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Retinoic acid-induced testosterone production and retinoylation reaction are concomitant and exhibit a positive correlation in Leydig (TM-3) cells

Paola Tucci · Erika Cione · Giuseppe Genchi

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Abstract Retinoic acid (RA) exerts diverse biological effects in the control of cell growth in embryogenesis and oncogenesis. The effects of RA are thought to be mediated by the nuclear retinoid receptors; however, not all the effects of RA can be explained by the nuclear receptor pathways. Indeed, retinoylation is another mechanism of action elicited by RA. In growing TM-3 Leydig cell cultures, the extent of retinoylation depends in a saturable manner on the initial concentration of ^{3}H-RA, time and cell number. In addition, dose-response curves for RA-induced testosterone production and retinoylation are concomitant and exhibit a positive correlation. In the present study we demonstrate that RA is able to influence a retinoylation reaction on protein(s) probably involved on steroidogenesis.

Keywords Retinoic acid · Testosterone · Retinoylation · TM-3 cells · Protein

Introduction

All-*trans*-retinoic acid (RA), a carboxylic acid derivative of vitamin A (retinol), exerts diverse biological effects in the control of cell growth, in embryonic development and oncogenesis (De Luca 1991; Sporn 1994). Clarification of the mechanisms of the RA action is of critical interest, since various biological effects of RA have been reported. It is thought that the effects of RA are mediated through two classes of nuclear receptors, the RA receptors and the retinoid X receptors (De Luca 1991; Sporn 1994). These receptors belong to the steroid/thyroid hormone receptor super family, which regulate gene transcription through binding to a specific DNA sequence, resulting in an increased or decreased synthesis of specific proteins. Nevertheless, recent evidence (Delia et al. 1993; O'Connell et al. 1996; Ahuja et al. 1997) suggests that other retinoid response pathways, independent of the nuclear receptors, may exist. Thus, despite our knowledge of the nuclear receptors for RA, how RA can exert a great diversity of biological effects is still not fully understood.

Other mechanisms, other than nuclear receptors, may be involved in RA's biological effects. Retinoylation (acylation of proteins by RA) is another mechanism of the RA action (Takahashi and Breitman 1989; Takahashi and Breitman 1991; Takahashi et al. 1991a, b). The retinoylation mechanism involves the formation of a retinoyl-CoA intermediate (Wada et al. 2001) and the subsequent transfer and the covalent binding of the retinoyl moiety to protein(s) (Renstrom and DeLuca 1989). Recently, the enzymatic formation of retinoyl-CoA from RA has been demonstrated in crude extracts of rat tissues (Renstrom and DeLuca 1989; Wada et al. 2001). Previously, the formation of retinoylated proteins from retinoyl-CoA in subcellular fractions (membrane, nuclei, mitochondria, microsome and cytosol) of vitamin A-deficient rat kidney was studied (Renstrom and DeLuca 1989). The latter study demonstrated that the incorporation of retinoyl-CoA into proteins was enzymatic judging from the heat inactivation and SDS denaturation of subcellular fractions containing enzyme or/and protein substrates (Kubo et al. 2005).

Paola Tucci and Erika Cione are equally contributed.

P. Tucci · E. Cione · G. Genchi
Dipartimento Farmaco-Biologico, Laboratorio di Biochimica,
Edificio Polifunzionale, Università della Calabria,
87036 Rende, CS, Italy

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

RA is incorporated into proteins of cells in culture (Takahashi and Breitman 1989; Takahashi and Breitman 1990; Takahashi and Breitman 1994; Breitman and Takahashi 1996; 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).

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 proteins. It is important to note that both db-cAMP (dibutryl-cyclicAMP) and forskolin increase the retinoylation level on the protein of TM-3 cells by about 70% and 80% respectively (Cione et al. 2005). These results suggested that the retinoylation reaction could be regulated by cAMP-activated enzymes.

In this study, we have seen that RA-induced steroidogenesis and retinoylation are parallel events and exhibit a positive correlation. Thus, retinoylation may be a true physiological reaction of RA, opening a new perspective on the studies investigating the mechanisms regulating the effects of retinoids in testicular steroidogenesis.

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), human chorionic gonadotropin (hCG) and $MnCl_2$ were obtained from Sigma-Aldrich (Milano, Italia); DMEM/F12, fetal calf serum (FCS), horse serum (HS), penicillin and streptomycin from Gibco (Invitrogen Life Technologies, Italia); scintillation cocktail from Packard Bioscience (Groningen, The Netherlands). All other chemicals used were of analytical reagent grade.

Cell cultures

The TM-3 cell line, derived from testes of immature BALB/c mice, was originally characterized, based on its morphology, hormone responsiveness and metabolism of steroids (Mather 1980). This cell line was kindly provided by Dr. S. Andò (University of Calabria) and cultured in DMEM/F12 medium supplemented with 2 mM glutamine, serum (5% HS and 5% FCS) and 1% of a stock solution containing 10,000 IU/ml penicillin and 10,000 μg/ml streptomycin. Cell cultures were grown on Petri plastic tissue culture dishes at 37 °C in a humidified atmosphere of 5% CO_2 in air. Cells from exponentially growing stock cultures were removed from the plate with trypsin (0.05% *w*/*v*) and EDTA (0.02% *w*/*v*). The trypsin/EDTA was inhibited with an equal volume of DMEM/F12 medium supplemented with serum. Cell number has been estimated with a Burker camera and cell viability by trypan blue dye exclusion. The medium was changed twice weekly, and the cells were subcultivated when confluent.

Incorporation of radioactive RA

TM-3 cells growing exponentially were removed by trypsin/EDTA, harvested by centrifugation and resuspended at a concentration of 8×10^4 cells/0.5 ml in serum-free DMEM/F12 medium, supplemented with 2 mM glutamine and 1% of a stock solution containing 10,000 IU/ml penicillin and 10,000 μg/ml streptomycin. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air for 24 h in the presence of 100 nM ^{3}H-RA (about 350,000 cpm). Tritiated all-*trans*-retinoic acid and unlabelled RA were dissolved in absolute ethanol and diluted into the growth medium such that the final concentration was no higher than 0.1%. The cells, washed twice in PBS (phosphate-buffered saline, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 136.9 mM NaCl, pH 7.2), were collected by scraping in 100 μl ice-cold lysis buffer [50 mM HEPES, pH 7.5, 150 mM NaCl, 10% (*v*/*v*) glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 μg/ml aprotinin, 50 mM phenyl methyl sulfonyl fluoride and 50 mM sodium orthovanadate]. The lysates were incubated for 30 min on ice with intermittent vortexing, followed by centrifugation in an Eppendorf centrifuge at 14,000 rpm/10 min. The supernatants were treated with cold acetone and centrifuged. The pellets were washed twice in PBS, dried, solubilized in 200 μl of 1% SDS, 40 mM Tris, 2 mM EDTA, pH 7.5, and counted in a TriCarb 1600TR liquid scintillation counter (Packard). The counting efficiency was about 70%.

RIA of testosterone

To assay the ability of Leydig cells to produce testosterone *in vitro*, TM-3 cells (1×10^6/ml) were incubated for 24 h in the presence of RA (0–200 nM). After the incubation period, media were collected and stored for subsequent assay of testosterone by RIA. Medium testosterone was assayed using the solid-phase RIA kit obtained from BIOGEMINA sas (Catania, Italia). The sensitivity of the testosterone assay was 0.001 ng/dl, the intra- and inter-assay coefficient of variations was 3.1% and 5.4%, respectively.

Statistical analyses

Statistical differences were determined by one-way analysis of variance (ANOVA) followed by Dunnet's method, and the

results were expressed as mean±SD from three independent experiments. Differences were considered statistically significant for $P<0.05$.

Results

Retinoylation of TM-3 cellular proteins

Retinoylation (acylation by RA of protein) is a non-genomic mechanism by which RA may act on cells. In order to determine the best conditions for the retinoylation reaction, TM-3 cells were plated at 8×10^4 cells/0.5 ml DMEM/F12 supplemented with 2 mM glutamine, 5% HS and 5% FCS and treated with different concentrations of ^{3}H-RA for 24 h. During exponential growth of TM-3 cells in the presence of ^{3}H-RA, there is an increase in the incorporation of ^{3}H-RA (Fig. 1a). This incorporation is characterized by a very rapid initial uptake that makes it difficult to obtain true zero time values. The uptake was shown to plateau at about 2.5–5 μM (2296±120 and 2496±132 fmoles/8×10^4cells) ^{3}H-RA. We have chosen 100 nM ^{3}H-RA with a binding activity of 597±30 fmoles/8×10^4cells, because Cione et al. (2005) showed this concentration (100 nM) to be the maximum binding activity after 24 h of incubation. Figure 1b shows that the extent of retinoylation activity as a function of cell number increases linearly until about 8×10^4 cells (24 h) and it is then constant with a value of 639±45 fmol/20×10^4 cells.

Therefore, dose-response studies were carried out in the following conditions: 8×10^4 cells were plated in 0.5 ml medium and were incubated at 24 h with ^{3}H-RA (100 nM final concentration) at 37 °C in a humidified atmosphere of 5% CO_2 in air.

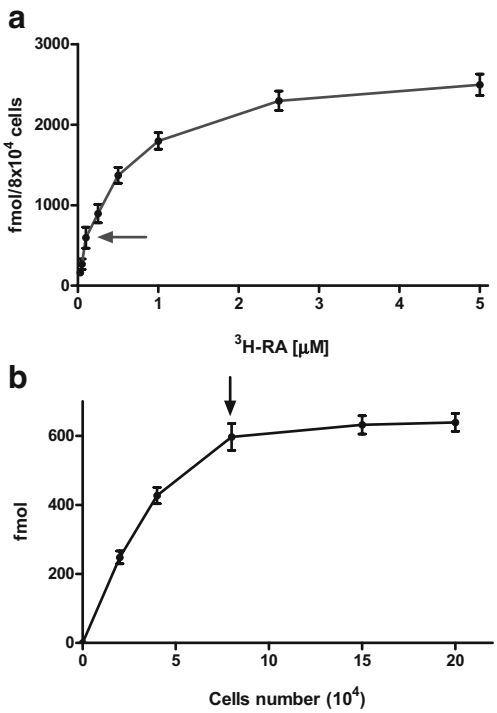

Fig. 1 Retinoylation reaction on TM-3 proteins. (**a**) TM-3 cells were plated at 8×10^4 cells/0.5 ml DMEM/F12 supplemented with 2 mM glutamine, 5% HS and 5% FCS at 37 °C in a humidified atmosphere of 5% CO_2 in air and treated with indicated concentrations of ^{3}H-RA for 24 h. (**b**) TM-3 cells were plated at 2, 4, 8, 15 and 20×10^4 cells/0.5 ml DMEM/F12 supplemented with 2 mM glutamine, 5% HS and 5% FCS and were incubated at 24 h with ^{3}H-RA (100 nM final concentration) at 37 °C in a humidified atmosphere of 5% CO_2 in air. Results are presented as the mean ± SD for three individual experiments

Dose-response

TM-3 cells were treated for 24 h at 37 °C with unlabeled RA (0–200 nM) and then, after washing procedure in PBS, the cells were incubated for 24 h with 100 nM ^{3}H-RA. In Fig. 2a this pre-incubation RA effect is shown. After pre-incubation with 10 nM cold RA, the retinoylation activity was 680±46 fmol/8×10^4 cells, about 20% ($P<0.05$) higher than the control (570±34 fmol/8×10^4 cells). Then, pre-incubating TM-3 cells with 100 nM cold RA, there was a further increase of retinoylation activity (941±50 fmol/8×10^4 cells; $P<0.01$), about 70% higher than the control.

The effect of increasing of human chorionic gonadotropin (hCG) concentration, that shows similar effects to that of luteinizing hormone (LH) in the regulation of reproductive function, has been studied on TM-3 binding activity of RA in our previous study. The retinoylation activity increased in the presence of hCG with a maximum binding activity at 250 ng/ml (Cione et al. 2005). In addition, RA had no effect on the increase of the hCG-induced retinoylation reaction. As shown in Fig. 2b the retinoylation activity after pre-incubation with RA 100 nM plus hCG 100 ng/ml for 24 h was the same as with hCG 100 ng/ml alone.

In the presence of 20 and 200 μM $MnCl_2$, that is an inhibitor of both StAR protein and steroidogenetic enzymes, the retinoylation reaction was reduced respectively 28% ($P<0.05$) and 43% ($P<0.01$) in respect to the control value (Fig. 3).

To assay the ability of Leydig cells to produce testosterone in vitro, 1×10^6 cells were incubated for 24 h in the presence of RA (0–200 nM) and medium testosterone was assayed by RIA. It was found that RA induced a statistically significant increase in testosterone production at a concentration of 100 nM (7.98±0.86 ng/1×10^6 cells; $P<0.01$) and plateaued thereafter up to a dose of 200 nM (Fig. 4).

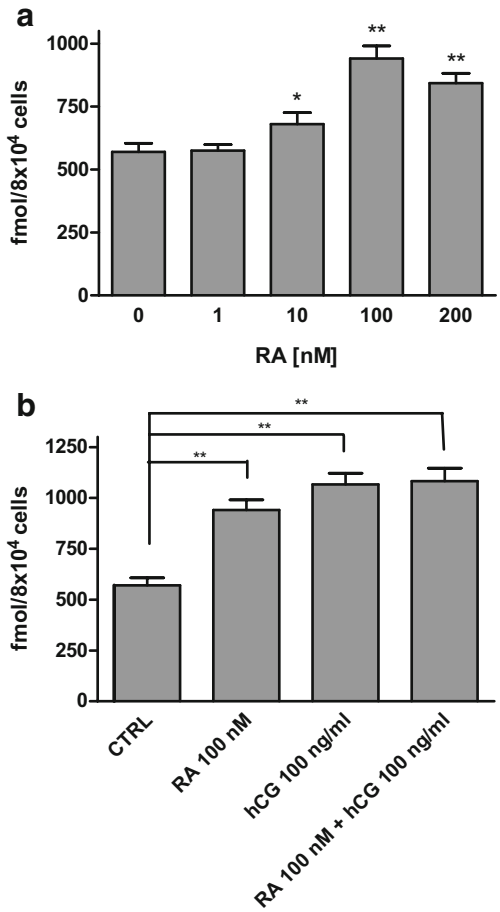

Fig. 2 Effect of RA and RA plus hCG on retinoylation reaction. After incubation for 24 h with (**a**) various concentration of RA and (**b**) RA 100 nM, hCG 100 ng/ml and RA 100 nM plus hCG 100 ng/ml, TM-3 cells were incubated with ^{3}H-RA (100 nM final concentration) for 24 h at 37 °C in a humidified atmosphere of 5% CO_2 in air. Results are presented as the mean±SD for three individual experiments. *P< 0.05 compared to the control; **P<0.01 compared to the control

Discussion

Several types of lipid modifications occur on many proteins. Some examples are myristoylation, palmitoylation, acetylation, phosphorylation, glycosylation and isoprenylation (Schultz et al. 1988; Towler et al. 1988). Retinoylation is one of these covalent modification reactions occurring on proteins. Biochemical similarities exist among retinoylation, palmitoylation and myristoylation. In fact, palmitic acid, myristic acid and RA covalently bind to pre-existing proteins via a thioester bond after the formation of a CoA-intermediate (Schultz et al. 1988; Towler et al. 1988; Renstrom and DeLuca 1989; Wada et al. 2001). Such binding of fatty acids is expected to change the physical properties of the proteins so that hydrophilic proteins can be converted into very hydrophobic ones. These modification reactions will influence the interactions of the proteins with cellular membranes, as well as the interactions with other proteins, lipids or even nucleic acids.

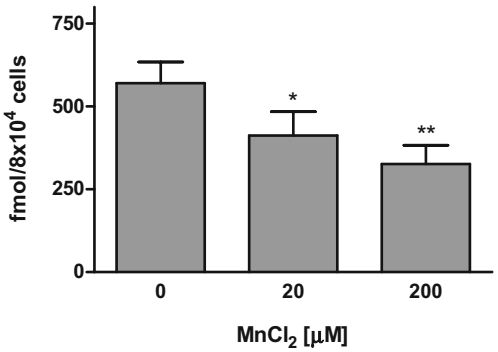

Fig. 3 Effect of $MnCl_2$ on retinoylation reaction. After incubation for 24 h with $MnCl_2$ 20 and 200 μM, TM-3 cells were incubated with ^{3}H-RA (100 nM final concentration) for 24 at 37 °C in a humidified atmosphere of 5% CO_2 in air. Results are presented as the mean±SD for three individual experiments. *P<0.05 compared to the control; **P<0.01 compared to the control

It is known that retinoids play an essential role in spermatogenesis in rodents. A vitamin A-deficient diet caused 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).

We have previously shown that in growing TM-3 cultures the retinoylation reaction occurs on pre-existing proteins of cells and involves the formation of a thioester bond (Cione et al. 2005). Our previous results have shown that in the presence of db-cAMP (a synthetic analogue of cAMP) and in the presence of 25 μM forskolin, that activates the adenylate cyclase and raises the cAMP intracellular concentration, the retinoylation reaction increased respectively of 70% and 80% with respect to the control value (Cione et al. 2005).

These results suggested that the retinoylation reaction increased with the concentration of the cAMP in the Leydig cells and could be regulated by cAMP-activated enzymes.

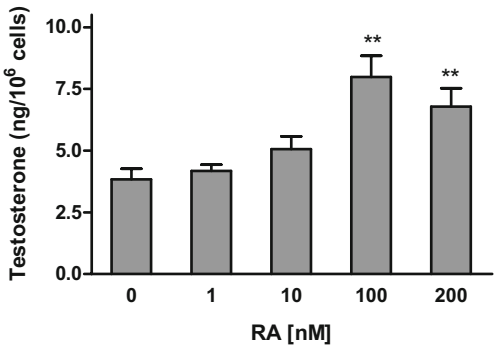

Fig. 4 Effect of RA on testosterone production in TM-3 cells. 1×10^6 cells were treated with various concentration of RA for 24 h and then the amount of testosterone in the medium was determined via RIA as described in "Materials and methods". Results are presented as the mean±SD for three individual experiments. **P<0.01 compared to the control

Several proteins in Leydig cells are regulated by a cAMP-dependent pathway and are involved in steroidogenesis. Among these, particularly interesting is the role of StAR protein that plays an essential role in the delivery of cytosolic cholesterol into the mitochondrial inner membrane, and is an acutely regulated and rate-limiting step for steroid hormone synthesis (Stocco 2001).

The objective of this study was to determine if the retinoylation reaction has a direct influence on steroidogenesis in cultured rat Leydig cells. It was found in the present study that the extent of retinoylation was dependent on the concentration of ^{3}H-RA and cell number (Fig. 1a,b). In particular the retinoylation activity was of 597±27 fmol/8×10^{4} cells after 24 h incubation with 100 nM ^{3}H-RA.

These findings were extended in this study by showing that the activation of LH receptors by hCG increased the retinoylation binding activity (Cione et al. 2005) and that a high rate of retinoylation occurs on protein(s) in TM-3 cells in the presence of RA (100–200 nM; Fig. 2a). In response, it was observed that RA was able to stimulate testosterone biosynthesis in these cells in the same doses where the retinoylation reaction was found to be enhanced (Fig. 4).

These results suggest that the retinoylation reaction has an important physiological role in the regulation of testicular steroidogenesis. RA in mouse testes is present constitutively at a concentration of 7–10 pmol/g or approximatively 7–10 nM (Deltour et al. 1997; Kane et al. 2005). After pre-incubation with unlabeled RA (Fig. 2a) and washing procedure with PBS, we did not check the final concentration of it within the cells. The aim of the pre-incubation was just to show that RA has the same effect as induced by db-cAMP, forskolin and hCG, that is, an increase of testosterone level and a corresponding increase of the retinoylation reaction (Cione et al. 2005). In addition in Fig. 2b, we have shown that the pre-incubation with RA plus hCG has no effect on the retinoylation reaction compared to the cells treated with RA or hCG alone. According to Chaudhary et al. (1989), RA had no effect on the rise of hCG-stimulated testosterone biosynthesis. Dose-response curves for the regulation of steroidogenesis by retinoic acid and retinoylation are similar and exhibit a positive correlation. In agreement with this, a decrease of retinoylation activity of 28% and 43% with respect to the control value in the presence of 20 and 200 μM $MnCl_2$ (steroidogenesis inhibitor) respectively was observed (Fig. 3).

Putative protein targets of the retinoylation reaction could be factors involved in the complex mechanisms activating StAR protein and regulating the delivery of cytosolic cholesterol, although other proteins cannot be excluded.

In conclusion, in the present study we have demonstrated that RA is able to influence a retinoylation reaction on protein(s) probably involved on steroidogenesis. At present, the protein that is retinoylated is not known; but further characterization and identification of the retinoylated protein are major objectives for the immediate future.

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