J Bioenerg Biomembr (2009) 41:215–221
DOI 10.1007/s10863-009-9220-z

Adrenal glands and testes as steroidogenic tissue are affected by retinoylation reaction

Attilio Pingitore · Erika Cione · Valentina Senatore · Giuseppe Genchi

Received: 18 January 2009 / Accepted: 24 May 2009 / Published online: 12 June 2009

Abstract This study was undertaken to better understand the physiological role of the retinoylation process in steroidogenic tissues. In adrenal gland mitochondria, the retinoylation extent was found equal to that of testes mitochondria but without ATP in the incubation buffer. We pointed out that the endogenous mitochondrial ATP in adrenal glands is much higher than in testes, about 1.3 x 10^{-2}M and 5.2 x 10^{-8}M, respectively. In addition, less CoASH is required for the maximal acylation activity of the retinoyl moiety to protein(s) compared to testes. The fatty acid analysis revealed a different composition of mitochondrial membranes of these two tissues. Among the different values of fatty acids, it is important to note that adrenal glands contain a much higher amount of C18:0 and a much lower amount of C22:5 ω6 and C22:6 ω3 than testes in the mitochondrial membranes. In addition, there were also differences in arachidonic acid (ARA, C20:4 ω6) content between adrenal glands and testes mitochondria. These different values in the fatty acids composition should explain the different extent of the retinoylation process between the two organs.

Keywords Rat · Adrenal glands · Testes · Mitochondria · Retinoylation reaction

Abbreviations

RA	all-*trans*-retinoic acid
ACTH	corticotrophin
LH	luteotropin
CoASH	coenzyme A
AMG	3-(4-aminophenyl)-3-ethyl-piperidine-2,6-dione
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid

A. Pingitore · E. Cione · V. Senatore · G. Genchi (✉)
Department of Pharmaco-Biology, University of Calabria,
87036 Rende, CS, Italia
e-mail: genchi@unical.it

Introduction

Adrenal glands and gonads synthesize and secrete steroid hormones in response to pituitary hormones such as corticotropin (ACTH) or luteotropin (LH) (Saez 1994; Waterman 1994). The binding of these peptide hormones to their cognate receptors is coupled to the formation of cAMP and activation of the protein kinase A signalling pathway (Waterman and Bischof 1997; Richards and Hedin 1988). In particular, adrenal glands are *vital* to health and have an important role in development and reproduction (Harvey and Everett 2003).

Retinoic acid (RA) acylation, also known as retinoylation, is a post-translational modification of proteins occurring in a variety of eukaryotic cell lines and subcellular compartments both in vivo and in vitro. Retinoylated proteins that have been identified so far include cAMP-binding proteins, vimentin, the cytokeratins, and some nuclear proteins (Takahashi and Breitman 1989; Takahashi and Breitman 1990; Takahashi and Breitman 1994; Tournier et al. 1996; Myhre et al. 1996; Renstrom and DeLuca 1996; Myhre et al. 1998). This process occurs in the presence of Mg^{2+}, ATP and CoASH. The omission of these substrates in the incubation buffer markedly reduced the extent of retinoylation (Genchi and Olson 2001; Cione and Genchi 2004) as the transfer mechanism involves the formation of a retinoyl-CoA intermediate (Kubo et al. 2005). Moreover, the incorporation of all-*trans*-retinoic acid and retinoyl-CoA into proteins is enzymatic judging from the inhibition of the process in the

presence of N-ethylmaleimide (NEM) and the inactivation by heating and SDS denaturation of subcellular fractions containing enzyme or/and protein substrates (Genchi and Olson 2001; Kubo et al. 2005). We have previously shown (Cione et al. 2005) that the retinoylation reaction occurred on protein(s) of TM-3 Leydig cell line by RA. The reaction involves the formation of a thioester bond and occurs on pre-existing protein. It is important to note that both db-cAMP and forskolin increase the retinoylation level on the protein of TM-3 cells of about 75% and 80% respectively (Cione et al. 2005). These results suggested that the retinoylation reaction could be regulated by cAMP-activated enzymes.

Previously, we have seen that RA-induced steroidogenesis and retinoylation are parallel events and exhibit a positive correlation (Tucci et al. 2008). Therefore in this context, we examined the retinoylation process on adrenal glands mitochondria to better understand whether the physiological role of the retinoylation process is steroidogenic tissues dependent.

Materials and methods

Chemicals

[11-12 ^{3}H] All-*trans*-retinoic acid ([^{3}H]RA) (50 Ci/mmol) was purchased from PerkinElmer (Boston USA). All-*trans*-retinoic acid (RA) and 3-(4-aminophenyl)-3-ethyl-piperidine-2,6-dione (AMG) were obtained from Sigma-Aldrich (Milano, Italia). All other chemicals used were of analytical reagent grade.

Isolation of mitochondria

Rats were killed by decapitation, according to practice procedures approved by the ethical committee, and adrenal glands and testes were immediately removed. Mitochondria were isolated by differential centrifugation as described by Genchi and Olson (2001) and suspended in a medium containing 250 mM sucrose, 10 mM Tris/HCl, pH 7.4, 1 mM EDTA at a concentration of 5–8 mg protein/ml. Protein concentration was determined by the Lowry procedure (Lowry et al. 1951) with bovine serum albumin (BSA) as the reference standard. These mitochondrial suspensions were either used immediately or frozen at −80 °C. The purity of the mitochondrial preparation was checked by assaying marker enzymes for lysosomes, peroxisomes and plasma membranes.

Incorporation of radioactive RA

[^{3}H]RA or RA was dissolved in ethanol under yellow safelight. The concentration of RA was spectrophotometrically determined, the ethanol was evaporated under nitrogen, and the dry residue resuspended in DMSO. The RA solution was diluted into the retinoylation buffer such that the final concentration of DMSO was no higher than 0.5%. The buffers were 5 mM ATP, 50 μM CoASH, 27 mM $MgCl_2$, 50 mM sucrose, 100 mM Tris, pH 7.4 for adrenal glands mitochondrial preparation, and 10 mM ATP, 150 μM CoA, 27 mM $MgCl_2$, 50 mM sucrose, 100 mM Tris, pH 7.4 for testes mitochondria; the retinoylation reaction was carried out at 37 °C for 90 min in a final ratio of 1 mg protein /1 ml. 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 precipitate was extracted seven times with 0.5 ml $CHCl_3$: CH_3OH (2:1) containing 0.005% BHT. The pellet was solubilized in 0.2 ml 1% SDS, 40 mM Tris, 2 mM EDTA, pH 7.5, at 50 °C and counted in a TriCarb 2100TR liquid scintillation counter (Packard). The counting efficiency was about 75%.

Mitochondrial ATP concentration

The amount of endogenous ATP in isolated mitochondria from testes and adrenal glands was determined using the bioluminescence method described by Drew and Leeuwenburgh (2003) with a commercial ATP kit (Molecular Probes) according to manufacturer instructions.

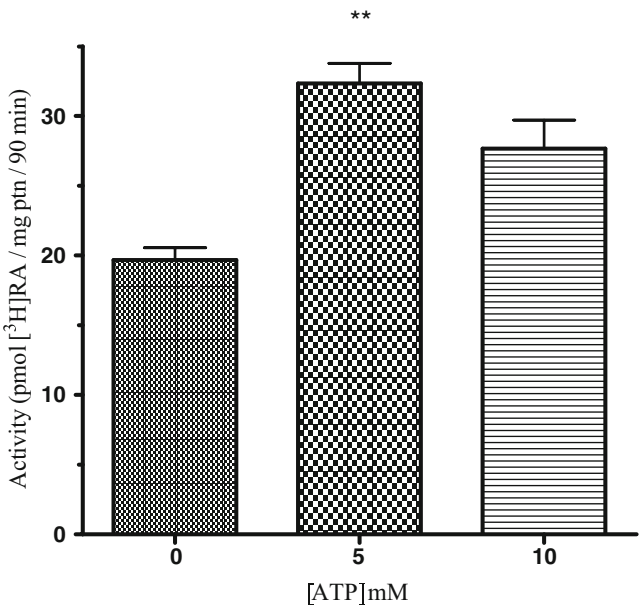

Fig. 1 Adrenal Glands Mitochondria are affected by RA. Retinoylation reaction ([^{3}H]RA, 100 nM) on rat adrenal glands mitochondria at 37 °C for 90 min in the absence and in the presence of 5 and 10 mM ATP. For other conditions see "Material and methods". The data represent the mean±SD of three independent experiments. ** P value<0.01 compared to the control (no ATP)

Fatty acid analysis of rat testes and adrenal gland mitochondrial membranes

Fatty acid composition of testes and adrenal gland mitochondrial membranes was determined. Briefly, mitochondria from both tissues were saponified with ethanolic KOH solution for 2 h at 90 °C. Fatty acids were extracted as described by Muci et al. (1992), 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.

Statistical analysis

Statistical analysis was performed by ANOVA followed by Dunnett's Multiple Comparison test. Values are shown as the mean ± SD of n independent experiments. Differences were considered significant at values of $P<0.05$.

Results

Retinoylation reaction on adrenal glands mitochondria

As shown in Fig. 1, the retinoylation extent (about 20 pmol/mg protein/90 min) for adrenal glands mitochondria without ATP in the incubation buffer reaches the maximum activity found for testes mitochondria (Genchi and Olson 2001; Cione and Genchi 2004) after the same incubation time. In addition, the concentration of CoASH, required to have the maximum activity in adrenal glands mitochondria without ATP in the incubation buffer, was lower than that used for testes mitochondria, as shown in Fig. 2 A–B.

Proteins retinoylated in cell fractions of adrenal glands homogenates and ATP quantification

In the standard conditions, without ATP in the incubation buffer, the mitochondrial fraction of the adrenal glands clearly was the most active to incorporate [^{3}H]RA (Table 1). All fractions other than the mitochondria showed relative activities lower than that of the homogenate. In addition endogenous ATP quantification in both mitochondrial preparations showed a very significant difference between testes and adrenal glands (Table 2).

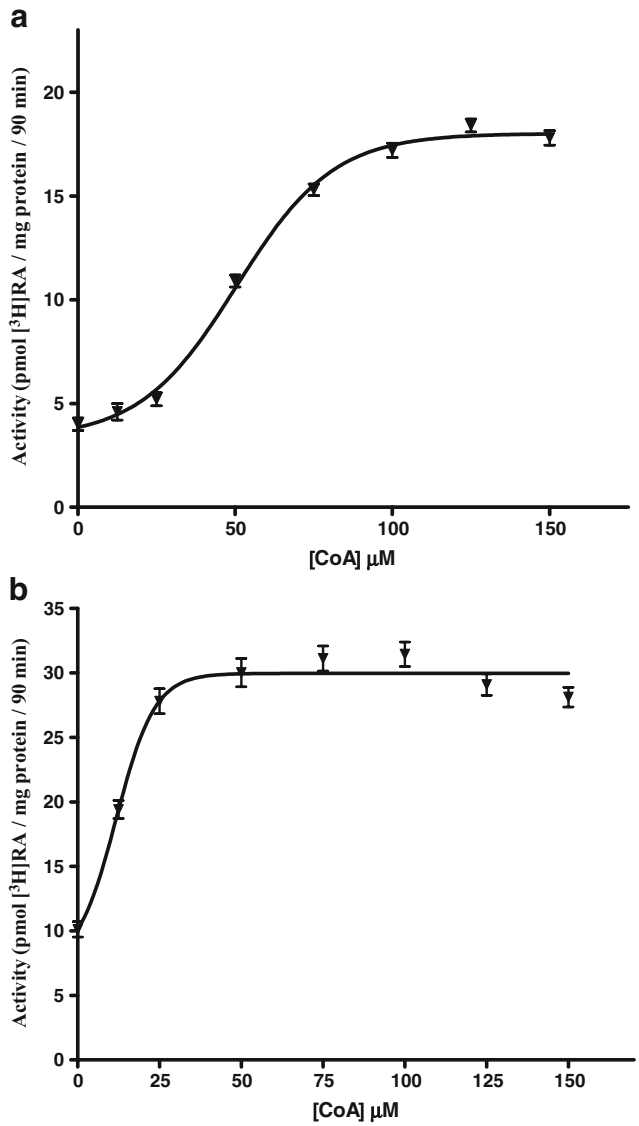

Fig. 2 Relationship between the retinoylation activity of testes and adrenal glands. The retinoylation activity of mitochondria from rat testes (A) and adrenal glands (B) is plotted as a function of indicated CoA concentrations under the standard conditions for 90 min at 37 °C, with 10 mM (A) and 5 mM ATP (B) respectively. The data represent the mean±SD of three independent experiments. Overall P value< 0.01 (**)

Table 1 Retinoylation reaction on adrenal glands cellular subfractions

Cell fraction	Incorporated radioactivity (pmol [^{3}H]RA/mg protein/90min)
Homogenate	8.32–8.09
Nuclei	6.89–6.53
Mitochondria	21.05–20.45
Microsomes	3.49–3.80
Cytosol	1.55–2.16

Incorporation of [^{3} H]RA (100 nM) into proteins by cellular fractions of adrenal glands in duplicate, incubated in a buffer without ATP for 90 min at 37°C, as described in "Materials and Methods"

Table 2 Mitochondrial ATP quantification

Adrenal Glands	1.3×10^{-2} M
Testes	5.2×10^{-8} M

Content of ATP in adrenal glands and testes mitochondria determined as in "Material and methods"

Time dependence of incorporation of [^{3}H]RA into protein(s) and activation energy determination

[^{3}H]RA incorporated into protein(s) increased with the time of incubation at 37°C. The incorporation rate was essentially linear for 20 min but then reached a plateau between 30 and 90 min, remaining constant until 150 min. Because the experimental intent was to compare the incorporation of radioactivity into protein(s) between testes and adrenal glands, a 90 min incubation time was selected for all subsequent studies (Fig. 3). The Arrhenius plot for adrenal glands mitochondria showed a straight line in the range of 5–40°C, and the value of activation energy for retinoylation reaction was 19.36 kJ/mol (not shown).

Effect of aminoglutethimide on retinoylation process

3-(4-aminophenyl)-3-ethyl-piperidine-2,6-dione, also known as aminoglutethimide, is a specific inhibitor of cytochrome P450scc (side chain cleavage) that had no effect on the retinoylation process in both testes and adrenal glands mitochondria, as shown in Fig. 4.

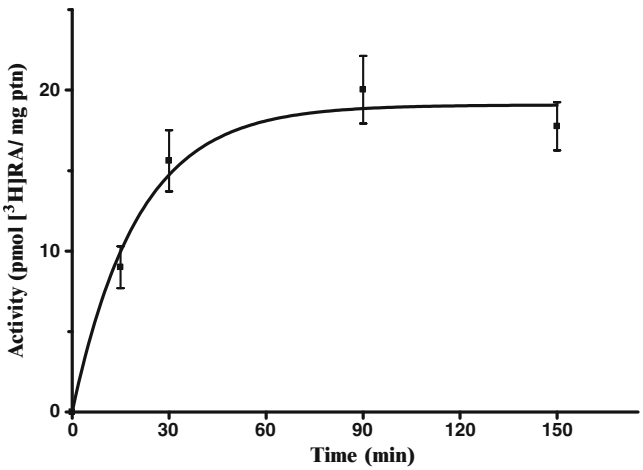

Fig. 3 Time-course of [^{3}H]RA Incorporation**.** Time-dependent incorporation of [^{3}H]RA (100 nM) into delipidated protein from rat adrenal glands mitochondria, incubated under standard assay conditions but without ATP at 37°C for the times indicated. The data represent the mean±SD of five independent experiments. Overall P value <0.01 (**)

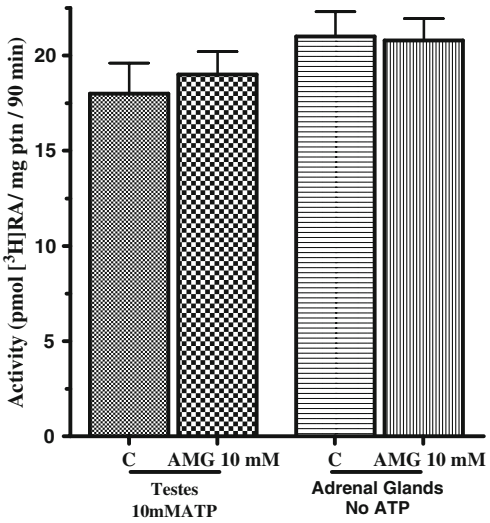

Fig. 4 Effect of aminoglutethimide on retinoylation reaction**.** Testes and adrenal glands mitochondria were incubated with 10 mM AMG but in the presence of 10 mM ATP (testes) and in the absence of ATP (adrenal glands). Other conditions are as described in "Material and methods". The data represent the mean±SD of three independent experiments

Fatty acid composition of adrenal glands and testes mitochondria

Significant differences in fatty acid composition of rat mitochondrial adrenal glands and testes were detected. A low percentage of docosahexaenoic acid (DHA, C22:6 ω3) and an even lower percentage of docosapentaenoic acid

Table 3 Fatty Acid composition (mol %) of rat mitochondrial membrane phospholipids from adrenal glands and testes

Fatty Acid	Adrenal Glands	Testes
14:0	0.48±0.01	0
16:0	11.52±0.35	28±0.41
18:0	31.96±0.85	4.7±0.14
18:1 ω9	9.94±0.02	6.2±0.19
18:2 ω6	9.25±0.11	3.4±0.10
20:4 ω6	34.30 ±1.12	23.4±0.96
20:5 ω3	n. d.	0.9±0.01
22:5 ω6	0.38±0.04	24.8±0.94
22:6 ω3	0.80±0.06	8.4±0.13
Σ saturated	44.11±1.19	32.7±1.02
Σ unsaturated	55.82±1.17	67.1±1.29
Σ sat./Σ unsat.	0.79±0.03	0.49±0.01

The fatty acid composition of adrenal glands and testes mitochondrial membranes was determined by gas-liquid chromatography as Muci et al. (1992) The data are the mean±SD of three independent determinations. Σ saturated=sum of saturated fatty acids; Σ unsaturated = sum of unsaturated fatty acids

(DPA, C22:5 ω6) were found in the membranes of mitochondria from adrenal glands compared to testes, together with a 85% increment of the stearic acid (C18:0) and a 30% increment of the arachidonic acid (ARA, C20:4 ω6) in adrenal glands. Therefore, significant changes were measured in the ratio of total saturated/unsaturated fatty acids between the two tissues as shown in Table 3. Figs. 5 A–C and 6 A–C highlighted the differences in saturated and unsaturated fatty acid composition, respectively, between adrenal glands and testes.

Discussion

Adrenal glands as steroidogenic tissue synthesize and secrete steroid hormones in response to pituitary hormones corticotrophin (ACTH) and/or luteotropin (LH) (Saez 1994; Waterman 1994). The binding of these peptide hormones to their receptors is coupled to the formation of cAMP that activate protein kinase A signalling pathway (Waterman and Bischof 1997; Richards and Hedin 1988). The adrenal glands are known for their help in regulating glucose levels through cortisol, for their natural anti-inflammatory activity, and for sex hormones supplementation to the organisms. Among these, the dehydroepiandrosterone (DHEA) can be converted into sex hormones, including estrone and testosterone. In this latter concern, it is known that retinoids are important in maintaining testes function. In fact, vitamin A-deficient diet causes the cessation of spermatogenesis, loss of mature germ cells and a reduction in testosterone level in mice and rat testes (Wolbach and Howe 1925; Ganguly et al. 1980; Appling and Chytil 1981). Previously we have shown that the retinoylation process occurs on pre-existing protein(s) growing of TM-3 cells and it is probably regulated by cAMP-activated enzymes; moreover under forskolin stimulus the retinoylation reaction was increased

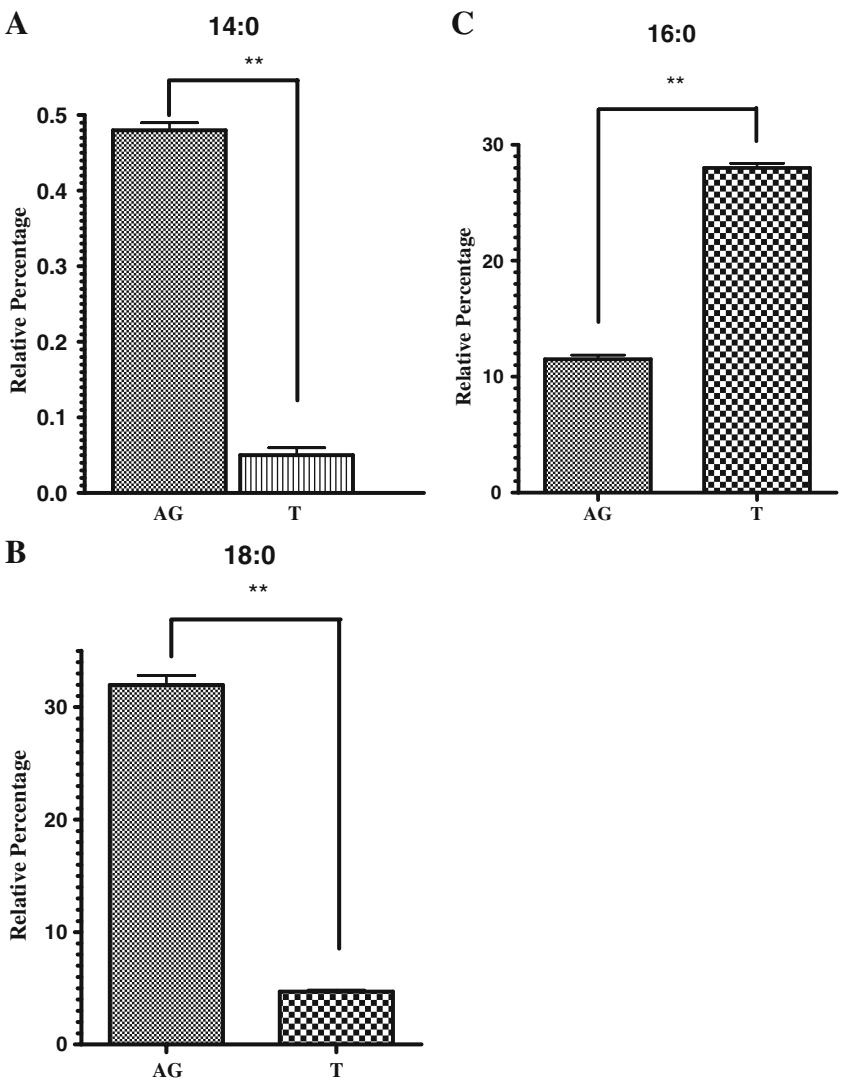

Fig. 5 Saturated fatty acid composition (mol %) of rat mitochondrial membrane phospholipids from adrenal glands and testes**.** The fatty acid composition of adrenal glands and testes mitochondrial membranes was determined by gas-liquid chromatography as Muci et al. (1992). Other conditions are as described in Table 3. The data represent the mean±SD of four independent experiments. ** P value <0.01

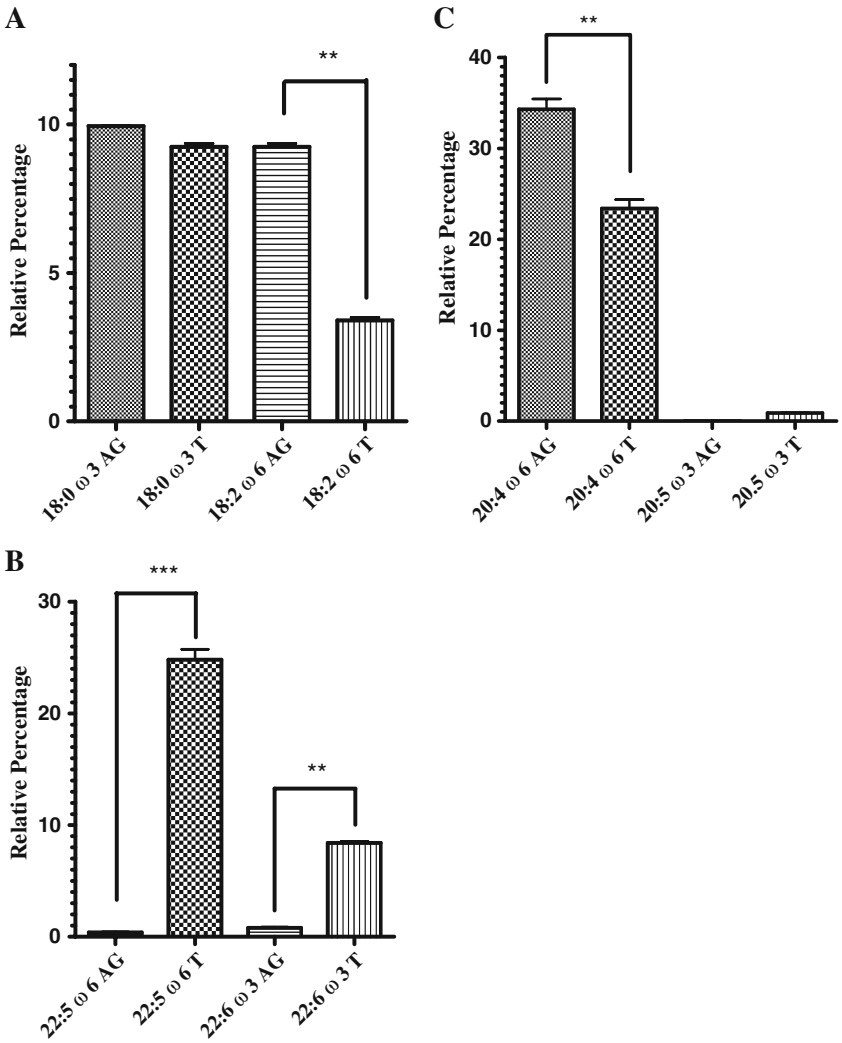

Fig. 6 Unsaturated fatty acid composition (mol %) of rat mitochondrial membrane phospholipids from adrenal glands and testes. Experimental conditions are as described in Table 3. The data represent the mean ± SD of three independent experiments. ** P value <0.01; *** P value <0.001

by 80% respect to the control value (Cione et al. 2005). In addition, we found that testosterone production and retinoylation reaction are concomitant and exhibit a positive correlation in Leydig cells (Tucci et al. 2008). Therefore, the aim of this paper is to evaluate the post translational modification triggered by RA in adrenal glands, since they are the second main steroidogenic organ in the male. In the mitochondrial compartment of adrenal glands after 90 min at 37°C, the value of [^{3}H]RA incorporated reached that of testes (Cione and Genchi 2004) with no need of ATP supplementation in the incubation buffer (Fig. 1). However among the cellular sub-fractions, adrenal glands mitochondria were 2.5-fold more active in binding retinoic acid than the homogenate (Table 1), providing a further evidence of a mitochondrial localization of the retinoylation system in steroidogenic tissues. The great difference in the endogenous ATP levels between the two organs, 1.3 x 10^{-2}M for adrenal glands and 5.2 x 10^{-8}M for testes, is the reason for which no extra ATP is required in the incubation buffer of the retinoylation reaction (Table 2). In our case, the Arrhenius plot of the retinoylation reaction showed that changes exist in the process of the transferring of the retinoyl moiety to the protein(s) between adrenal glands and testes: the adrenal glands activation energy was found equal to 19.36 kJ/mol in contrast with the 43.5 kJ/mol value determined for testes (Cione and Genchi 2004). Most likely our retinoylated protein(s), such as the enzymatic complex that allows the transfer of the retinoyl moiety, are embedded within the inner mitochondrial membrane and/or are localized on both sides of the membrane because the retinoylation does not require external ATP supplementation to occur. A further evidence of it comes from Genchi and Olson (2001) as mitoplasts from testes are still labelled with ^{3}HRA. In addition, the concentration of CoASH, required for the retinoylation process was lower in adrenal glands mitochondria than in testes as shown in Fig. 2 A–B. Several proteins in Leydig cells are regulated by a cAMP-dependent pathway and are involved in steroidogenesis;

first of all, the cytochrome P450scc (side chain cleavage) (Mellon and Vaisse 1989) that catalyzes the conversion of cholesterol to pregnenolone. In our experimental procedures the aminoglutethimide a specific inhibitor of cytochrome P450scc had no effect on the retinoylation process in both testes and adrenal glands mitochondria giving us the proof that this enzyme is not involved in the retinoylation process as shown in Fig. 4.

Moreover, differences in fatty acid composition in mitochondrial rat preparation of adrenal glands and testes were highlighted. Fig. 5 (A–C) shows the differences in saturated fatty acid composition. In particular a lower percentages of myristic acid (14:0), as well as stearic acid (18:0), were found in the testes mitochondrial membranes than in adrenal gland, about 10-fold and 7-fold less, respectively as shown in Fig. 5A–B. In addition, a 3-fold higher amount of palmitic acid (16:0) was found only in testes mitochondria (Fig. 5C). The differences in unsaturated fatty acid composition are shown in Fig. 6 (A–C). A much lower percentage of docosapentaenoic acid (DPA, C22:5 ω6) about 65-fold less and a lower percentage of docosahexaenoic acid (DHA, C22:6 ω3) about 10-fold less were found in the adrenal glands membranes of mitochondria compared to testes (Fig. 6B). In the meanwhile, about a 30% higher amount of arachidonic acid (ARA, C20:4 ω6) was found in adrenal gland mitochondria (Fig. 6C). ARA has the important physiological function of maintaining membrane content and permitting the activity of enzymes of the respiratory chain (Vazquez-Memije et al. 2005): in the case of the retinoylation reaction the difference in the ARA content could contribute to the different behaviour of the two tissues in incorporating RA. Therefore, the about 2-fold change in the ratio of total saturated/unsaturated fatty acids between adrenal glands and testes leads us to postulate a relationship between fatty acids and the retinoylation process (Table 3). The different fluidity of the membranes, deriving from the fatty acids composition, should justify the different values of the retinoylation and the activation energy between the adrenal glands and the testes. It should be stressed that the membrane lipid composition, the degree of unsaturation and the length of the fatty acid chains play important roles in determining the influence of membranes lipids with respect to the specific enzyme activities in the mitochondria (Brenner 1984; Daum 1985; Crider and Xie 2003).

In conclusion, in the present study we demonstrated that RA action by the retinoylation process on protein(s) of rat adrenal glands and testes mitochondria is steroidogenic tissue dependent, that cytochrome P450scc is not affected by the process and that probably docosapentaenoic acid (DPA, C22:5 ω6) should play a fundamental role to allow the transfer of the retinoyl moiety. However, since adrenal glands are arguably the neglected organ in endocrine toxicology (Harvey et al. 2007), it cannot be excluded that perhaps the retinoylation process is toxic/protective for that organ.

Acknowledgements We thank Dr G.V. Gnoni (University of Lecce) for performing fatty acids analysis. This research was supported by grants from Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR, Italia) and IRCCS Associazione Oasi Maria SS.

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