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Xanthine oxidase-dependent ROS production mediates vitamin A pro-oxidant effects in cultured Sertoli cells

ALFEU ZANOTTO-FILHO, RAFAEL SCHRÖDER, & JOSÉ CLÁUDIO F. MOREIRA

Centro de Estudos em Estresse Oxidativo, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil

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Abstract
Several studies have suggested that vitamin A (retinol, ROH) presents pro-oxidant properties in biological systems. Recent studies point out that xantine oxidase, a ROS-generating enzyme, catalyses ROH oxidation to RA *in vitro*. These works stimulated the authors to investigate whether xanthine oxidase could be involved on the ROH pro-oxidative effects reported in cultured Sertoli cells. *In vitro*, it was demonstrate that xanthine oxidase generates superoxide in the presence of ROH as assessed by superoxide mediated-NBT reduction. Superoxide production is potentiated in the presence of NADH and inhibited by allopurinol. In Sertoli cells, ROH treatment increased xanthine oxidase activity and inhibition of the enzyme with allopurinol attenuated ROH-induced ROS production, protein damage and cytotoxicity. Moreover, inhibition of ROH oxidation to RA by retinaldehyde dehydrogenase inhibitor potentiated both xanthine oxidase-dependent ROS production and cell damage in ROH-treated cells. The data show that xanthine oxidase may play a role on vitamin A pro-oxidant effects.

Keywords: *Retinol, xanthine oxidase, ROS production.*

Introduction

The recently published observation that xanthine oxidase is capable of converting ROH (retinol) to RAL (retinaldehyde) and RAL to RA (retinoic acid) [1,2] should be taken into account, not only because the enzyme catalyses the oxidation of an alcohol to the respective acid, but also because the alcohol involved is ROH and the produced acid is RA which is a major regulator of cell proliferation, differentiation and morphogenesis [3,4]. Nonetheless, it has been well established that xanthine oxidase in the presence of the substrates hypoxanthine and xanthine reduces molecular oxygen to form superoxide anion and hydrogen peroxide [5]. In biological systems, the reactive oxygen species (ROS) produced by xanthine oxidase could in turn react with proteins, cell membranes and DNA causing cellular injury. Xanthine oxidase reaction has been shown to be a central mechanism of the oxidative cellular damage in post-isquemic tissues, playing a key role in the pathophysiology of myocardic infarct, stroke and exercise injury [5,6].

In spite of being one of the first vitamins to be discovered, the full range of biological activities mediated by vitamin A (ROH) remains to be better elucidated. A growing body of evidence has been suggesting that ROH and other retinoid derivatives have pro-oxidant properties, which might lead to cell oxidative damage, neoplasic transformation and/or cell death [7–9]. We have described that ROH treatment induces ROS production, lipoperoxidation, protein oxidative modifications and DNA damage in

Correspondence: Msc Alfeu Zanotto Filho, Depto Bioquímica (ICBS-UFRGS); Rua Ramiro Barcelos, 2600/Anexo, CEP 90035-003, Porto Alegre, Rio Grande do Sul, Brazil. Email: ohalceu@yahoo.com.br

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Sertoli, chromaffin and PC12 cells [10–21]. Moreover, we recently reported that both acute (3-days) and chronic (28-days) supplementation with retinol palmitate (ROH-palmitate) increase lipid peroxidation, protein damage and mitochondrial dysfunction in cerebral cortex, striatum, hippocampus and substantia nigra which were accompanied by anxiety-like behaviour and decreased locomotion in Wistar rats [22–25]. In this view, redox-active properties of ROH and other retinoids need to be better investigated since the mechanisms underlying ROH-induced ROS production and cellular oxidative damage are still unclear and diverse vitamin A supplementation protocols are currently used as 'antioxidant therapies' in individuals without vitamin deficiency [7–9].

In particular, the recently reported ability of the xanthine oxidase to metabolize ROH to RA *in vitro* [1,2], the well described involvement of the enzyme as a source of superoxide and hydrogen peroxide [3,4] and our previous results showing that ROH (a xanthine oxidase substrate) induces ROS production in biological systems [10–25] stimulated us to investigate the involvement of xanthine oxidase on ROH-induced ROS production and oxidative damage in cultured Sertoli cells, a well characterized model to study both physiological as well as pro-oxidant actions of ROH [13–21]. Data presented in this work showed that *in vitro* incubation of milk purified xanthine oxidase with ROH generates superoxide anion at a rate comparable to xanthine, a classical xanthine oxidase substrate. The rate of superoxide anion production was enhanced in the presence of NADH and was inhibited by allopurinol, a well described xanthine oxidase inhibitor. In Sertoli cells, the pre-treatment with the xanthine oxidase inhibitor attenuated ROH-elicited ROS production, protein damage and cytotoxicity. Inhibition of the ROH oxidation to RA by the retinaldehyde dehydrogenase inhibitor citral increased the rate of the allopurinol-inhibited ROS production by xanthine oxidase reaction. Data presented here show evidence that xanthine oxidase-dependent ROS production may be involved in Vitamin A pro-oxidant effects observed in previous studies from our group and others.

Materials and methods

Materials

Type I collagenase, medium 199, HBSS, all trans-retinol (t-ROH), xanthine, milk xanthine oxidase (EC 1.1.3.22), allopurinol (4-hydroxypyrazolo [3,4-d] pyrimidine), NBT (nitrotetrazolium blue chloride), MTT (3-(4,5-dimethyl)-2,5-diphenyl tetrazolium bromide), 2′,7′-dichlorohydrofluorescein diacetate (DCFH-DA) and Tween-20, were purchased from Sigma Chemical Co. (St. Louis, MO). Trypsin was purchased from Difco (Detroit, MI). NADH disodium salt was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Tissue culture reagents were from Sigma Chemical Co. and were of analytical or tissue culture grade.

Xanthine oxidase and ROH solution preparation

Milk xanthine oxidase from Sigma (22 U/mL) was 10-times diluted with 50 mM Tris-HCl, pH 7.4, containing 1 mM EDTA as described [1]. After, the sample was dialysed against the same buffer (1 L) for 6 h to remove ammonium sulphate and sodium salicylate. Xanthine oxidase activity was determined by measuring the rate of oxidation of xanthine to uric acid spectrophotometrically at 295 nm, using an absorption coefficient of 9.6 mM^{-1} cm^{-1} [26]. Assays were performed at 37°C in air-saturated Tris-HCl buffer, pH 7.4, containing 100 μM xanthine. ROH solution was freshly prepared in DMSO, in the dark to minimize photo-oxidation, and the concentration was determined spectrophotometrically at 325 nm, using an absorption coefficient of 52.77 mM^{-1} cm^{-1}. For *in vitro* superoxide production assay, ROH solutions were diluted with 50 mM Tris-HCl, pH 7.4, containing 1 mM EDTA and 0.012 mM Tween 20.

In vitro assays for xanthine oxidase-mediated superoxide production, NADH consumption and ROH effect on uric acid formation

Superoxide generation in xanthine oxidase-catalysed ROH oxidation was determined spectrophotometrically by monitoring superoxide-dependent NBT reduction to the blue chromogen formazan at 560 nm in the presence of different ROH concentrations [27]. Briefly, a 180 μl mixture of 0.1 mM NBT and 0.02 units/mL xanthine oxidase in 50 mm Tris-HCl, pH7.4, containing 0.012 mM Tween 20 and 1 mM EDTA was warmed at 37°C. Then, 20 μl of a 10-fold concentrated ROH solution was added to a final volume of 200 μL and NBT reduction was monitored for 1 h with 2 min intervals in a 96-well microplate reader. The rate of superoxide formation was calculated assuming a NBT absorption coefficient of 12.8 mM^{-1} cm^{-1} at 560 nm [27]. In some assays, NADH or/and allopurinol were added to the incubation.

NADH consumption by xanthine oxidase reaction was monitored by the decrease of the absorption at 340 nm in the same buffer above described in the absence of NBT. The rate of NADH oxidation to NAD was calculated using an absorption coefficient of 6.3 mM^{-1} cm^{-1} at 340 nm. In addition, one enzyme assay was performed for the study of the inhibitory effect of ROH on uric acid formation from xanthine. Uric acid formation was determined by

measuring the rate of 10 μM xanthine oxidation by xanthine oxidase (final concentration of 0.02 U/mL) in the presence or absence of ROH, using an absorption coefficient of 9.6 mM^{-1} cm^{-1} at 295 nm [26].

Sertoli cells isolation and cultures

Sertoli cells were isolated as previously described [13]. Briefly, testes of 15-day-old rats were removed, decapsulated and digested enzymatically with trypsin 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, hyaluronidase and deoxyribonuclease 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 the supernatant containing peritubular and germ cells was discarded. After counting, Sertoli cells were plated in multi-well plates (3×10^5 cells/cm^2) in Medium 199 pH 7.4, 1% FBS, and maintained in a humidified 5% CO_2 atmosphere at 34°C for 24 h to attach. The medium was then changed to serum-free medium and cells were taken for assay after 48 h of culture.

Determination of intracellular ROS production

Intracellular ROS production was detected using 2′, 7′-dichlorodihydrofluorescein diacetate, DCFH-DA [28]. This reagent is known to enter the cells and react with ROS producing the fluorophore dichlorofluorescein (DCF). This method has been used to monitoring xanthine oxidase-dependent ROS production in cell culture models [29]. Briefly, 6×10^4 cells were seeded in 96-well plates and treated with ROH or ROH plus co-treatments as described above. Before incubations, 100 μM DCFH-DA dissolved in medium containing 1% FBS was added to the cell culture and incubated for 30 min to allow cellular incorporation. After, the medium was discarded and DCF fluorescence was read at 37°C during 24 h with 6 h intervals with an emission wavelength set at 535 nm and an excitation wavelength set at 485 nm in a 96-well plate fluorescence reader (model F2000, Hitachi Ltd., Tokyo, Japan). ROS production was estimated by measuring the rate of DCF fluorescence (delta DCF fluorescence/hour) over 24 h.

Xanthine oxidase activity

The xanthine oxidase activity was measured spectrophotometrically at 295 nm and 37°C using xanthine as the substrate [30]. The formation of uric acid from xanthine increases the absorbance. One unit of activity was defined as 1 μmol uric acid formed per minute at 37°C and pH 7.4 and was expressed in units/mg protein.

Measurement of protein carbonyl groups

The oxidative damage to proteins was measured by the quantification of carbonyl groups based on the reaction with dinitrophenylhydrazine (DNPH) as previously described [25]. At the end of ROH treatment, cells were scraped and proteins were precipitated by the addition of 20% TCA, redissolved in 10 mM DNPH and the absorbance was read in a spectrophotometer at 370 nm. Results were calculated using an absorption coefficient of 22 mM^{-1} cm^{-1} at 370 nm; data were expressed as nmol carbonyl/mg protein.

MTT assay

Cell viability was estimated by the quantification of the MTT reduction to a blue formazan product by cellular dehydrogenases [11]. At the end of treatments, the medium was discarded and a new medium containing 0.5 mg/mL MTT was added. The cells were incubated for an additional 30 min at 37°C in humidified 5% CO_2 atmosphere. After, medium was removed, cells were washed three times with phosphate buffered saline (PBS) and DMSO was added for 10 min. Formazan salt formation was determined at 560 nm. Hydrogen peroxide (200 μM) was used as positive control for cell death. Data were expressed as percentage of the formazan formation in the untreated cells.

Protein quantification

Protein contents of each sample were measured as described by Lowry et al. [31] for data normalization.

Statistical analysis

Data are expressed as means ± standard error (SE) and were analysed by one-way ANOVA followed by Duncan's post hoc test. Differences were considered significant at $p \leq 0.05$.

Results

In vitro superoxide formation by xanthine oxidase in the presence of ROH

Purified xanthine oxidase incubated with increasing ROH concentrations was able to produce superoxide anion, which was measured by the rate of NBT reduction *in vitro*. We observed that xanthine oxidase-catalysed ROH oxidation increased significantly superoxide production from 1 μM ROH and a dose-dependent effect was observed up to 100 μM (Figure 1A). Retinoic acid (RA), which was described as a final product of ROH oxidation by the enzyme [1,2], did not induce superoxide formation (Figure 1A). In Figure 1A we include a positive control showing the

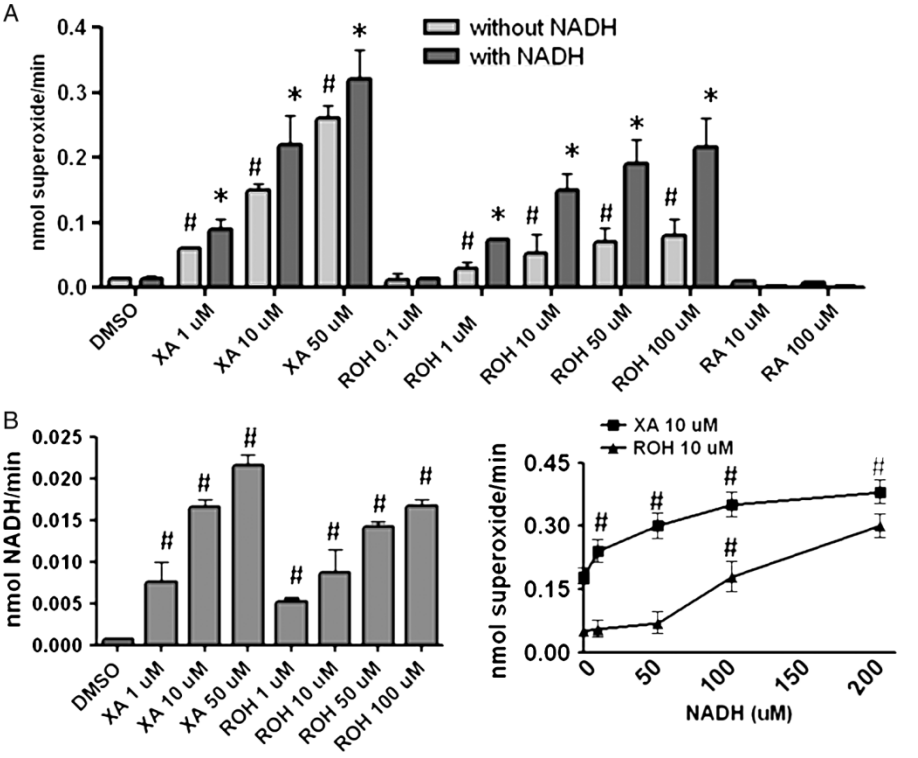

Figure 1. *In vitro* superoxide generation by xanthine oxidase in the presence of ROH. (A) Purified xanthine oxidase was incubated with different concentrations (μM) of ROH, RA and xanthine, in the presence or absence of 100 μM NADH. The rate of superoxide formation was estimated by superoxide-mediated NBT reduction over 1 h of incubation as described in the Materials and methods section. (B) NADH oxidation by xanthine oxidase reaction was evaluated by measuring the decrease of absorption at 340 nm. (C) Superoxide formation was determined in the presence of different NADH concentrations (μM). ROH 10 μM or xanthine 10 μM-induced superoxide formation was monitored by NBT reduction. Legends: DMSO (dimethylsulphoxide, vehicle), XA (xanthine); ROH (retinol); RA (retinoic acid). Data from four independent experiments in quadruple ($n=4$). #Different from basal levels, *different from its respective incubations in the absence of NADH, $p<0.05$, ANOVA.

rate of superoxide generation in the presence of the classical xanthine oxidase substrate, xanthine. The addition of 100 μM NADH to these enzyme assay systems caused a 3-fold increment on superoxide formation by ROH and a 1.4-fold increase in xanthine-induced superoxide production (Figure 1A).

We confirmed the oxidation of NADH to NAD by xanthine oxidase-catalysed ROH oxidation by measuring the decrease of NADH absorption at 340 nm in the presence of different ROH concentrations (Figure 1B). NADH consumption was dose-dependent in the range of 1–100 μM ROH. Variations on NADH concentrations showed that, *in vitro*, the NADH concentration was more important to ROH-elicited than xanthine-induced superoxide formation by xanthine oxidase reaction (Figure 1C). At low NADH concentrations (i.e. 0, 1 and 50 μM), the rate of 10 μM xanthine-elicited superoxide formation by xanthine oxidase was 6-fold higher than that induced by 10 μM ROH. In addition, we observed that a 4-fold increase in NADH concentration in the enzyme assay (i.e. 50 to 200 μM) reflects only in a 1.4-fold increase in superoxide formation with 10 μM xanthine as substract. The same increase in NADH concentration promotes a 6-fold increase in superoxide generation by ROH incubation (Figure 1C). Thus, further assays were performed in the presence of 100 μM NADH.

Influence of ROH on uric acid formation from xanthine and the effect of allopurinol on superoxide formation by xanthine oxidase in the presence of ROH

The effect of ROH on xanthine oxidase-catalysed xanthine conversion to uric acid was evaluated by incubating 10 μM xanthine, 0.02 units/mL xanthine oxidase and 10 or 20 μM ROH in the presence of 100 μM NADH. The rate of uric acid formation was monitored during 20 min at 295 nm. Results showed that 10 and 20 μM ROH significantly inhibited xanthine oxidase-dependent uric acid formation from xanthine by $21\pm3\%$ and $45\pm4.2\%$, respectively (Figure 2A). In another xanthine oxidase assay system, we observed that 1 and 10 μM allopurinol exerted a well-known effect inhibiting $51\pm5.1\%$ and $91\pm7\%$, respectively, the 10 μM ROH-elicited superoxide-dependent NBT reduction *in vitro* (Figure 2B). These results altogether suggest that xantinhe oxidase is able to generate superoxide anion in the presence of ROH. Superoxide formation is over-stimulated in the presence of NADH and is inhibited by allopurinol. In

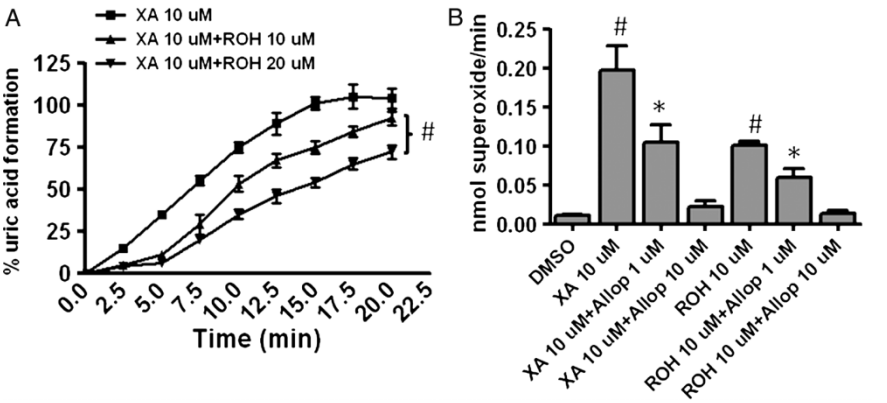

Figure 2. Influence of ROH on uric acid formation from xanthine and the effect of allopurinol on superoxide formation by xanthine oxidase in the presence of ROH. (A) Xanthine oxidase-catalysed uric acid formation from xanthine was evaluated in the presence of 10 and 20 μM ROH as described in Materials and methods. (B) Allopurinol effect on ROH-elicited superoxide formation. *In vitro* enzyme system was incubated in the presence of 1 and 10 μM of allopurinol and the rate of superoxide-mediated NBT reduction was monitored at 560 nm. Legends: DMSO (dimethylsulphoxide, vehicle), XA (xanthine), Allop (allopurinol). Data from four independent experiments in quadruple ($n=4$). #Different from basal level; *different from its respective positive control (10 μM xanthine or 10 μM ROH) and from basal levels. $p < 0.05$, ANOVA.

addition, ROH seems to compete with the classical xanthine oxidase substrate, xanthine.

Inhibition of xanthine oxidase decreases ROH-induced ROS production, protein oxidative damage and cytotoxicity in Sertoli cells

Previous works from our group and others reported that ROH presents strong pro-oxidative properties in biological systems [10–25]. Since *in vitro* experiments suggest that xanthine oxidase generates ROS in the presence of ROH and our previous data reported oxidative damage to lipids, protein and DNA in ROH-treated Sertoli cells, we decided to investigate the involvement of the xanthine oxidase on ROH-elicited ROS production in these cells. Thus, cells were treated for 24 h with different ROH concentrations in the presence or absence of the xanthine oxidase inhibitor allopurinol at 50 μM. Allopurinol treatment resulted in a reduction of over 35% on 7–20 μM ROH-induced ROS production during 24 h treatment (Figure 3A). Another set of experiments were performed by incubating Sertoli cells with RA (0.1–20 μM) for 24 h. RA treatment did not increase ROS production at any tested concentration (data not shown). As expected, 50 μM allopurinol did not inhibit 50 μM hydrogen peroxide-elicited ROS production, confirming that allopurinol did not act as ROS scavenger *per se* (Figure 3A). Moreover, we detected increased protein carbonyl damage following 10 μM ROH treatment at 24 h which was also inhibited by allopurinol (Figure 3B). The evaluation of xanthine oxidase activity showed that the enzyme is 1.6-fold more active in 10 μM ROH-treated cells when compared to untreated cells at 12 h treatment (Figure 4A). Following 10–20 μM ROH treatment, Sertoli cell viability was reduced as assessed by the MTT assay (Figure 4B). In agreement with DCF and

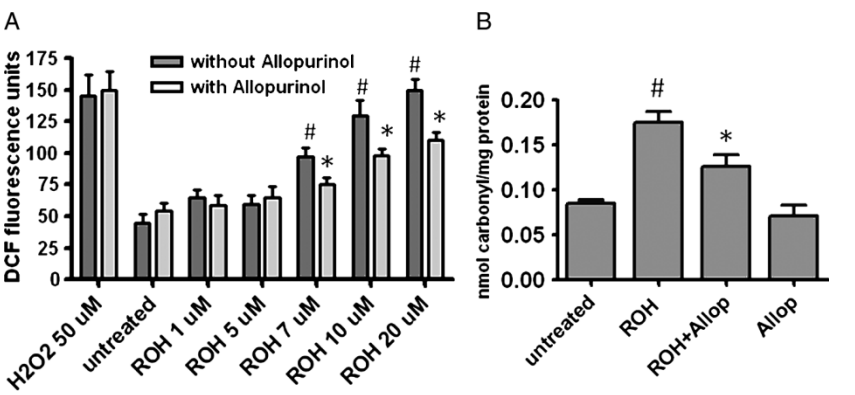

Figure 3. Xanthine oxidase mediates ROS production and oxidative damage in Sertoli cells. (A) ROS production in Sertoli cells treated with different ROH concentrations (μM) for 24 h in the presence or absence of 50 μM allopurinol. (B) Allopurinol effect on protein oxidative damage (carbonyl groups) in 10 μM ROH-treated cells. Cells were incubated with 10 μM ROH during 24 h and carbonyl groups were quantified as described in the Materials and methods section. Legends: ROH (10 μM retinol); Allop (50 μM Allopurinol), H_2O_2 (50 μM Hydrogen peroxide). Data from three independent experiments in triplicate ($n=3$). #Different from untreated cells; *different from its respective ROH-treated and from untreated cells. $p<0.05$, ANOVA.

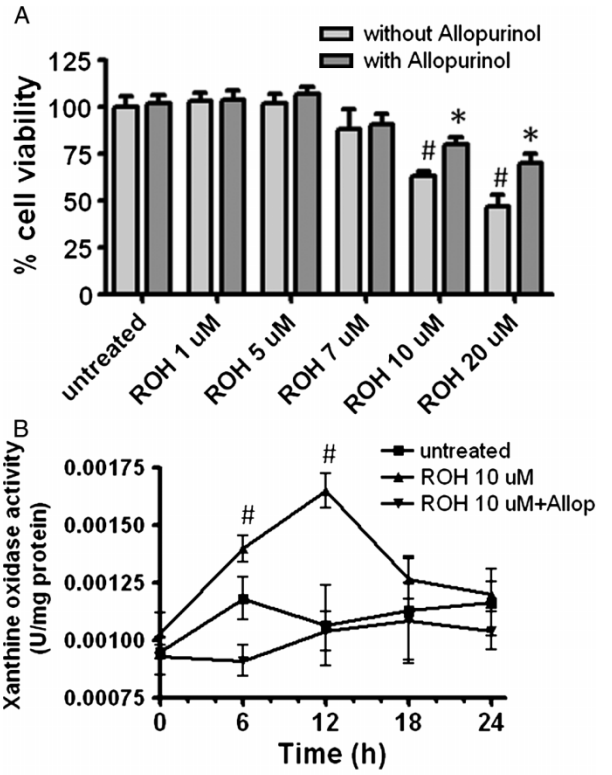

Figure 4. ROH increases xanthine oxidase activity and induces allopurinol-inhibited cytotoxicity. (A) Time course effect of ROH on xanthine oxidase activity in Sertoli cells. Cells were incubated with 10 μM ROH for different times (h), extracts were obtained by potter homogenization and xanthine oxidase activity was measured by the rate of the enzyme-catalysed uric acid formation from 100 μM xanthine at 295 nm. (B) ROH-mediated decrease in cell viability was evaluated by MTT assay after cell incubation with different ROH concentrations (μM) for 24 h in the presence or absence of allopurinol. Legends: Allop (50 μM allopurinol). Data from three independent experiments in triplicate ($n=3$). #Different from untreated cells; *different from its respective ROH-treated and from untreated cells. $p<0.05$, ANOVA.

carbonyl assays, at the end of 24 h treatment, the decrease in cell viability by 10 and 20 μM ROH was ~40% inhibited by 50 μM allopurinol (Figure 4B).

Inhibition of retinaldehyde dehydrogenases potentiates xanthine oxidase-dependent ROS production and cytotoxicity

In order to block ROH metabolism by retinaldehyde dehydrogenases and to increase bioavailability of ROH and retinaldehyde to xanthine oxidase, we treated Sertoli cells with 200 μM citral, a classical selective competitive retinaldehyde dehydrogenase inhibitor [32,33], for 15 min prior to 10 μM ROH addition. At the end of 24 h, the pre-treatment with citral potentiated 10 μM ROH-induced ROS production as compared to 10 μM ROH alone (Figure 5A). The over-stimulation of ROS production by the retinaldehyde dehydrogenase inhibitor in the presence of ROH was blocked by 50 μM allopurinol.

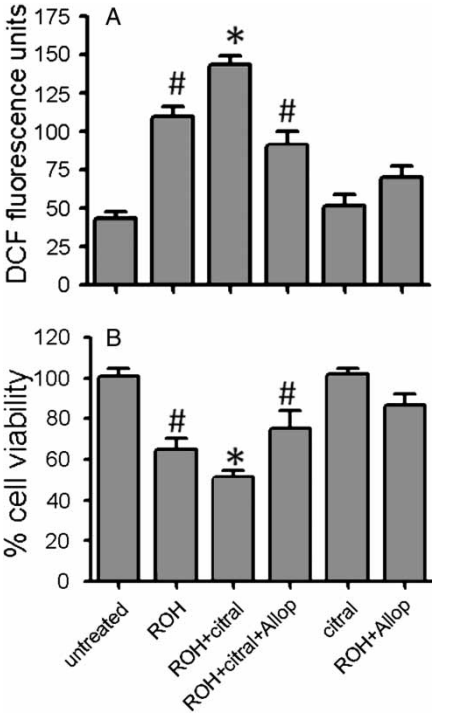

Figure 5. Retinaldehyde dehydrogenase inhibition potentiates xanthine oxidase-dependent ROS production and cytotoxicity in ROH-treated cells. (A) Effect of 200 μM citral pre-treatment on 10 μM ROH-induced ROS and cytotoxicity in the presence or absence of 50 μM allopurinol. Citral or citral plus allopurinol were added 15 min prior to 10 μM ROH incubation and ROS were monitored during 24 h by DCF fluorescence. (B) ROH-induced decrease in cell viability is potentiated in the presence of citral in an allopurinol-inhibited manner. Legends: ROH (10 μM retinol), citral (200 μM citral), Allop (50 μM allopurinol). #Different from untreated cells; *different from ROH-treated and from untreated cells. $p<0.05$, ANOVA.

Citral alone had no effect on ROS production. In agreement, the 10 μM ROH-induced decrease in cell viability was also potentiated in the presence of citral; this effect was also attenuated by allopurinol (Figure 5B). These data altogether suggest that the enhancement in both ROS production and cell death induced by citral in ROH-treated cells was mediated by xanthine oxidase reaction.

Discussion

Previous studies from our group and others have reported that ROH and its derivatives, retinoids, present redox-active properties in biological systems [7–25]. Clinical trials have been carried out based on the potential antioxidant role of retinoids and carotenoids, but in some studies retinoid supplementation had to be discontinued due to increased mortality related to lung cancer and cardiovascular disease incidence [7]. It is possible that many of the deleterious side effects observed in some clinical trials with retinoids may be related to free radical and oxidants generation in vitamin A supplementation.

Recently, some works from our group reported that ROH-palmitate supplementation induces lipoperoxidation, protein carbonylation, thiol oxidation and mitochondrial damage in the cortex, cerebellum and substantia nigra of Wistar rats [22–25]. However, the mechanisms by which ROH and retinoids induce oxidative stress remain uncertain.

Testicular Sertoli cells are one of the principal physiological ROH targets in mammalian [4,34,35]. It has been well established that ROH and its derivatives regulate many reproductive related functions in these [4,16,34], although some studies reported that supplementation with high doses of vitamin A leads to testicular lesions, cytotoxicity to Sertoli cells and spermatogenesis disorders [13,36]. ROH concentrations in Sertoli cells may vary between 2–5 μM depending on plasma ROH levels [4,16,34,37]. We previously reported that slight variations in the ROH concentrations may trigger important changes in the cellular redox state [10–21]. ROH concentrations above 5 μM are able to increase intracellular ROS production and this led to DNA oxidative damage and apoptosis in primary Sertoli cells. The recently published work reporting that purified xanthine oxidase catalyses ROH oxidation to RA *in vitro* stimulated us to investigate whether the enzyme could be involved in the pro-oxidant effect of ROH in our cellular model. The work conduced by Taibi and Nicotra [1] did not contemplate the xanthine oxidase-catalysed ROH oxidation as a possible source of ROS in ROH supplementation. Thus, initially, we investigated some *in vitro* parameters regarding superoxide production by xanthine oxidase in the presence of ROH. Data showed that ROH concentrations as low as 1 μM were able to increase superoxide production by milk xanthine oxidase in an allopurinol-inhibited manner. RA, the described final product of the enzyme reaction, did not induce superoxide formation. The inhibitory effect of allopurinol on superoxide production *in vitro* was also taken into account in order to indirectly deduce that the mechanism of superoxide production by xanthine oxidase-catalysed ROH oxidation is probably the same as that for purine substrates. The inhibitory effect of ROH on uric acid formation from xanthine suggests that ROH and xanthine are metabolized at the same enzyme site [2]. Interestingly, superoxide formation during ROH oxidation by the enzyme was strongly enhanced (3-fold) in the presence of NADH as compared to ROH alone. In contrast, superoxide production by the enzyme when xanthine was used as substrate increased only 1.4-fold in the presence of 100 μM NADH. The importance of NADH on superoxide production by xanthine oxidase-catalysed ROH oxidation is in agreement with the classical reaction mechanism in which the enzyme may to use NADH to reduce molecular oxygen and to form superoxide anions [3,4].

In cultured Sertoli cells, ROH stimulated xanthine oxidase activity and the pre-treatment with 50 μM allopurinol attenuated ROH-induced ROS production suggesting a role for xanthine oxidase in ROH pro-oxidant effect. Not only ROS production, but also the increase in protein damage and the decrease in cell viability promoted by ROH were inhibited by allopurinol pre-treatment. Pharmacological inhibition of the ROH oxidation to RA by citral, a retinaldehyde dehydrogenase inhibitor, potentiated the ROH-induced ROS production. This effect was also inhibited by allopurinol. Retinaldehyde dehydrogenases are important enzymes involved in RA synthesis from the ROH-derived retinaldehyde in the cell [32]. Inhibition of retinaldehyde dehydrogenases accumulates retinaldehyde and ROH, two xanthine oxidase substrates [1,2]. Possibly, ROH and retinaldehyde metabolism by xanthine oxidase was stimulated in the presence of the retinaldehyde dehydrogenase inhibitor since allopurinol totally blocked the effects of citral on ROH-induced ROS production.

On the other hand, RA—the final product of xanthine oxidase-catalysed ROH oxidation—did not present any ROS-generating effect on *in vitro* enzyme assays and in cultured Sertoli cells. In fact, previous works reported that ROH and retinaldehyde, but not RA, induce ROS-dependent cell death in human fibroblasts and PC-12 cells [11,12]. Possibly, some steps of the oxidative metabolism of ROH to retinaldehyde and retinaldehyde to RA may be involved in ROH and retinaldehyde-dependent ROS generation. Our data showed direct evidence that xanthine oxidase may be involved, at least in part, in ROH-induced oxidative stress. However, the herein performed assays showed that total enzyme inhibition with allopurinol (as showed in Figure 4) presented only a partial inhibitory effect in ROH-induced ROS production, protein damage and cytotoxicity. Our previous data suggest that other free radical sources such as mitochondria also mediate free radical production in ROH-treated Sertoli cells and rat brain [13,19,24]. In addition, it was observed that ROH and retinaldehyde auto-oxidation generates superoxide *in vitro* [38]. Thus, others free radical sources different from xanthine oxidase reaction are involved in the ROS-generating properties of ROH.

In spite of being a recently described function, the studies suggest that xanthine oxidase-catalysed ROH and retinaldehyde oxidation is a feasible process *in vivo* which also may play a role in situations in which enzyme activity is stimulated such as surgical stress and hypoxia/reperfusion injury [39,40]. Xanthine oxidase-dependent decreases in retinaldehyde and increases in RA levels were observed after surgical stress in small intestine cells, suggesting a possible

role of the enzyme on RA formation [40]. In addition, the determined Km value for retinaldehyde (0.29 μM) may indicate that the processing of retinaldehyde by xanthine oxidase possibly occurs *in vivo* [2]. In our experimental model, inhibition of the ROH metabolism by dehydrogenases inhibitor, which in turn accumulates ROH and retinaldehyde [32], have increased the xanthine oxidase-dependent ROS production, suggesting the existence of ROH/retinaldehyde metabolism by xanthine oxidase as a source of cytosolic ROS.

Overall, data presented in this work showed that xanthine oxidase *in vitro* generates superoxide anions in the presence of ROH. Superoxide formation is enhanced in NADH presence and inhibited by allopurinol, a classical xanthine oxidase inhibitor. In Sertoli cells, ROH stimulated xanthine oxidase activity and ROH oxidative-related effects were attenuated by allopurinol treatment, suggesting the involvement of the xanthine oxidase in ROH-induced ROS production in our model. This is the first study demonstrating that xanthine oxidase mediates vitamin A pro-oxidant effects in a biological system.

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