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Original Article

Retinol induces morphological alterations and proliferative focus formation through free radical-mediated activation of multiple signaling pathways

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Aim: Toxicity of retinol (vitamin A) has been previously associated with apoptosis and/or cell malignant transformation. Thus, we investigated the pathways involved in the induction of proliferation, deformation and proliferative focus formation by retinol in cultured Sertoli cells of rats.

Methods: Sertoli cells were isolated from immature rats and cultured. The cells were subjected to a 24-h treatment with different concentrations of retinol. Parameters of oxidative stress and cytotoxicity were analyzed. The effects of the p38 inhibitor SB203580 (10 μmol/L), the JNK inhibitor SP600125 (10 μmol/L), the Akt inhibitor LY294002 (10 μmol/L), the ERK inhibitor U0126 (10 μmol/L) the pan-PKC inhibitor GÖ6983 (10 μmol/L) and the PKA inhibitor H89 (1 μmol/L) on morphological and proliferative/transformation-associated modifications were studied.

Results: Retinol (7 and 14 μmol/L) significantly increases the reactive species production in Sertoli cells. Inhibition of p38, JNK, ERK1/2, Akt, and PKA suppressed retinol-induced [^{3}H]dT incorporation into the cells, while PKC inhibition had no effect. ERK1/2 and p38 inhibition also blocked retinol-induced proliferative focus formation in the cells, while Akt and JNK inhibition partially decreased focus formation. ERK1/2 and p38 inhibition hindered transformation-associated deformation in retinol-treated cells, while other treatments had no effect.

Conclusion: Our results suggest that activation of multiple kinases is responsible for morphological and proliferative changes associated to malignancy development in Sertoli cells by retinol at the concentrations higher than physiological level.

Keywords: retinol; vitamin toxicity; Sertoli cell; oxidative stress; cell deformation; p38; JNK; Akt; ERK; PKC; PKA

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Introduction

Vitamin A (retinol) affects several biological processes through diverse mechanisms. Classically, it was believed that most actions of retinol were mediated only by different isomers of retinoic acid (RA), which is obtained from retinol enzymatic dehydrogenation[1]. RA modulates the transcription of several genes associated to cell cycle regulation through the action of the so-called retinoid receptors, which belong to the superfamily of steroid/thyroid receptors[2]. Gene transcription regulated by retinoid receptors influences cell death/survival, differentiation and growth, and is believed to exert an important role in neural development[3].

Besides this genomic action, there has been some recent attention to a potential role in redox modulation of oxidative balance at cellular and systemic levels by retinol[4]. Based on epidemiologic studies, retinol was suggested to be an important dietary antioxidant, preventing pathologies associated to oxidative stress such as cancer, atherosclerosis, neurodegenerative diseases and other age-related conditions[5]. On the other hand, extensive trials of retinol supplementation resulted in increased incidence of lung cancer and cardiovascular diseases[6, 7], and more recent works on the redox properties of retinol and other retinoids revealed a pro-oxidant action at specific concentrations and experimental conditions[8–10]. Besides, retinol and RA have been reported to regulate cellular processes not related to retinoid receptor-mediated gene transcription, in which has been denominated a "non-genomic" action[11, 12]. These actions were observed to influence synaptic transmission, catecholamine production and also cell cycle regulation through mitogen-activated protein kinase (MAPK),

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Akt and Protein Kinase C (PKC) activation[11, 13–15]. Since oxidative stress and protein kinase activation have been extensively associated with cancer induction, it has been speculated that non-genomic actions of vitamin A may account for many of these pro-neoplastic effects.

We have previously observed that retinol is able to enhance reactive species production and induce extensive oxidative damage in Sertoli cells, a physiological target of retinol[16–21]. In these cells, retinol and RA are endocrine factors that regulate diverse reproductive-related functions in a constitutive fashion. Although Sertoli cells are "professional" vitamin A targets, retinol is also able to induce cell cycle impairment and DNA damage via reactive species production at concentrations slightly above the reported physiological limit[19–21]. In this regard, primary Sertoli cell cultures constitute an excellent model to study the mechanisms and effects of vitamin A supplementation at cellular level.

Considering that the role of retinol in diet supplementations or in new therapies to treat or prevent malignant processes is still a matter of debate, here we investigated the effects of retinol on morphological parameters associated to pre-neoplasic morphological transformation and mitosis in Sertoli cell cultures, aiming to determine the role of the MAPKs ERK1/2, p38 and Jun-activated kinase (JNK), the protein kinase Akt, the cAMP-activated Protein Kinase (PKA) and PKC.

Material and methods

Chemicals and animals

Pregnant Wistar rats were housed individually in Plexiglas cages. Litters were restricted to eight pups each. Animals were maintained on a 12-h light/dark cycle at a constant temperature of 23 °C, with free access to commercial food and water. Male immature rats (15 days old) were killed by cervical dislocation. All experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publication number 80–23 revised, 1996). Our research protocol was approved by the Ethical Committee for animal experimentation of the Federal University of Rio Grande do Sul. All-trans retinol alcohol, epinephrine, thiobarbituric acid, dinitrophenylhydrazine, Trolox, Tween-20, and β-mercaptoethanol were purchased from Sigma Chemical Co (St Louis, MO, USA). U0126 was from Promega Corporation (Madison, WI, USA), GÖ6983 and SB203580 were from Merck Biosciences (Darmstadt, Germany) and H89 was from Biomol Research Laboratories (Plymouth Meeting, PA, USA). Other kinase inhibitors were kind gifts from Professor Peter DUNKLEY (University of Newcastle, NSW, Australia). Rabbit polyclonal antibodies against phosphorylated forms of p38, JNK, ERK1/2 and Akt were obtained from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA), and monoclonal anti-β-actin was from Sigma. West Pico chemiluminescent kit was obtained from Pierce (Rockford, IL, USA). Concentrated stocks of solutions were prepared immediately before experiments by diluting retinol into ethanol and determining final stock concentration by UV absorption; solution was kept protected from light and temperature during all procedures. Appropriate solvent controls were performed for each condition. Treatments were initiated by adding concentrated solutions to reach final concentrations in the well. The final ethanol concentration did not exceed 0.2% in any experiment. Electrophoresis reagents and equipment were from BioRad (Hercules, CA, USA). Tissue culture reagents were from Gibco (Invitrogen Corporation, Carlsbad, CA, USA) and were of tissue culture grade.

Isolation and culture of Sertoli cells and assays

Sertoli cells were isolated as previously described[18]. Briefly, testes of 15-day-old rats were removed, decapsulated and digested enzymatically with trypsin and deoxyribonuclease for 30 min at 37 °C, and centrifuged at 750×*g* for 5 min. The pellet was mixed with soybean trypsin inhibitor, then centrifuged and incubated with collagenase and hyaluronidase for 30 min at 37 °C. After incubation, this fraction was centrifuged (10 min at 40×*g*). The pellet was taken to isolate Sertoli cells and supernatant was discarded. After counting, Sertoli cells were plated in multiwell plates (3×10^5 cells/cm^2) in Medium 199 pH 7.4 1% FBS, and maintained in humidified 5% CO_2 atmosphere at 37 °C for 24 h for attachment. The medium was then changed to serum-free medium and cells were maintained for more 24 h. Medium was then replaced by fresh medium containing treatments and cells were incubated for more 24 h. Morphology was examined at the end of the 24 h treatments.

Measurement of mitochondrial superoxide production

Mitochondrial superoxide production was assessed as previously described[18]. To isolate submitochondrial particles (SMP) from Sertoli cell cultures, cells were homogenized in an isolation buffer (230 mmol/L mannitol, 70 mmol/L sucrose, 10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 7.4). Homogenates were centrifuged (750×*g*, 10 min) to eliminate nuclei and cell debris and the pellet was washed to enrich supernatant; the supernatant was then centrifuged at 7000×*g* for 10 min. The pellet was washed and resuspended in the same buffer, and then SMP were obtained by freezing and thawing (three times) this fraction. The resulting SMP are washed twice with buffer consisting of 140 mmol/L KCl and 20 mmol/L Tris-HCl (pH 7.4) and resuspended in the same medium for determination of superoxide production, which was assessed by mixing SMP solution (0.3–0.1 mg protein/ml) to a reaction medium consisting of 230 mmol/L mannitol, 70 mmol/L sucrose, 20 mmol/L Tris-HCl (pH 7.4), plus 0.1 mmol/L catalase and 1 mmol/L epinephrine. Succinate (7 mmol/L) was used as substrate, and the superoxide-dependent oxidation of epinephrine to adrenochrome at 37 °C (Extinction molar coefficient at 480 nm and nm and 4020 mmol·L^{-1}·cm^{-1}) was followed by spectrophotometry detection. SOD was used at 0.1–0.3 mmol/L final concentration to assess assay specificity.

Thiobarbituric acid reactive species (TBARS)

As an index of lipid peroxidation, we quantified the formation of TBARS formed in an acid-heating reaction of cell

lipid extracts with thiobarbituric acid[22]. Briefly, the cell homogenates were mixed with 0.6 mL of 10% trichloroacetic acid (TCA) and 0.5 mL of 0.67% thiobarbituric acid, and then heated in a boiling water bath for 25 min. TBARS were determined by the absorbance in a spectrophotometer at 532 nm. Results were normalized against the content of cell protein and expressed as TBARS/mg of protein.

Measurement of protein carbonyls

The oxidative damage to proteins was measured by the quantification of carbonyl groups based on the reaction with dinitrophenylhidrazine (DNPH)[23]. Briefly, proteins were precipitated by the addition of 20% TCA, redissolved in DNPH and the absorbance was read in a spectrophotometer at 370 nm. Results were expressed as nmol carbonyl/mg of protein.

Proliferation focus assessment

Cell focus assay was carried as previously described[18]. Briefly, Sertoli cells were treated during 24 h with retinol in the presence or absence of different protein kinase inhibitors. The incubation medium was then replaced by Medium 199 (pH 7.4) supplemented with 10% FBS in all groups. Cells were maintained in humidified 5% CO_2 atmosphere at 37 °C for 14 d, with medium replacement every 3 d. Morphology was examined during this period and cell foci were scored at the end of the experiment under a light microscope (Nikon Eclipse TE 300). Photomicrography images were captured at the end of the 24 h-period of incubation with retinol and also at the end of the 14 d-period of proliferative focus induction.

Cell proliferation assay

As an index of cell proliferation we used the incorporation of [methyl-^{3}H] thymidine ([^{3}H]dT). Briefly, after the first 24 h of culture in Medium 199 at 1% FSB, the medium was replaced for serum-free Medium 199 supplemented with 2.5 μCi/mL of [^{3}H]dT (248 GBq/mmoL; Amersham International, Amersham, UK). After 24 h, the medium was replaced by the same medium containing retinol and different protein kinase inhibitors, and cells were incubated for more 24 h. Cells were then washed, harvested, suspended in nucleus isolation buffer (50 mmol/L $NaPO_4$, 2 mol/L NaCl, pH 7.4) and centrifuged (5 min, 14 000×*g*) to extract nuclear DNA. An aliquot was used to determine [^{3}H]dT incorporation into DNA in a Packard Tri-Carb Model 3320 scintillation counter, and another aliquot was utilized for protein quantification.

Deformation coefficient *D* measurement

The morphological relation between spreading and confluent cells was measured to assess modifications in cell plasticity. The increase in the difference between spread and confluent cells implies an increase of coefficient D[24]. Morphometrical measurements were obtained by analysis of the scanned phase-contrast photomicrographs of cells plated as dispersed and confluent densities. At least 35 cells from each experimental group in three independent experiments were measured to evaluate shape parameters of each cell. Data are reported as mean±SEM, with the level of significance set at $P<0.05$.

Immunoblot

To perform immunoblot experiments, Sertoli cell cultures were lysed in Laemmli-sample buffer (62.5 mmol/L Tris-HCl, pH 6.8, 1% (*w/v*) SDS, 10% (*v/v*) glycerol) and equal amounts of cell proteins (approximately 35 μg/lane) were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) and electroblotted onto polyvinyledilene difluoride (PVDF) membranes. Protein loading and electrobloting efficiency were verified by Ponceau S staining, and the membrane was then blocked in Tween-Tris buffered saline (TTBS; 100 mmol/L Tris-HCl, pH 7.5, containing 0.9% NaCl and 0.1% Tween-20) containing 5% albumin and incubated overnight with the primary antibody to be tested. The membrane was washed in TTBS and incubated with horseradish peroxidase coupled anti-IgG antibody, washed again and the immunoreactivity was detected by enhanced chemiluminescence. Densitometric analysis of the films was performed with GraphPad Software Inc; San Diego, CA, USA. Blots were developed to be linear in the range used for densitometry.

Data normalization and statistics

Data were normalized by protein content, which was measured by the Lowry method. Normalized data was analyzed with GraphPad software by one-way ANOVA with Duncan's *post-hoc* test. Differences were considered significant when $P<0.05$.

Results

Previously, we reported that retinol induced oxidative stress at 7 μmol/L during 24 h of incubation[17]. To confirm this observation, here we incubated Sertoli cells with retinol for 24 h to establish the concentrations at which retinol was able to induce oxidative stress. Cytosolic concentrations of retinol at physiological conditions have been reported to range between 0.2 to 5 μmol/L[25]; for this reason, we incubated Sertoli cells with concentrations ranging from physiological (2 and 5 μmol/L) to elevated levels (7 and 14 μmol/L). As previously observed[26], mitochondrial superoxide production (Figure 1A), carbonyl levels (Figure 1B) and lipid peroxidation (Figure 1C) were significantly increased by retinol at 7 μmol/L; retinol at 2 μmol/L or 5 μmol/L had no effect on these parameters. In previous reports, we also observed that retinol enhances intracellular free radical production at 7 and 14 μmol/L, but the concentration of 14 μmol/L also led to extensive cell death after 24 h of incubation[27]. Altogether, these results confirmed that retinol increases reactive species production at 7 μmol/L and 14 μmol/L, and increasing concentrations of retinol may induce cytotoxic effects probably due to uncontrolled oxidative damage to cells.

Retinol did not cause any detectable morphological changes in cell monolayers at 2 μmol/L and 5 μmol/L (Figure 2). On the other hand, retinol 7 μmol/L induced visible morphologic modifications in the cell. At the concentration of 14 μmol/L

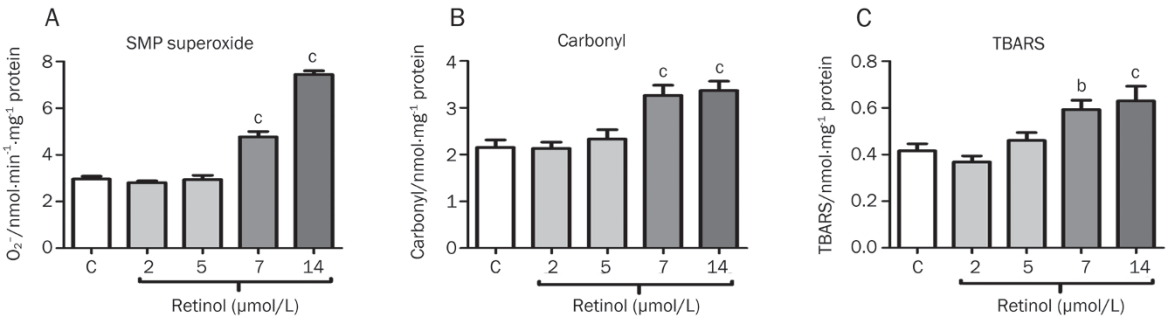

Figure 1. Sertoli cells were treated with retinol for 24 h and different parameters related to oxidative stress and cell damage were assessed. (A) Superoxide production by submitochondrial particles isolated from retinol-treated cells was measured, and oxidative damage was assessed by quantification of (B) carbonyl and (C) TBARS levels. C=control in all graphs. Bars represent mean±SEM from three independent experiments (triplicate); data were analyzed by one-way ANOVA with Duncan's *post hoc* test. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control.

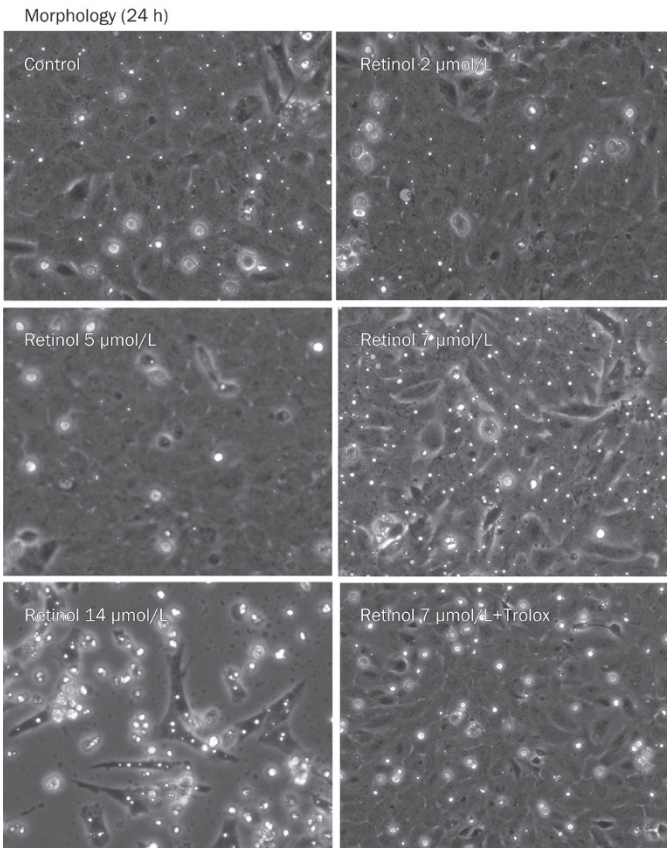

Figure 2. Cell morphology was examined by phase-contrast microscopy (Nikon Eclipse TE 300, ×100). Sertoli cells were treated with retinol at 2, 5, 7, and 14 μmol/L for 24 h. Retinol 7 μmol/L induced mild body cell shrinkage and retinol 14 μmol/L caused extensive cell detachment. Trolox (100 μmol/L) inhibited the effect of retinol 7 μmol/L.

most cells were detached. Co-incubation with the antioxidant Trolox (0.1 mmol/L) prevented the changes in morphology induced by retinol, indicating the involvement of oxidative stress in this effect. Based on these results and on the results presented at Figure 1 we used the concentration of 7 μmol/L to test the pro-oxidant effects of retinol on morphological and proliferation parameters of Sertoli cells throughout this work.

In previous works, we demonstrated that retinol led to the activation of different signaling pathways in an oxidant-dependent fashion, and that the antioxidant Trolox inhibited this effect[15, 18]. Here, we wanted to establish the possible involvement of different signaling pathways in the induction of morphological and proliferative changes in Sertoli cells by analyzing parameters related to pre-neoplasic transformation. With this aim, we performed assays using pharmacological inhibitors of different MAPKs involved in cell cycle activation and transformation, such as p38, ERK1/2, and JNK, as well as inhibitors of PKA, PKC, and Akt. Sertoli cells were incubated with retinol and protein kinase inhibitors for 24 h, and the cell morphology was examined by phase-contrast microscopy (Figure 3A).

We observed here that the JNK inhibitor SP600125 (10 μmol/L) blocked the morphological alterations induced by retinol (Figure 3A). The other protein kinase inhibitors had no effect or caused new patterns of morphology modification in retinol-treated cells, such as observed for the p38 inhibitor SB203580 (10 μmol/L) and the Akt inhibitor LY294002 (10 μmol/L). To better understand how these morphological modifications were associated to malignant deformation, we calculated the deformation coefficient *D* (Figure 3B), a morphological relationship among spreading and confluent cells, related to cell malignancy, invasiveness and contact inhibition applied to cancer cell lines[24]. The coefficient *D* for cells treated with retinol for 24 h (D=2.812±0.229) was significantly lower than in control cells (D=5.258±0.538), indicating loss of substrate adhesion and contact inhibition[24]. Besides Trolox, the p38 inhibitor SB203580 and the ERK1/2 inhibitor U0126 (10 μmol/L) also inhibited this effect, while other protein kinase inhibitors did not. Cells co-treated with retinol and the PKA inhibitor H89 presented a negative value for the coefficient *D*, which is probably related to a loss of cell integrity resulting in cell detachment observed at later time periods (data not shown). These results suggest that p38 and ERK1/2 are involved in the activation of signaling pathways leading to morphological changes related to loss of contact inhibition and modulation of cell adhesion.

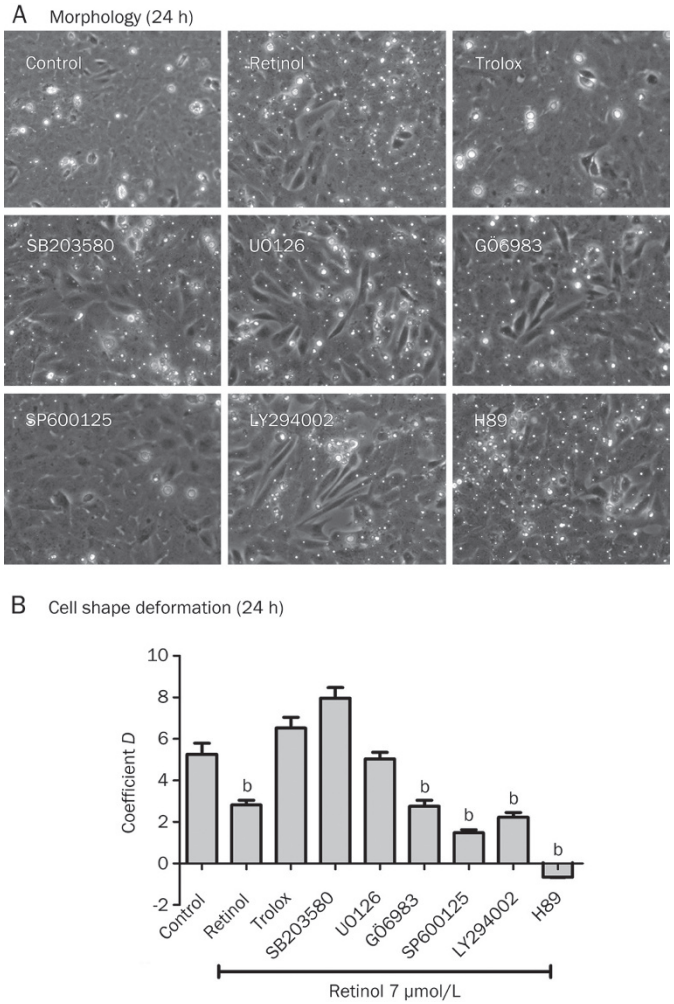

Figure 3. Effect of different protein kinase inhibitors on cell morphology modification by retinol. (A) Sertoli cells were treated with retinol 7 μmol/L in the presence or absence of the antioxidant Trolox (100 μmol/L), the p38 inhibitor SB203580 (10 μmol/L), the JNK inhibitor SP600125 (10 μmol/L), the Akt inhibitor LY294002 (10 μmol/L), the ERK inhibitor U0126 (10 μmol/L) the pan-PKC inhibitor GÖ6983 (10 μmol/L) and the PKA inhibitor H89 (1 μmol/L) for 24 h, and the cell morphology was examined by phase-contrast microscopy (Nikon Eclipse TE 300, ×100). (B) Cell deformation after 24 h was also analyzed by calculation of the coefficient *D*, an index of cell transformation-related deformation, obtained by morphometrical measurement of scanned phase-contrast photomicrographs of cells plated at dispersed and confluent densities. Bars represent mean±SEM from 22 individual cells analyzed per each group; data were analyzed by one-way ANOVA with Duncan's *post hoc* test. $^{b}P<0.05$ in relation to control.

In previous works, we found that Sertoli cell cultures treated with retinol for 24 h developed spots of proliferative focus when subjected to a 14 d-treatment with 10% Fetal Bovine Serum (FBS)-supplemented culture medium[18, 20]. Here, we studied the inhibition of different kinases on this effect. With this aim, Sertoli cells were incubated for 24 h with retinol in the presence or absence of the different protein kinase inhibitors; after this treatment, the medium in all groups was replaced for fresh 10% FBS medium (without retinol and protein kinase inhibitors), and cells were cultured in these conditions for 14 d with medium replacement every 3 d. In cells treated with retinol we observed a significant increase in proliferative focus formation, which was blocked by Trolox (Figure 4 and 5A). The ERK1/2 inhibitor U0126 also blocked retinol-induced proliferative focus formation, and p38 inhibition significantly decreased the number of proliferative focus in the plate well. PKC, Akt, and JNK inhibition had no effect on retinol-induced proliferative focus formation. Cells treated with retinol plus the PKA inhibitor H89 (1 μmol/L) for 24 detached from culture dishes within 1 week of 10% FBS-medium incubation.

Previously, we observed that retinol induced an increase in [^{3}H]dT incorporation that was associated to activation of cell division[18]. Here, we investigated the effect of the different protein kinases on this parameter. As previously observed, Sertoli cells incubated with retinol for 24 h had increased [^{3}H]dT (Figure 5B), and this effect was prevented by the antioxidant Trolox. The p38 inhibitor SB203580, the JNK inhibitor SP600125, the Akt inhibitor LY294002 and the ERK inhibitor U0126 decreased [^{3}H]dT incorporation to control levels when co-incubated with retinol. The pan-PKC inhibitor GÖ6983 (10 μmol/L) did not affect [^{3}H]dT incorporation, while the PKA inhibitor H89 decreased [^{3}H]dT incorporation in retinol-treated cells.

Altogether, our results suggest that the protein kinases p38, Akt, JNK, and ERK1/2 are associated to morphological changes, proliferative focus formation and activation of cell division induced by pro-oxidant concentrations of retinol. We then evaluated the activation states of these protein kinases by the immunodetection of their phosphorylated (i.e., active) isoforms by western blot (Figure 6). We observed an increase in the phosphorylation of p38, Akt, JNK, and ERK1/2 within 60 min of incubation with retinol 7 μmol/L (Figure 6A); phosphorylation levels of all kinases peaked around 15–30 min. We evaluated the effect of Trolox on the phosphorylation state of each protein kinase in Sertoli cells treated with retinol by 15 min, and compared with the effect of each specific protein kinase inhibitor. Trolox was able to completely or partially inhibit the effect of retinol on the phosphorylation of all kinases; besides, our results confirm that the protein kinase inhibitors SB203580, LY294002, SP600125, and U0126 were all effective in inhibiting, respectively, p38, Akt, JNK, and ERK1/2 phosphorylation.

Discussion

Retinol and retinoic acid are widely recognized as potent morphogens[1]. Retinoids regulate gene transcription through nuclear receptors belonging to the superfamily of steroid/thyroid/vitamin D-related hormone receptors known as retinoid receptors[3]. Such nuclear action is considered a key point in brain development and establishment of morphological patterns during embryonic and fetal development[2]. The observation of antioxidant properties of vitamin A *in vitro* and *in vivo* also led to the hypothesis that retinol and derivatives could act in cancer prevention/treatment, due to combined

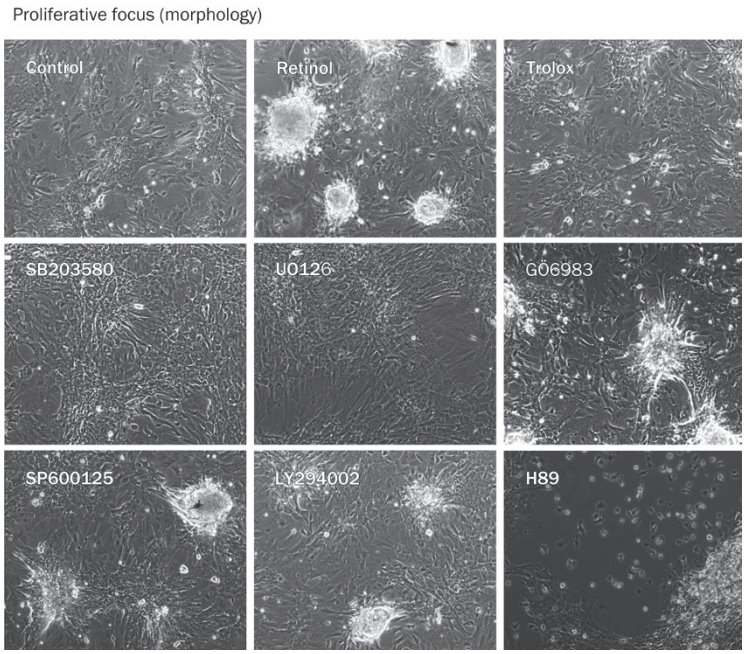

Figure 4. Proliferative focus morphology. Sertoli cells were treated for 24 h with retinol in the presence or absence of the antioxidant Trolox (100 μmol/L), the p38 inhibitor SB203580 (10 μmol/L), the JNK inhibitor SP600125 (10 μmol/L), the Akt inhibitor LY294002 (10 μmol/L), the ERK inhibitor U0126 (10 μmol/L) the pan-PKC inhibitor GÖ6983 (10 μmol/L) and the PKA inhibitor H89 (1 μmol/L); medium was then replaced in all groups for 10% FBS-supplemented 199 medium without any other compound for 14 d, with exchange for fresh medium every 3 d. At the end of the 14 d period, morphology was examined in a phase-contrast microscope (representative micrographs at ×40 are depicted) and proliferative foci were counted in each well (see Figure 5A for scores).

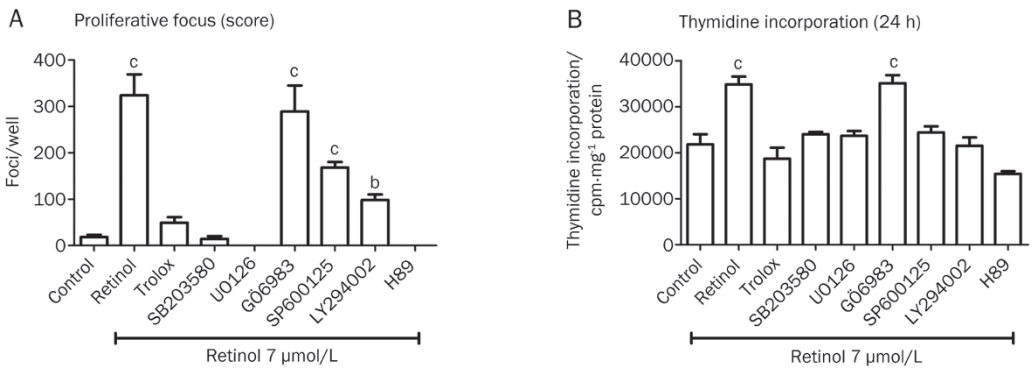

Figure 5. Proliferative focus scores and [^{3}H]dT incorporation in Sertoli cells. (A) Sertoli cells were treated for 24 h with retinol in the presence or absence of the antioxidant Trolox (100 μmol/L), the p38 inhibitor SB203580 (10 μmol/L), the JNK inhibitor SP600125 (10 μmol/L), the Akt inhibitor LY294002 (10 μmol/L), the ERK inhibitor U0126 (10 μmol/L) the pan-PKC inhibitor GÖ6983 (10 μmol/L) and the PKA inhibitor H89 (1 μmol/L); medium was then replaced in all groups for 10% FBS-supplemented 199 medium without any other compound for 14 d, with exchange for fresh medium every 3 d. At the end of the 14 d period, proliferative foci were counted in each well. (B) Sertoli cells were previously incubated with medium containing [^{3}H]dT, and then treated for 24 h in the same medium with retinol 7 μmol/L in the presence or absence of the above-mentioned compounds. Nuclei were isolated and incorporation of [^{3}H]dT was counted. Bars represent mean±SEM from three independent experiments (triplicate); data were analyzed by one-way ANOVA with Duncan's *post hoc* test. $^bP<0.05$, $^cP<0.01$ *vs* control.

free radical scavenging and control of cell cycle by retinoid receptor activity modulation[4].

More recently, extensive clinical trials and experimental works evidenced a pro-neoplasic effect of retinol and derivatives[28–31]. Retinoids have been more properly considered "redox-active" molecules rather than solely antioxidants; its redox actions in biological systems are certainly dependent on a variety of factors, such as its intracellular concentration and interaction with other redox-active molecules[32]. Although a role for retinoid receptors may not be completely discharged in such malignant effect, there is strong evidence pointing to a more prominent role of free radicals and other nuclear receptor-independent pathways in the mechanism of neoplasic transformation by retinoids. Free radicals and related species have been extensively recognized as potential inducers of different types of cancer[33], and MAPK-controlled signaling pathways and related proteins have been increasingly implicated in the control of cell cycle and triggering of oncogenic processes[34]. Retinoids have been described to evoke both types of responses in Sertoli cells, a well-recognized physiological target of retinol and RA[35, 36].

We have previously demonstrated that concentrations above 5 μmol/L are able to induce deleterious effects on cells due to enhanced free radical production and oxidative dam-

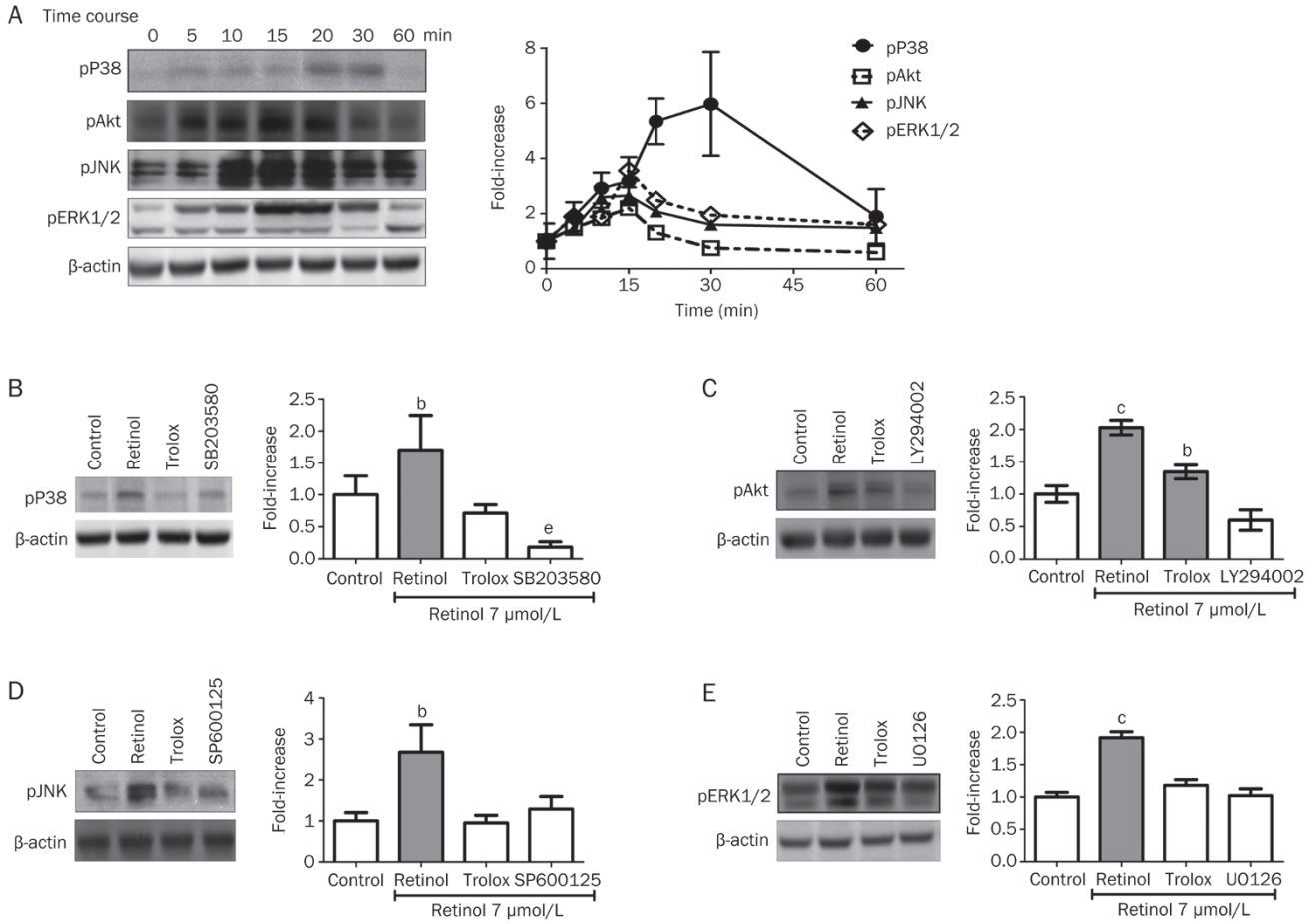

Figure 6. Representative immunoblots and quantification of levels of phosphorylated p38, Akt, JNK, and ERK1/2 in retinol-treated Sertoli cells. (A) Sertoli cells were treated with retinol 7 μmol/L and the time course of p38, Akt, JNK, and ERK1/2 during 60 min was evaluated by western blot using antibodies against phosphorylated forms of these protein kinases. Beta-actin was used as internal control. (B) The effect of the antioxidant Trolox (100 μmol/L) on p38 phosphorylation at 15 min of incubation with retinol was compared with the effect of the p38 inhibitor SB203580 (10 μmol/L). The effect of Trolox on Akt (C), JNK (D), and ERK1/2 (E) phosphorylation was also compared, respectively, with the effect of the inhibitors LY294002 (10 μmol/L), SP600125 (10 μmol/L), and U0126 (10 μmol/L). Bars represent mean±SEM from three independent experiments (triplicate); data were analyzed by one-way ANOVA with Duncan's *post hoc* test; asterisks denote significantly higher than control. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control. $^{e}P<0.05$ *vs* Trolox.

age to biomolecules[17, 19, 21, 31, 37]. We also observed that retinol induces the rapid activation of phosphorylation-controlled signal pathways involving ERK1/2, JNK, and Src in a redox-dependent fashion, indicating the involvement of reactive species in the so-called non-genomic signaling by vitamin A[18, 38]. Since kinase activation by retinol displays a peak of phosphorylation followed by a return to control levels within the period of 60 minutes, it seems that free radical production during the initial moments of retinol incubation are important for this effect. Normally, retinoid receptor activation in Sertoli cells is part of the paracrine signaling involved in the support and control of spermatogenesis, along with testosterone and Follicle-stimulating hormone (FSH).

Sertoli cells cease mitosis after birth and remain as fully differentiated and non-proliferative supportive cells. Physiological concentrations of retinoids do not affect the Sertoli cell cycle homeostasis in normal conditions[36]. However, pro-oxidant concentrations of retinol may induce proliferative effects related or not to malignancy, as free radicals are recognized to be involved in the regulation of cell cycle, especially in cell division and cell death[39]. Here, we observed that specific concentrations of retinol increase free radical production and parameters related to cell division and deformation. Cell cycle activation induced by pro-oxidant concentrations of retinol is probably related to cell transformation, since [^{3}H]dT incorporation was accompanied by proliferative focus formation and cell shape deformation, and these effects were reversed by antioxidant treatment. MAPK and Akt activation is also involved in the redox-dependent promotion of cell division by retinol, but not all these kinases are necessarily involved in cell transformation. Although the involvement of MAPKs in cell division control and transformation is well recognized, as mentioned earlier, different members of the MAPK family and related kinases may regulate different path-

ways associated to activation of normal mitosis and malignant transformation in a given cell type, and this may vary under different conditions.

Many of the cell cycle regulatory effects promoted by different members of the MAPK family have also been observed to be evoked or strongly influenced by free radicals and related species. ERK was demonstrated to be involved in the free radical-mediated promotion of transformation or in the increase to transformation susceptibility[40, 41]. The p38 MAPK was observed to be a key contributor for the redox signaling involved in UVA-mediated transformation[42] and was postulated to exert a selective role in promoting free radical-mediated tumorigenesis[43].

The extent of the role of cell phenotype in modulation of cell growth is a central question in the biomedical sciences[44]. In human non-small cell lung carcinoma cell lines, two- and three-dimensional growth patterns were described to follow a common growth dynamic associated to malignancy, such as in the case of the association with the Gompertz model for growing[45, 46]. It has been suggested that cell-shape compression control the inhibitory feedback as a geometrical arrest of mitosis, reflecting the influence of the cytoskeletal network organization[47]. As cells become rounded, DNA synthesis gradually stops[48]; inversely, most cells require spreading on extracellular matrix substrate for proper growth and normal function[49]. Such relationships among growth and shape have been studied by regression analysis and resulted in a cell deformation parameter — the deformation coefficient *D*, which describes the amplitude of cell shape variation, showing positive correlation with kinetic parameters of growth described by the Gompertz model[24].

Decrease in coefficient *D* value is related to decreased cell-to-cell recognition, adhesion and contact inhibition, and retinol treatment led to a decrease in this parameter. ERK1/2 and p38 inhibition reversed this effect, indicating that pathways controlled by these kinases are involved in the modulation of morphological parameters related to malignant transformation in these cells. Other kinase-controlled pathways such as the JNK and Akt seem to be involved in other effects promoted in a redox-dependent fashion by retinol in these cells; JNK inhibition blocked proliferative focus formation, while Akt inhibition hindered [^{3}H]dT incorporation, indicating a role in DNA synthesis. While inhibition of some kinases blocked or partially inhibited specific parameters related to proliferation or morphology change, our results indicate that ERK1/2 are involved in all aspects related to redox-dependent modifications on such parameters. ERK1/2 are highly sensitive to regulation by reactive species[50], and previous works from our group indicated that these kinases are involved in the activation of matrix metalloproteinase-2 by retinol in a redox-dependent fashion[51].

It has been increasingly clear that the concentration of retinoids influences their actions on biological systems, and this seems to be related to changes in their redox profile. As a redox active molecule, retinol may take part in different oxidation-reduction cycles of several biomolecules and micronutrients that change their oxidation states according the redox conditions of their microenvironment[5]. It was observed both in cell cultures and animal models[10, 52] that retinol-induced oxidative stress is caused by the administration of concentrations above the reported physiological limit, which would increase the availability of this molecule to take part in oxidation-reduction cycles inside cells. On the other hand, it was recently reported that retinol is able to induce the mitochondrial activity by enhancing pyruvate dehydrogenase activity through a nongenomic mechanism of PKC activation, thus resulting in increased free radical formation[53]. This would explain why increased concentrations of retinol induce reactive species production in both cell cultures and animal models.

Concluding, reactive oxygen species production induced by retinol activates different protein kinase pathways, which we found to be related to different morphologic and proliferative alterations in Sertoli cells. Inhibition of p38, JNK, ERK1/2, Akt, and PKA decreased retinol-induced [^{3}H]dT incorporation; p38, ERK1/2, JNK, and Akt are also involved in retinol-induced proliferative focus formation. ERK1/2 and p38 inhibition reversed transformation-associated deformation, while other treatments did not show any effect. Co-treatment with the antioxidant Trolox inhibited phosphorylation of p38, ERK1/2, JNK, and Akt, indicating the activation of these kinases during retinol treatment is dependent on oxidative stress. These results indicate that consumption of vitamin A at high levels must be better evaluated at clinical and epidemiological levels, as concentrations of retinol slightly above cellular physiological levels induces the activation of several pathways associated to cell deformation and pre-neoplasic transformation, as observed here. Experimental therapies or supplementation protocols using vitamin A should be more extensively studied in regard to their safety, possible toxicological effects and long-term side-effects.

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Author contribution

Daniel Pens GELAIN performed research, analyzed data and wrote the manuscript. José Claudio Fonseca MOREIRA supervised research and designed experiments. Matheus Augusto de Bittencourt PASQUALI, Fernanda Freitas CAREGNATO, and Mauro Antonio Alves CASTRO performed research.

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