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All-*trans*-retinoic acid induces apoptosis in Leydig cells via activation of the mitochondrial death pathway and antioxidant enzyme regulation

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Abstract In addition to playing a fundamental role in diverse processes, such as vision, growth and differentiation, vitamin A and its main biologically active derivative, retinoic acid (RA), are clearly involved in the regulation of testicular functions. The present study was undertaken to examine the direct effect of RA treatment on Leydig (TM-3) cells. TM-3 cells were cultured and treated with varying concentrations of RA for 24h. High doses of RA (1–20μM) induced a decrease in cell vitality and an increase in lipid peroxidation. RA treatment also induced a corresponding increase in apoptosis in the same cells in a dose-dependent manner. Apoptosis proceeded via the mitochondrial dependent pathway, as demonstrated by the release of cytochrome c, caspase-3 enzymatic activation and DNA fragmentation. Conversely, at physiological doses (0.1–500nM) RA did not increase lipid peroxidation or cell death and resulted in an increase of antioxidant enzyme activity.

Keywords Retinoic acid · Apoptosis · Leydig cells · Mitochondria · Antioxidant enzymes

Abbreviations

RA	All-*trans*-retinoic acid
CAT	Catalase
GST	Glutathione *S*-transferase
SOD	Superoxide dismutase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MPMS	1-methoxyphenazine methosulfate
HS	Horse serum
FCS	Foetal calf serum
PBS	Phosphate buffered saline
TBARS	Thiobarbituric acid reactive species
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid

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Introduction

Retinoids are natural and synthetic substances structurally related to vitamin A (retinol). They exert antiproliferative and differentiation-inducing effects on cancer cells and are used in the prevention and treatment of certain types of human cancer and precancerous lesions (Gudas et al. 1994; Lotan 1996). Their action is mediated by two types of receptors, the retinoic acid receptors (RARs) and retinoid X receptors (RXRs) (Chambon 1996) belonging to the steroid/thyroid hormone receptors superfamily. There is substantial evidence *in vitro* that retinoids exert their effect through the induction of apoptosis in tumor cells including hepatoma, leukemia, breast cancer and embryonal carcinoma cell lines (Nagy et al. 1995; Nakamura et al. 1995; Horn et al. 1996; Kim et al. 1996; Li et al. 1999). The action of retinoids in promoting apoptosis may explain the anti-carcinogenic properties of these compounds.

Apoptosis, a specific form of programmed cell death, is a biological process that plays a crucial role in normal development and tissue homeostasis (Woodle and Kulkarni 1998). Apoptosis is characterized by morphological changes including progressive cell shrinkage with condensation, fragmentation of nuclear chromatin and membrane blebbing (Kerr et al. 1997). The ability of the cell to undergo or resist apoptosis is modulated by several factors

including the expression of mitochondrial-associated pro- and anti-apoptotic Bcl-2 family member proteins. Interestingly the expression of Bcl-2 has been observed to be decreased in cells treated with retinoids (Delia et al. 1992; Agarwal and Mehta 1997; Lu et al. 1997; Toma et al. 1997).

Recent observations indicate that mitochondria play an important role in the induction of apoptosis (Liu et al. 1996; Li et al. 1997); indeed Bcl-2 is localized in the outer mitochondrial membrane (Nakai et al. 1993) and, among other actions, prevents the release of apoptogenic factors such as cytochrome c (Liu et al. 1996). Cytochrome c, after its displacement to cytosol, stimulates the formation of a complex with Apaf-1 and caspase-9 in the presence of dATP (Liu et al. 1996). This association leads to the activation of caspase-9 which, in turn, cleaves and activates caspase-3 and hence triggering DNA fragmentation (Li et al. 1997). Caspases are known to mediate a crucial stage of the apoptotic process and are expressed in many mammalian cells (Garcia-Calvo et al. 1999). Of particular interest is caspase-3, the most widely studied member of the caspases family and one of the key executors of apoptosis, which is responsible for several proteolytic cleavage of many proteins (Cohen 1997; Nagata 1997).

In this work we investigated the effect of RA on Leydig cells, using the TM-3 cell line. We found that high doses of RA induce apoptosis in TM-3 cells and that this effect is primarily associated with the mitochondrial pathway involving cytochrome c release and caspase-3 activation. As higher RA doses induced lipid peroxidation whilst decreasing cell viability we also investigated the effects of physiological RA treatment (at nM concentration) in TM-3 cells. In order to detect changes in the antioxidant defences we measured the glutathione *S*-transferase (GST), the superoxide dismutase (SOD) and the catalase (CAT) activities in RA treated and non-treated TM-3 cells. RA increased the activity of antioxidant enzymes under physiological doses, thereby preventing oxidative cell damage as shown by the malondialdehyde (MDA) content and cell viability.

Materials and methods

Chemicals

All-*trans*-retinoic acid, EGTA, EDTA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 1-methoxyphenazine methosulfate (MPMS) were obtained from Sigma-Aldrich (Milano, Italia). DMEM/F12, foetal calf serum (FCS), horse serum (HS), penicillin and streptomycin were obtained from Gibco (Invitrogen Life Technologies, Italia). GST, SOD and CAT enzymatic activities were assayed using kit obtained from Sigma-Aldrich (Milan, Italia). All other chemicals used were of analytical reagent grade. RA was prepared prior to use by dilution in 100% ethanol, evaporated under nitrogen, suspended in the same volume of DMSO and diluted into medium at the indicated concentrations. All experiments involving RA were performed under yellow light, and the tubes and culture plates containing RA were covered with aluminium foils.

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. This cell line was kindly provided by Dr. S. Andò (University of Calabria) and cultured in DMEM/F12 medium supplemented with 2mM glutamine, serum (5% HS and 5% FCS) and 1% of a stock solution containing 10,000IU/ml penicillin and 10,000μg/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 adding to the plates an equal volume of DMEM/F12 medium supplemented with serum. The medium was changed twice weekly, and the cells were subcultivated when confluent. Cell number was estimated using a Burker camera.

MTT cytotoxicity assay

Cell viability was determined by MTT assay (Mosmann 1983). In order to determine the cytotoxicity of RA cells (2×10^5cells/ml) were treated with RA at different concentrations for 3 and 24h. Control cells were treated with vehicle alone. One hundred microlitres of MTT (5mg/ml) was added to each well and the plates incubated for 4h at 37°C. Subsequently 1ml 0.04N HCl in isopropanol was added to solubilize the cells. The absorbance was measured with the Ultrospec 2100 pro spectrophotometer (Amersham-Biosciences) at a test wavelength of 570nm with a reference wavelength of 690nm. The optical density (O.D.) was calculated as the difference between the absorbance at the reference wavelength and that at the test wavelength. Percent viability was calculated as (O.D. of drug-treated sample/control O.D.) ×100.

Oxidative stress parameters

Following sonication of TM-3 cells in PBS (phosphate-buffered saline, 1.5mM KH_2PO_4, 8.1mM Na_2HPO_4, 136.9mM NaCl, pH7.2) buffer the crude homogenate was divided into two equal parts. One part was processed for assay of lipid peroxidation, while the second part was centrifuged at 10,000rpm for 5min and the supernatant

utilized in assays for glutathione *S*-transferase, superoxide dismutase and catalase activities.

Lipid peroxidation and antioxidant enzymes activity

The level of lipid peroxidation was assayed through the formation of thiobarbituric acid reactive species (TBARS) during an acid-heating reaction as previously described (Esterbauer and Cheeseman 1990). Briefly, the samples were mixed with 1ml of 10% trichloroacetic acid (TCA) and 1ml of 0.67% thiobarbituric acid (TBA), then heated in a boiling water bath for 15min. TBARS were determined by the absorbance at 535nm and were expressed as malondialdehyde equivalents (nmol/mg protein).

The enzymatic activities in the cell samples of SOD, CAT and GST were determined by the methods of Das et al. (2000), Aebi (1974) and Habig et al. (1973) respectively. Protein concentration in samples was estimated by the Lowry method (Lowry et al. 1951).

Immunoblot analysis of cytochrome c release

Cytochrome c was detected by western blotting in mitochondrial and cytoplasmatic fractions. The cells were harvested by centrifugation at 1,200 × g for 10min at 4°C. The pellets were suspended in 36μl lysis buffer (250mM sucrose, 1.5mM EGTA, 1.5mM EDTA, 1mM $MgCl_2$, 25mM Tris-HCl, pH6.8, 1mM DTT, 10μg/ml aprotinin, 50mM phenylmethylsulfonylfluoride and 50mM sodium orthovanadate) and then 4μl of 0.1% digitonine was added. The cells were incubated for 15min at 4°C and centrifuged at 12,000rpm for 30min at 4°C. The resulting mitochondrial pellet was resuspended in 3% Triton X-100, 20mM Na_2SO_4, 10mM PIPES and 1mM EDTA, pH7.2, and centrifuged at 12,000rpm for 10min at 4°C. Equal amounts of protein (2–5μg) were resolved by 15% SDS-PAGE and electrotransferred to nitrocellulose membranes. The membranes were incubated in blocking buffer over night at 4°C, followed by incubation with 1:1,000 sheep polyclonal antihuman cytochrome c antibody (2h, room temperature) and then with HRP-conjugated (horse radish peroxidase-conjugated) secondary antibody (1:2,000) for 2h at 4°C. Peroxidase activity was visualized with the Amersham Pharmacia Biotech ECL system according to manufacturer's instructions. The cytochrome c protein content was determined densitometrically. Immunoblotting for β-actin was carried out to confirm equal loading.

Western blot determination of caspase-3

Leydig cells were lysed with ice-cold PBS (pH7.4) containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitors (1mM phenylmethylsulfonylfluoride, 10mg/ml aprotinin and 10mg/ml leupeptin). Lysates were centrifuged (13,000rpm at 4°C for 30min) and supernatant protein content determined. Equal amounts of protein (20μg) were resolved by 15% SDS-PAGE, electrotransferred to nitrocellulose membranes and immunoblotted for caspase-3 as previously described.

DNA extraction and agarose gel electrophoresis

Agarose gel electrophoresis of extracted DNA was performed to detect damage to nuclear chromatin, a characteristic biochemical feature of apoptosis, indicated by laddering of oligonucleosomal fragments (180–200bp) as described by Mu et al. (2001).

Briefly, 1×10^6 cells, treated without and with 5, 10 and 20μM of RA for 24h, were trypsinized and washed with PBS. Then the cells were spun down and resuspended in 0.5ml of lysis buffer (50mM Tris-HCl, pH 7.8, 10mM EDTA, and 0.5% SDS). RNase A was added to a concentration of 0.5mg/ml and incubated at 37°C for 60min. The protein content was degraded with proteinase K (0.5mg/ml) at 50°C for 60min. DNA was obtained with two extractions. The first extraction was carried out with an equal volume of phenol–chloroform–isoamylic alcohol (25:24:1) and the second with chloroform–isoamylic alcohol (24:1). DNA was precipitated from the upper aqueous phase with 0.1vol. of 3M sodium acetate, 2.5vol. of ice-cold ethanol and left at −20°C over night. After centrifugation the DNA pellet was solubilized in 25μl sterile water. DNA fragments were separated by electrophoresis in 1.2% agarose gel and visualized by staining with ethidium bromide.

Lactate dehydrogenase assay

The amount of lactate dehydrogenase (LDH) released by the cells was determined as described by Abe and Matsuki (2000) with little modifications. The culture supernatant (250μl) was mixed with 250μl of the LDH substrate mixture (2.5mg/ml L-lactate lithium salt, 2.5mg/ml NAD^+, 100μM MPMS, 600μM MTT, and 0.1% Triton X-100 in 0.2M Tris-HCl buffer, pH 8.2). The reaction was carried out for 5min at 37°C and stopped by adding 0.5ml of 0.04N HCl in isopropanol. The absorbance was measured with the spectrophotometer at a test wavelength of 570nm, and a reference wavelength of 655nm. In these assay conditions, MTT was converted into MTT formazan in proportion to LDH activity. LDH release was calculated as (sample LDH-blank)/(total LDH-blank) ×100.

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 n independent experiments. Differences were considered statistically significant for $P < 0.05$.

Results

Effect of RA on vitality of TM-3 cells

In order to examine the cytotoxic effect of RA on TM-3 cells, they were cultured with RA in a concentration range from 0.1 to 20μM for 24h and MTT assay was carried out with cells cultured in RA-free media as control. No significant change in viability was observed in TM-3 treated in 0–0.5μM RA concentration range (Fig. 1A). Upon incubation with RA concentration of 1–20μM a significant reduction of vitality, however, observed. As shown in Fig. 1A, the cell viability was less than 20% after exposure to 20μM RA for 24h. The effect of RA at μM doses on cell viability was also time-dependent, since the cell survival declined drastically following an increase in the treatment time from 3 to 24h in cells incubated with 10 and 20μM RA (Fig. 1B). The present results show that RA exerts a cytotoxic effect on TM-3 cells in a concentration and time dependent manner (Fig. 1A,B).

Biochemical markers of apoptosis

In order to examine whether apoptosis was the cause of the loss of cell viability, the cells were treated with μM doses of RA and subjected to various biochemical analyses to detect biomarkers of apoptosis. Mitochondria are vulnerable targets for toxic injury and act as crucial executors of apoptosis by releasing cytochrome c into the cytoplasm (Cai et al. 1998). 24h post-exposure TM-3 cells were collected and the cytosolic protein fraction was assayed for cytochrome c release. As shown in Fig. 2A,B, cytochrome c was detectable in cytoplasm following exposure to 0.5μM RA and the protein increased significantly at higher RA exposure (10–20μM) when compared to time matched

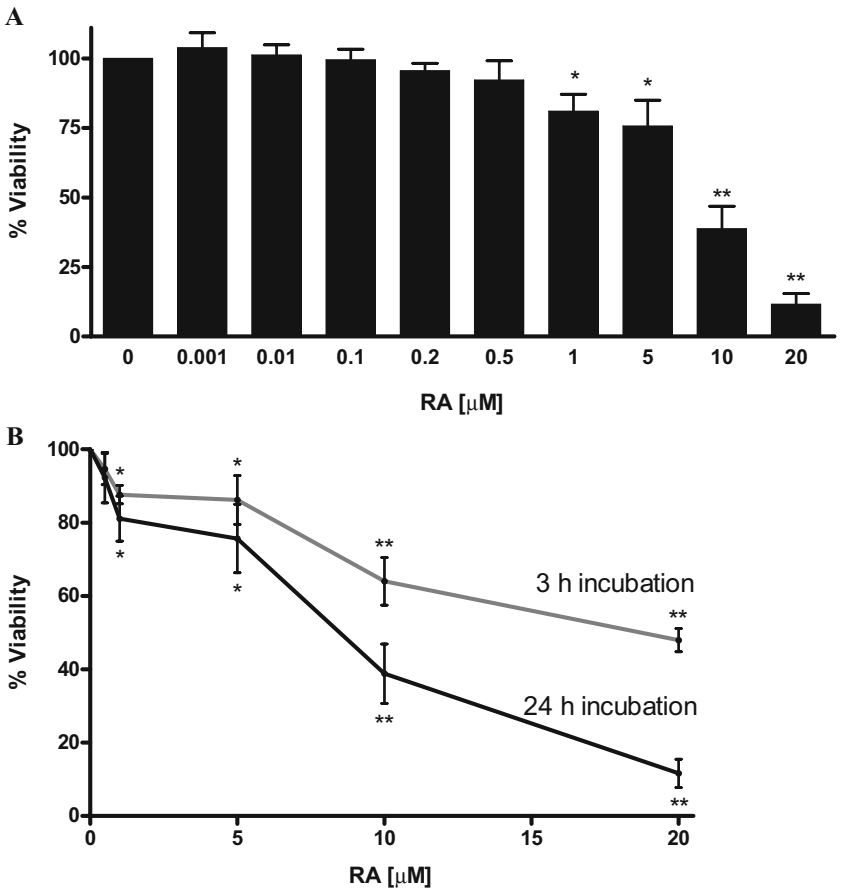

Fig. 1 The effect of RA on TM-3 cell survival. **A** Cells were incubated with indicated concentrations of RA and then the cell viability was determined by MTT assay as described in "Materials and methods". **B** The cell viability was evaluated at 3 and 24 h post-exposure with RA (at μM doses). The data represent means±SD of four independent experiments with triplicate well and are presented as the percentage of the control cell number. *P<0.05 compared to the control. **P<0.01 compared to the control

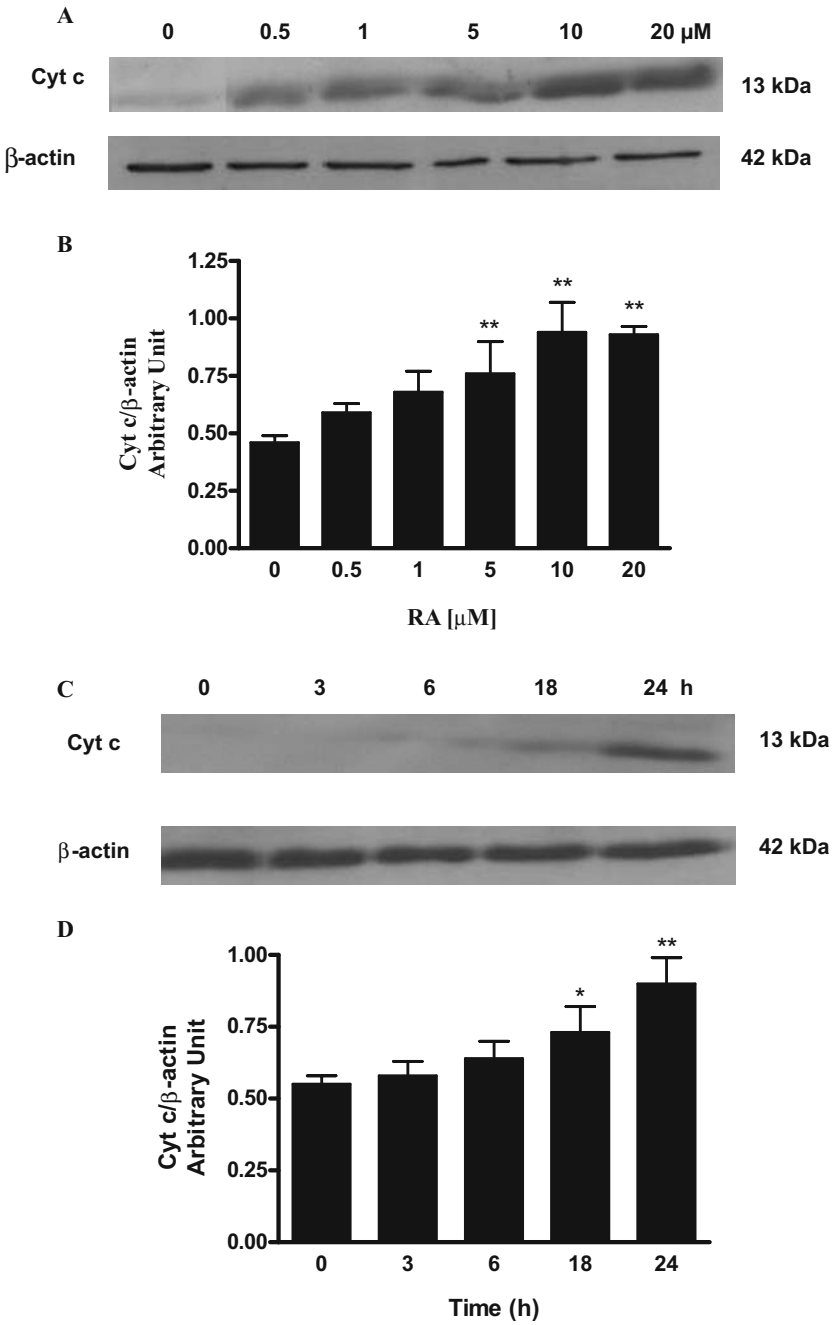

Fig. 2 Biochemical markers of apoptosis. **A** TM-3 cells were incubated both in the absence and in the presence of 0.5, 1, 5, 10 and 20 μM RA for 24 h. After incubation, cells were washed with PBS; equal amounts of cytosolic proteins (2–5 μg) were separated by 15% SDS-PAGE, transferred to a nitrocellulose membrane, and probed as described in "Materials and methods". Cytochrome c was detected by chemiluminescence. β-actin, used as internal control, was detected at the position corresponding to a molecular weight of 42 kDa. **B** The cytochrome c protein content was determined densitometrically. Results are presented as the mean±SD of three independent experiments. *$P<0.05$ compared to the control; **$P<0.01$ compared to the control. **C** TM-3 cells were incubated with 20 μM RA and release of cytochrome was evaluated at 0, 3, 6, 18 and 24 h post-exposure. **D** The cytochrome c protein content was determined densitometrically. Results are presented as the mean±SD of three independent experiments. *$P<0.05$ compared to the control; **$P<0.01$ compared to the control. **E** Cells were incubated with 0, 0.5, 1, 5, 10 and 20 μM RA. At 24 h post-exposure, cells were washed with PBS. Equal amounts of cytosolic proteins (20 μg) were separated by 15% SDS-PAGE, transferred to a nitrocellulose membrane, and probed as described in "Materials and methods". Procaspase-3 and p17 fragment were detected by chemiluminescence. β-actin, used as internal control, was detected at the position corresponding to a molecular weight of 42 kDa. **F** The cells were treated with various concentrations of RA for 24 h; then the DNA was extracted, separated by electrophoresis on 1.2% agarose gel and visualized by staining with ethidium bromide. *Lane 1*, control; *lane 2*, 5 μM RA; *lane 3*, 10 μM RA; *lane 4*, 20 μM RA

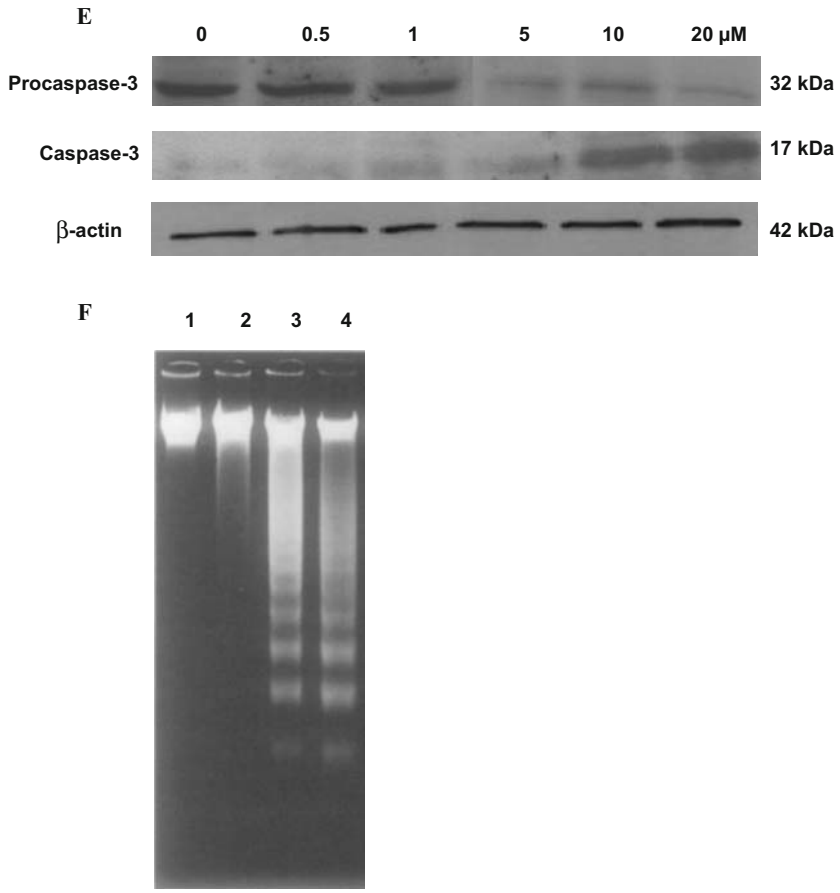

Fig. 2 (continued)

controls. The time-course of cytochrome c release from the mitochondria into the cytosol was also determined after 3, 6, 18 and 24h of incubation in the presence of 20μM RA. As shown in Fig. 2C,D, detectable release of cytochrome c was, only apparent, after 18h of treatment.

The activation of the caspase family members is a hallmark of apoptosis. After treatment for 24h with RA, western blot analysis, using a caspase-3 antibody that recognizes the caspase-3 holoenzyme as well as the p17 cleavage product of caspase-3, was performed to investigate whether enzymatic processes had been activated. Procaspase-3 is synthesized as a precursor of 32kDa which is then proteolitically cleaved. Immunoblotting analysis revealed that procaspase-3 levels decreased in cells treated with 5, 10 and 20μM RA for 24h in a dose-dependent manner; while a band, corresponding to the activated form of caspase-3 (17kDa), was increased over the same dose-response curve (Fig. 2E).

In order to evaluate the induction of apoptosis by RA we measured DNA fragmentation using DNA electrophoresis and fluorescent staining. The genomic DNA extracted from cells, treated with 5, 10 and 20μM RA for 24h, was subjected to 1.2% agarose gel electrophoresis. DNA ladders, which are typical of apoptosis, were detected only in the cells treated with 10 and 20μM RA (Fig. 2F).

LDH activity in the culture media was measured spectrophotometrically as an index of plasma membrane damage and loss of membrane integrity and therefore a parameter of cytotoxicity. RA treatment for 24h resulted in a dose-dependent induction of LDH release (Fig. 3A). To summarise, we have demonstrated that RA-induced cell death occurred by classical apoptosis, whilst at higher concentrations there is also evidence of necrotic death.

RA-induced apoptosis is not mediated by an increase of ceramide generation

Ceramide, as a second messenger, is generated by the hydrolysis of the cell membrane sphingomyelin or is derived from *de novo synthesis* in response to inducers of apoptosis (Bose et al. 1995). Previous studies have shown that ceramide induces cell apoptosis by the mechanisms of activation of a ceramide-activated protein phosphatase (CAPP); moreover, ceramide up-regulates the apoptosis effector, Bax, or down-regulates the apoptosis inhibitor, Bcl-2, leading to caspase activation (Pinton et al. 2001;

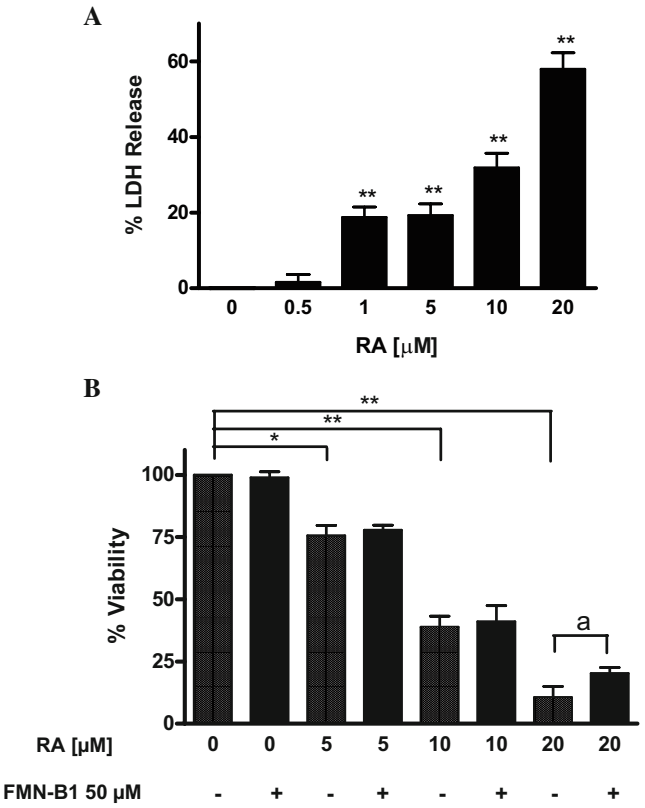

Fig. 3 The effect of RA on LDH release and the effect of ceramide synthase inhibitor on TM-3 cell survival. **A** The TM-3 cells were treated with various concentrations of RA as indicated for 24 h, and the amount of LDH released was determined as described in "Materials and methods". **B** The cells were treated with various concentrations of RA (0, 5, 10 and 20 μM) with and without 50 μM fumonisin B1 (FMN-B1), a ceramide synthase inhibitor, for 24 h, and the cell viability was determined by MTT assay as described in "Materials and methods". The data represent means±SD of four independent experiments with triplicate well and are presented as a percentage of viable cells in the control sample. *$P<0.05$ compared to the control. **$P<0.01$ compared to the control. *a* $P<0.05$ compared to identical RA group

Kolesnick 2002; Von Haefen et al. 2002). To identify the possible mechanisms mediating RA-induced Leydig cell apoptosis we measured the cytotoxicity of RA in cell cultures in the presence or absence of the ceramide synthase inhibitor, fumonisin B1. As shown in Fig. 3B, fumonisin B1 treatment alone had no effect on cell survival. The apoptotic effect of RA was not mediated by ceramide because 50μM of this compound could not prevent the apoptotic effect of various concentrations of RA (5–10μM range). At 20μM RA there was an increase in ceramide generation providing evidence of necrotic death.

Oxidative stress

TM-3 cells were treated with RA and the lipid peroxidation was estimated by TBARS formation as described in "Materials and methods". RA treatment (0–1μM concentration range, for 24h treatment) did not considerably increase TBARS content in cultured TM-3 cells, while RA doses higher than 1μM increased lipid peroxidation levels (Fig. 4A). In agreement with this, RA at higher doses (at μM doses) induced a decrease in cell viability (Fig. 1A). As higher RA doses induced lipid peroxidation and apoptosis, we decided to investigate only the effects of RA treatment at nM physiological concentrations in TM-3 cells. In order to investigate changes in antioxidant defences we measured the GST, SOD and CAT activities in RA treated and non treated TM-3 cells. GST activity increased with 10, 100 and 200nM RA (Fig. 4B), while SOD and CAT activities increased only with 100 and 200nM RA (Fig. 4C,D).

Discussion

Over the last decade retinoids have been the object of intense investigation. Vitamin A and carotenoids have been considered as physiologically important antioxidants. Despite our understanding on important physiological functions of retinoids (Livrea and Packer 1993), the effects of retinoid supplementation at supra-physiological doses, in addition to their known physiological actions, are not well defined. Retinoid action is mediated by specific nuclear retinoic acid receptors (RARs) and retinoid X receptors (RXRs) belonging to the steroid/thyroid super-family of transcription factors (Giguere 1994). The wide spectrum of physiological and pharmacological retinoids effects is, however, attributed to both receptor-dependent and receptor-independent mechanisms (Radominska-Pandya et al. 2000).

In this context, the mechanism of the antioxidant effect of RA remains unclear. We report here that supplementation with RA caused lipid peroxidation. This damage seems to be induced only by supra-physiological doses, since physiological doses did not induce TBARS. In response, a decrease in cell viability was observed at the same doses where TBARS was found to be enhanced.

Our data strongly suggest that RA induces apoptosis as determined by cytochrome c release from the mitochondria to bind to Apaf-1, which in turn initiates a caspase cascade, the executionary machinery of apoptotic cell death. Our results showed there was a measurable release of cytochrome c into the cytoplasm following exposure to RA. This release of cytochrome c may result in the activation of members of the caspase family of proteases, another hallmark of apoptosis. Caspases, and in particular caspase-3, play a central role in the terminal biochemical events that ultimately lead to apoptotic cell death. We observed a dose-dependent increase in caspase-3 activity after incubation for

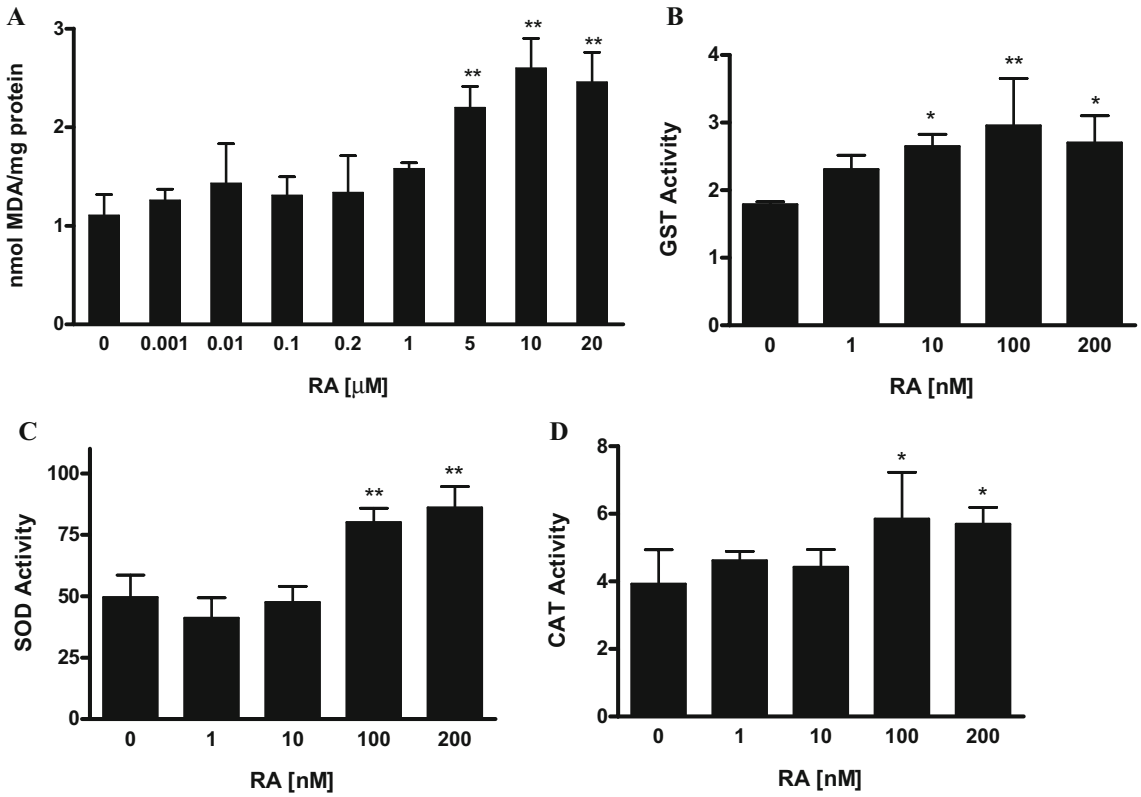

Fig. 4 Determination of TBARS and antioxidant enzymes activities in TM-3 treated with RA for 24 h. **A** Cells were incubated with increasing concentrations of RA as indicated and lipid peroxidation was evaluated by TBARS assay as described in "Materials and methods". Cells were treated with the indicated concentrations of RA for 24 h and GST (**B**), SOD (**C**) e CAT (**D**) activities were measured as described in "Materials and methods". Enzymes activities are expressed as μmol substrate/min/mg protein. Results are presented as the mean±SD of three individual experiments. *$P<0.05$ compared to the control. **$P<0.01$ compared to the control

24h with RA. As a consequence, DNA was cleaved by endonucleases, as observed by condensation and fragmentation of chromatin. Taken together, these observations suggest that RA activates the classic mitochondrial dependent apoptotic process.

Since higher doses of RA (μM) induced lipid peroxidation and cell death, we decided to investigate only the effects of RA treatment at physiological doses in TM-3 cells. In order to investigate changes in antioxidant defences we measured the SOD, CAT and GST activities in TM-3 cells treated with RA. The SOD, CAT and GST activities increased with 100 and 200nM RA treatment. Interestingly, at these same doses RA did not increase lipid peroxidation and no effect on cell viability was observed. These findings suggest that RA increased the activity of antioxidant enzymes at physiological doses, thereby preventing oxidative cell damage which is manifest at higher doses of RA as may be evidenced by TBARS content and cell viability. In accordance with earlier studies (Livera et al. 2000) we observed no significant effect of 1μM RA on DNA fragmentation (date not shown). This concentration of RA was able, however, to decrease cell viability by 15–18% and trigger a low level of cytochrome c release indicating that 1μM RA could represent a threshold limit for non apoptosis inducing RA treatments.

Hurnanen et al. (1997) have shown that low RA concentrations stimulated cell growth and proliferation but that high concentrations inhibited cell proliferation. Moreover, higher RA concentrations increased lipid peroxidation. There was a significant negative correlation between lipid peroxidation and cell proliferation, which suggests that RA may generate free radicals. The mechanism of RA-mediated lipid peroxidation is not fully understood. There are, however, at least two possible mechanisms: (i) RA can stimulate the activity of Δ-6-desaturase resulting in an increase of polyunsaturated fatty acids (PUFA), which are then easily oxidized (Alam et al. 1984). Δ-6-desaturase is the enzyme responsible for inserting double bonds during PUFA synthesis. A loss or decreased activity of this enzyme has been found in some malignant tumors. (ii) RA can also directly increase free radical generations which could result in increased lipid peroxidation (Davis et al. 1990). Moreover, 13-*cis*-RA was shown to directly increase levels of superoxide anion, hydrogen peroxide and hydroxyl anions in isolated chick neural crest cells (Davis et al. 1990). It is noteworthy that every antioxidant is in fact a

redox agent, protecting against free radicals in some circumstances and promoting free radicals generation in others (Herbert 1996).

Our results suggest the importance of keeping vitamin status within the normal range, as a deficit or administration of greater than the upper physiological limits could explain in part the adverse effects found in the literature. The concentrations of RA used in this study range from physiological to pharmacological plasma concentrations. RA is present constitutively in the plasma at a concentration of 4–14nM (De Leen Heer et al. 1982; Kane et al. 2005). Pharmacological RA doses result in transient plasma concentration in the same μM range at which we observed TBARS formation and decreased cell viability.

In conclusion, the above findings indicate that retinoic acid at μM doses raises oxidative stress and induces apoptosis in Leydig cells through cytochrome c release and caspase-3 activation. Conversely, at physiological doses (0.1–500nM) RA modulates antioxidant enzymes through an increase of GST, SOD and CAT activities. An understanding of the RA behavior will, therefore, be necessary to define how it may be used both alone as a chemopreventive agent and also in combination with chemotherapeutic agents for cancer treatment.

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