder EW, Black PH (1982) J Cell Physiol 110:318–322

Renstrom B, DeLuca HF (1989) Biochim Biophys Acta 998:69–74

Siculella L, Sabetta S, Giudetti AM, Gnoni GV (2006) J Biol Chem 281:19072–19080

Takahashi N, Breitman TR (1989) J Biol Chem 264:5159–5163

Takahashi N, Breitman TR (1990) J Biol Chem 265:19158–19162

Takahashi N, Breitman TR (1994) J Biol Chem 269:5913–5917

Taylor WA, Xu FY, Ma BJ, Mutter TC, Dolinsky VW, Hatch GM (2002) BMC Biochem 3:9–14

Torun AN, Kulaksizoglu S, Kulaksizoglu M, Pamuk BO, Isbilen E, Tutuncu NB (2009) Clin Endocrinol 70:469–474

Tournier S, Raynaud F, Gerbaud P, Lohmann SM, Anderson WB (1996) J Cell Physiol 167:196–203

Vazquez-Memije M, Cardenas-Mendez MJ, Tolosa A, Hafidi ME (2005) Exp Gerontol 40:482–490

Wada M, Fukui T, Kubo Y, Takahashi N (2001) J Biochem 130:457–463

Wolf G (2000) Nutr Rev 58:151–153

Zamoner A, Barreto KP, Filho DW, Sell F, Woehl VM, Guma FCR, Pessoa-Pureur R, Silva FR (2008) J Mol Endocrinol 40:125–135