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ScienceDirect
Toxicology in Vitro 22 (2008) 553–558
Toxicology in Vitro
www.elsevier.com/locate/toxinvit

Evidence of increased reactive species formation by retinol, but not retinoic acid, in PC12 cells

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Received 24 August 2007; accepted 6 November 2007
Available online 17 November 2007

Abstract

The biological effects of vitamin A (retinol) are generally ascribed to the activation of nuclear retinoid receptors by retinoic acid (RA), considered the most biologically active retinoid. However, it is not established whether the cytotoxic effects of vitamin A are due to retinoid receptors activation by RA. Vitamin A-related toxicity is associated with cellular redox modifications, often leading to severe oxidative damage, but the role of RA in this effect is also uncertain. We therefore studied the formation of intracellular reactive species induced by retinol and retinoic acid in PC12 cells, using an *in vitro* dichlorofluorescein (DCFH) fluorescence real-time assay. We observed that retinol, but not retinoic acid, induced a steady increase in DCF-based fluorescence over 60 min of incubation, and this increase was reversed by antioxidant (*N*-acetyl-cysteine and α-tocopherol) pre-treatment. This effect was also inhibited by the iron chelator 1,10-phenantroline and the impermeable calcium chelator EGTA. These results suggest that vitamin A-associated cytotoxicity is probably related to an oxidant mechanism dependent on iron and calcium, and the formation of intracellular reactive species is related to retinol, but not to RA.

Keywords: Retinol; Retinoic acid; Intracellular reactive species; DCFH; PC12

1. Introduction

Vitamin A (retinol) is an essential factor for regulation of cell cycle and differentiation during early stages of development. Retinol can be converted to retinoic acid (RA), which is the main activator of nuclear steroid receptors known as retinoid receptors. These receptors are subdivided into retinoic acid receptors (RAR) and retinoid X receptors (RXR) and it is generally accepted that most of the biological actions of retinol are mediated by the modulation of these receptors, after the conversion of retinol into RA. However, other studies have shown that retinol and RA can exert some of their effects by triggering cellular events not related to retinoid receptor activation; these events have been referred to as non-genomic or extranuclear effects of retinoids (Liao et al., 2004; Elliot, 2005).

Retinoids have redox-related properties and influence the oxidant status of the cell (Murata and Kawanishi, 2000; Klamt et al., 2003). Many authors suggested that retinol and related molecules, such as beta-carotene, act in biological systems as antioxidants and thus could be potential clinical agents in antioxidant therapies for treatment and prevention of malignant and neurodegenerative diseases (Okuno et al., 2004). However, clinical trials have observed that retinoids can also be deleterious and are associated with activation of proto-oncogenes, leading to an increased incidence of neoplasias (Omenn, 2007). The existence of these apparently conflicting data implies that more studies are necessary to address this issue. This is especially so when it is considered that retinoid-based therapies present potential treatments for a range of diseases related to cell cycle disruption/cell death and

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0887-2333/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tiv.2007.11.007

increased reactive oxygen species (ROS) formation, including skin cancer, lung cancer, Parkinson's disease and Alzheimer disease (Njar et al., 2006; Greenwald et al., 2006; Ono and Yamada, 2007).

Our group has been studying the effects of retinol on oxidative stress-related parameters, and we observed that retinol induced several cytotoxic parameters. We reported that retinol increased lipoperoxidation, DNA oxidative damage, mitochondrial superoxide production and led to mitochondrial membrane swelling and cytochrome c release, causing cell death (Dal-Pizzol et al., 2000; Dal-Pizzol et al., 2001; Klamt et al., 2005). Furthermore, retinol activated ERK1/2 and CREB, causing proliferative focus formation, and these effects were reversed by antioxidant treatment (Gelain et al., 2006). Interestingly, these results were always observed with the administration of 7 μM retinol, while concentrations up to 5 μM – which is considered to be within the physiological range for cells (Ross, 1993) – were never observed to induce deleterious effects. This suggested that slight variations in the concentrations of retinol may trigger changes in the cellular redox state. These results indicate that retinol can also induce toxic effects, not compatible with the genomic action of retinoid receptors, and these effects are, at least in part, mediated by intracellular reactive oxygen species.

It is not known whether the pro-oxidant properties observed with retinol are due to its conversion to RA. The fact that this conversion is by many different non-specific cellular dehydrogenases (Lidén et al., 2003) also made difficult an approach using enzymatic inhibitors.

In the present study, we report that retinol, but not RA, increases intracellular reactive species formation in cultured PC12 cells, using a real-time DCFH-DA based assay for intact, living cells (Wang and Joseph, 1999). This is the first time that increased production of intracellular reactive species by retinol is demonstrated in PC12 cells, and also that RA is shown not to increase the formation of these species by the same approach. Also, we observed that EGTA (calcium chelator) and 1,10-phenantroline (iron chelator) inhibited the effect of retinol, suggesting that the presence of calcium and iron is essential for retinol-induced toxicity.

2. Material and methods

2.1. Chemicals

EGTA, *N*-acetyl-cysteine (NAC), all-*trans* retinol alcohol, all-*trans* retinoic acid (RA), 2′,7′-dichlorohydrofluorescein diacetate (DCFH-DA) and 1,10-phenantroline were from Sigma Chemical Co. (St Louis, MO, USA). Retinol and RA were dissolved in ethanol. Concentrated stocks were prepared immediately before experiments by diluting retinol or RA into ethanol and determining final stock concentration by UV absorption; solutions were kept protected from light and temperature during all procedures. Appropriate solvent controls were performed for each condition. Retinol and RA 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. Tissue culture reagents were from Sigma Chemical Co. and were of analytical or tissue culture grade.

2.2. Cell culture

PC12 cells were originally obtained from the ATCC (VA, USA) and were kindly donated by Dr. Peter R Dunkley (University of Newcastle, Australia) and Dr. Alicia Kolwaltolwski (Universidade de Sao Paulo, Brazil). Cells were plated onto plastic culture plates and maintained under a 5% CO_2-containing atmosphere at 37 °C in the presence of complete medium (Dulbecco's modified Eagle's medium supplemented with 4 mM L-glutamine, 5.5 mM glucose, 10% fetal bovine serum, 5% fetal horse serum, 15 mM HEPES, pH 7.4, 100 mg/mL penicillin G, 100 mg/mL streptomycin, 50 mg/mL gentamicin and 5 mg/mL Mycostatin) for 24–48 h, or until cells reached 70–80% confluence. Preliminary MTT viability assay indicated that cell viability was not affected during incubations.

2.3. DCFH-DA assay

Intracellular ROS production was determined by the DCFH-DA-based real-time assay using intact living cells, as described by Wang and Joseph (1999). Briefly, PC12 cells were plated onto 96-well plates and incubated for 1 h with DCFH-DA 100 μM (stock solution in DMSO, 10 mM) in 1% FBS culture medium at 5% CO_2 and 37 °C. Then cells were washed and treatments were carried out. During treatment, changes in the fluorescence by the oxidation of DCFH into the fluorogen DCF were monitored in a microplate fluorescence reader (F2000, Hitachi Ltd., Tokyo, Japan) for 1 h at 37 °C. H_2O_2 1 mM was used as positive control for ROS production. Excitation filter was set at 485 ± 10 nm and the emission filter was set at 530 ± 12.5 nm. Data were recorded every 30 s and plotted in Excel software.

2.4. MTT assay

Following retinol treatment, PC12 cells viability was assessed by the MTT assay. This method is based on the ability of viable cells to reduce MTT (3-(4,5-dimethyl)-2,5-diphenyl tetrazolium bromide) and form a blue formazan product. MTT solution (sterile stock solution of 5 mg/mL) was added to the incubation medium in the wells at a final concentration of 0.2 mg/mL. The cells were left for 45 min at 37 C in a humidified 5% CO_2 atmosphere. The medium was then removed and plates were shaken with DMSO for 30 min. The optical density of each well was measured at 550 nm (test) and 690 nm (reference). H_2O_2 1 mM was used as positive control.

3. Results

Real-time DCFH-DA assay showed that retinol induced a concentration-dependent steady increase in intracellular reactive species in PC12 cells over 1 h of incubation (Fig. 1A). While concentrations up to 2.5 μM retinol did not have any effect, we observed increased formation of intracellular reactive species with 5 μM and 10 μM. The rate of increase in DCF fluorescence shown in Fig. 1B was calculated over this first hour of incubation. This range of concentrations was chosen based on physiological levels reported for cells (Ross, 1993). A similar experiment using RA (0.1–10 μM) was performed. However, RA failed to produce any effect upon PC12 cells over 1 h of incubation (not shown). Fig. 1C and D shows DCF-based fluorescence in cells treated with retinoic acid (10 μM) in comparison to cells incubated with retinol (10 μM) and H_2O_2 (1 mM, used as a positive control for DCF fluorescence). Vehicle (ethanol 0.2%) had no effect on this assay.

Next, we verified the effect of different antioxidant pre-treatments (30 min) on the retinol-induced increase in DCF fluorescence. Both *N*-acetyl-cysteine (NAC) 1 mM and α-tocopherol 0.1 mM inhibited the effect of retinol (Fig. 2A and B), indicating that antioxidant pre-treatment is able to inhibit the pro-oxidant effect of retinol. This result agrees with our previous observations, where antioxidants inhibited DNA damage and apoptosis caused by retinol treatment (Dal-Pizzol et al., 2001).

We previously observed that DNA damage induced by retinol is dependent on iron uptake, and that iron chelators inhibited DNA damage by retinol treatment (Dal-Pizzol et al., 2001). Recent observations also suggested that calcium might be involved in the pro-oxidant effects of retinol (Klamt et al., 2005). In this regard, we verified the effect of pre-incubation with the iron chelator 1,10-phenantroline (0.1 mM) or the calcium chelator EGTA (4 mM) on the effect of retinol. Both treatments inhibited retinol-induced DCF fluorescence (Fig. 2C and D). Combination of both treatments further decreased intracellular reactive species production. This result suggests that iron and calcium were involved in the production of cellular free radicals induced by retinol. Viability measurements by the MTT assay

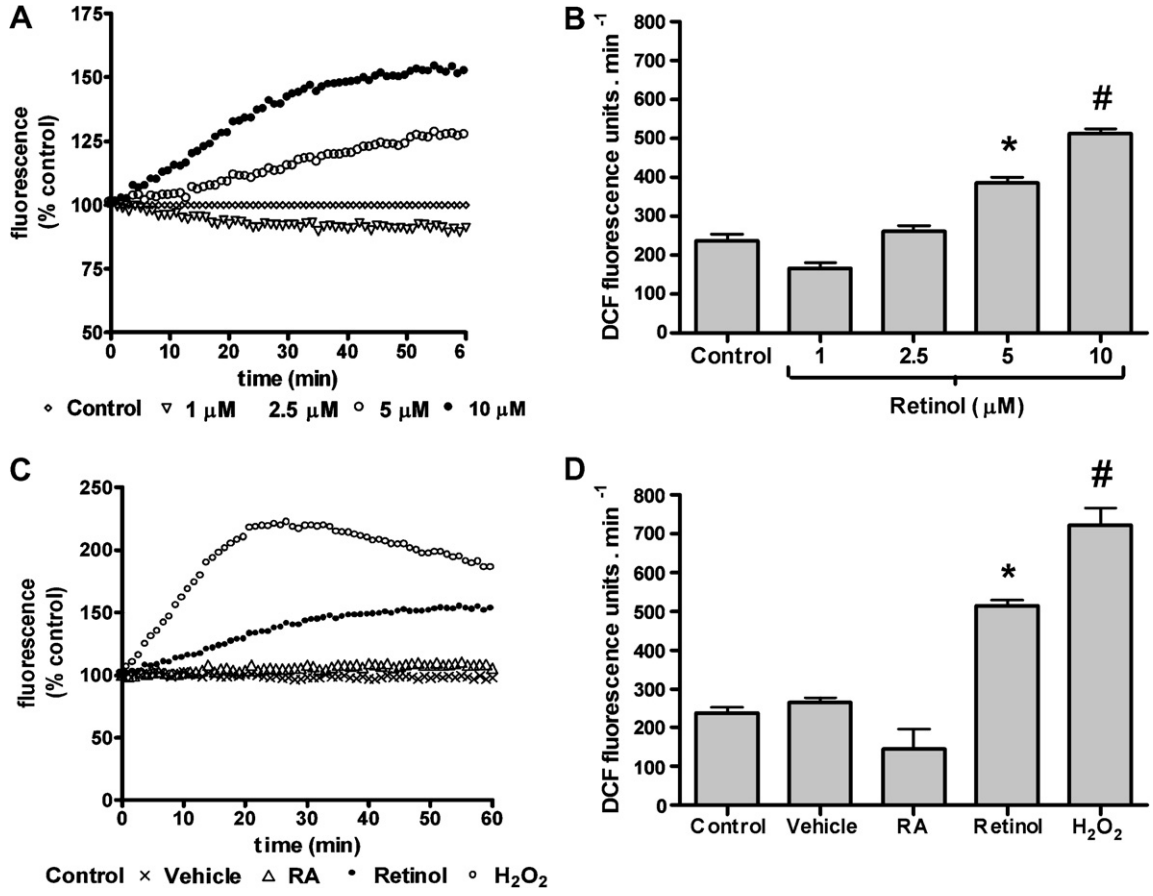

Fig. 1. Kinetics of intracellular reactive species production in PC12 cells evaluated by the DCFH-DA real-time fluorescence assay. (A) PC12 cells were incubated with increasing concentrations of retinol (up to 10 μM) for 60 min. (B) The rate of DCF-based fluorescence production over this period was plotted and analysed. (C) Kinetics and (D) rate of DCF-based fluorescence production were compared between PC12 cells treated with only ethanol 0.2% (vehicle), retinoic acid 10 μM (RA), retinol 10 μM (retinol) and H_2O_2 1 mM. Data are representative of 3 different experiments (A and C, each line is the mean of 16 different wells) or expressed as mean + S.E.M and were analysed by one-way ANOVA followed by Duncan's post hoc test using SPSS 11.0 software (B and D). $^{*}p < 0.05$, $^{\#}p < 0.01$.

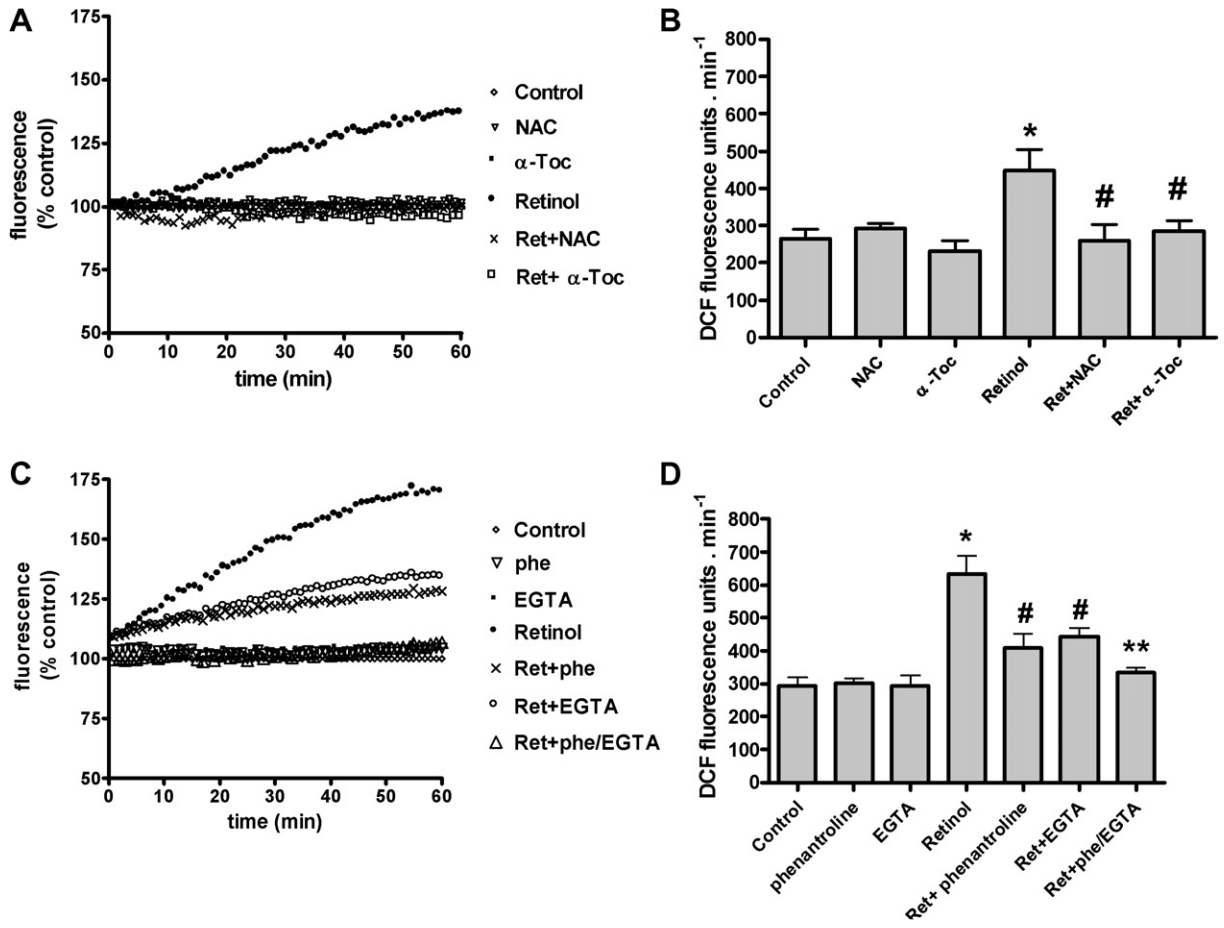

Fig. 2. Effect of antioxidants and iron and calcium chelators on retinol-induced intracellular reactive species formation. The effect of 30 min pre-incubation with (A) *N*-Acetyl-cysteine 1 mM (NAC) and α-tocopherol 0.1 mM, or (C) the iron chelator 1,10-phenantroline 0.1 mM (phe) and the calcium chelator EGTA 4 mM on DCF-based fluorescence over 60 min of retinol 10 μM incubation is shown. The rate of DCF-based fluorescence increase during this time course was analysed (B and D). Data are representative of 3 different experiments (A and C, each line is the mean of 16 different wells) or expressed as mean + S.E.M and were analysed by one-way ANOVA followed by Duncan's post hoc test using SPSS 11.0 software (B and D). $^{*}p < 0.01$, $^{\#}p < 0.05$, $^{**}p < 0.001$.

indicated no alteration on cell viability by retinol (10 μM) or RA (1 μM) after 60 min of incubation; however, after 24 h, a decrease in cellular viability was observed in retinol-treated cells (Fig. 3).

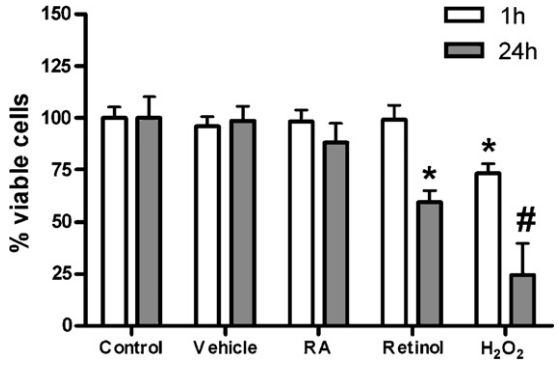

Fig. 3. MTT assay. Cellular viability was assessed by MTT assay after 1 h or 24 h of ethanol 0.2% (vehicle), retinoic acid 10 μM (RA), retinol 10 μM (retinol) and H_2O_2 1 mM incubation. Data are expressed as mean + S.E.M and were analysed by one-way ANOVA followed by Duncan's post hoc test using SPSS 11.0 software. $^{*}p < 0.05$, $^{\#}p < 0.01$.

4. Discussion

The ability of retinol and related molecules to act as antioxidant or protective agents in biological systems has been strongly supported in recent years. Several studies have reported a correlation between low serum levels of retinoids, or carotenoids, and the incidence of pathologies that are related in part to increased ROS production, such as neurodegenerative diseases and neoplasias (Jimenez-Jimenez et al., 1993; Greenwald et al., 2006). The mechanisms underlying these apparent protective properties are still not well understood. Clinical trials were undertaken aimed at establishing the efficiency of retinoid/carotenoid-based antioxidant therapies against lung cancer (the ATBC and the CARET trials); however these studies had to be discontinued due to the increased mortality related to lung cancer and cardiovascular disease incidence caused by the experimental treatments (Omenn et al., 1994). More recent studies observed that retinol or related molecules could exert pro-oxidant and cytotoxic effects, both *in vitro* and *in vivo* (Murata and Kawanishi, 2000; Pennis-

ton and Tanumihardjo, 2006). The mechanisms of these effects are also poorly understood.

This is the first time that reactive species production is demonstrated to be increased by retinol incubation in intact living cells. DCFH is commonly used as a probe to evaluate intracellular ROS formation; it was first described as a hydrogen peroxide-specific probe, but later it was shown to be sensitive to other reactive species as well, including hydroxyl and peroxyl radicals (LeBel et al., 1992). Nevertheless, it is demonstrated to be an excellent index for quantification of cellular oxidative stress, since it is oxidized intracellularly and the emitted fluorescence was demonstrated to be directly proportional to the production of intracellular reactive species. This is an advantage in relation to other methods that evaluate oxidative stress indirectly by the quantification of damaged biomolecules (Wang and Joseph, 1999). The assay used in this study is an adaptation to the original technique, which allows it to work with intact living cells, and to assess the production of ROS, besides other reactive species, in real time (Wang and Joseph, 1999). In this regard, the data presented here strongly indicate suggest that retinol is able to induce oxidative stress in cell systems by increasing the production of free radicals in a concentration-dependent manner.

It has been reported by different groups that other retinoids than vitamin A itself have pro-oxidant and/or toxic properties. Beta-carotene was observed to stimulate human pulmonary adenocarcinoma cells by activation of PKA and ERK1/2 pathways (Al-Wadei et al., 2006), to induce haem oxygenase-1 in HFP-1 cells (Obermuller-Jevic et al., 1999) and to increase ROS production and decrease cellular oxidised glutathione content in cancer cell lines (Palozza et al., 2003). Murata and Kawanishi (2000) observed the occurrence of DNA oxidative damage *in vitro* caused by retinol and retinal. In this study we observed that retinol had a very clear effect increasing ROS production in a time- and concentration-dependent manner. In contrast, a wide range of concentrations of RA failed to produce any effect. However, RA did not seem to exert antioxidant effects in these cells, since it has not decreased basal reactive species production; also, RA co-incubation with retinol had not affected retinol-induced intracellular reactive species production (data not shown).

It has long been postulated that RA is the most active metabolite of retinol in biological systems; many authors refer to RA simply as the "metabolically active vitamin A" (Zile, 2001). This concept is related to the fact that, among the metabolites of retinol, only all-*trans* RA and 9-*cis* RA are able to activate gene transcription through the activation of the RAR and RXR retinoid receptors (Chambon, 1994). However, it has becoming increasingly clear that vitamin A-related compounds, including RA, exhibit biological activities other than RAR/RXR-mediated gene transcription (Ochoa et al., 2003; Aggarwal et al., 2006). Despite the observations that retinol administration to cells and vitamin A supplementation protocols may lead to increased oxidative damage or toxicity (Dal-Pizzol et al., 2001; Omenn et al., 1994), the question about whether these effects are caused by the action of retinol itself or by the RA generated after retinol administration/intake has never been explored. Here, we have not observed any similarities between the effect of retinol and RA on cellular reactive species production by the DCFH assay. This suggests that vitamin A may exert different effects on cell systems by the action of different derivatives from retinol, including retinol itself. These actions may well involve both genomic and non-genomic mechanisms.

We observed that retinol-dependent ROS production was inhibited by iron and calcium chelators. We had previously noted that iron was involved in the oxidant-mediated cytotoxicity by retinol (Dal-Pizzol et al., 2000), but this is the first time we observed the involvement of calcium on intracellular reactive species production by retinol. Since EGTA is not permeable to cell membranes, this effect is probably dependent on extracellular calcium. The involvement of iron in ROS production is generally associated to the transition metal-catalyzed Fenton reaction, which accounts for the production of highly reactive hydroxyl radicals from previously formed hydrogen peroxide, leading to severe oxidative damage (Terman and Brunk, 2006). However, there are no reports linking extracellular calcium to increased ROS formation in cells. DCFH reacts intracellularly with both hydroxyl radicals and hydrogen peroxide, thus the inhibition of iron-mediated radicals production by the iron chelator 1,10-phenantroline is probably diminishing the DCF fluorescence associated only to hydroxyl radicals, but not to hydrogen peroxide. As hydrogen peroxide production is also probably increased by retinol, since this compound is necessary for iron-mediated hydroxyl formation, the remaining DCF fluorescence in cells pre-treated with the iron chelator is most likely due to hydrogen peroxide formed by a mechanism dependent on extracellular calcium, since combined EGTA and 1,10-phenantroline further inhibit ROS production by retinol.

In conclusion, this is the first work demonstrating intracellular reactive species production by retinol using intact, living cells, by a real-time fluorescence-based assay, and the first time that retinol-induced ROS formation was observed to be dependent on both iron and calcium. We did not observe any pro-oxidant effect by RA, indicating that most pro-oxidant and/or redox activities associated to vitamin A are not related to the *in vivo* production of RA from retinol. This may also explain the decreased cellular viability observed after 24 h of retinol incubation, which was not observed with RA.

Acknowledgement

This work was supported by CAPES, FAPERGS, CNPq and PROPESQ/UFRGS. We would like to thank Professor Peter R. Dunkley for reviewing the manuscript.

References

Aggarwal, S., Kim, S.W., Cheon, K., Tabassam, F.H., Yoon, J.H., Koo, J.S., 2006. Nonclassical action of retinoic acid on the activation of the cAMP response element-binding protein in normal human bronchial epithelial cells. Molecular Biology of the Cell 17, 566–575.

Al-Wadei, H.A., Takahashi, T., Schuller, H.M., 2006. Growth stimulation of human pulmonary adenocarcinoma cells and small airway epithelial cells by beta-carotene via activation of cAMP, PKA, CREB and ERK1/2. International Journal of Cancer 118, 1370–1380.

Chambon, P., 1994. The retinoid signaling pathway: molecular and genetic analyses. Seminars in Cell Biology 5, 115–125.

Dal-Pizzol, F., Klamt, F., Frota Jr., M.L., Moraes, L.F., Moreira, J.C., Benfato, M.S., 2000. Retinol supplementation induces DNA damage and modulates iron turnover in rat Sertoli cells. Free Radical Research 33, 677–687.

Dal-Pizzol, F., Klamt, F., Benfato, M.S., Bernard, E.A., Moreira, J.C., 2001. Retinol supplementation induces oxidative stress and modulates antioxidant enzyme activities in rat Sertoli cells. Free Radical Research 34, 395–404.

Elliot, R., 2005. Mechanisms of genomic and non-genomic actions of carotenoids. Biochimica et Biophysica Acta 1740, 147–154.

Gelain, D.P., Cammarota, M., Zanotto-Filho, A., de Oliveira, R.B., Dal-Pizzol, F., Izquierdo, I., Bevilaqua, L.R., Moreira, J.C., 2006. Retinol induces the ERK 1/2-dependent phosphorylation of CREB through a pathway involving the generation of reactive oxygen species in cultured Sertoli cells. Cellular Signalling 18, 1685–1694.

Greenwald, P., Anderson, N., Nelson, S.A., Taylor, P.R., 2006. Clinical trials of vitamin and mineral supplements for cancer chemoprevention. American Journal of Clinical Nutrition 85, 3147S–3317S.

Jimenez-Jimenez, F.J., Molina, J.A., Fernandez-Calle, P., Vazquez, A., Cabrera-Valdivia, F., Catalan, M.J., Garcia-Albea, E., Bermejo, F., Codoceo, R., 1993. Serum levels of beta-carotene and other carotenoids in Parkinson's disease. Neuroscience Letters 157, 103–106.

Klamt, F., Dal-Pizzol, F., Rohers, R., Oliveira, R.B., Dalmolin, R.J., Henriques, J.A., Andrades, H.H., Paula Ramos, A.L., Saffi, J., Moreira, J.C., 2003. Genotoxicity, recombinogenicity and cellular preneoplasic transformation induced by vitamin A supplementation. Mutation Research 539, 117–125.

Klamt, F., Roberto de Oliveira, M., Moreira, J.C., 2005. Retinol induces permeability transition and cytochrome c release from rat liver mitochondria. Biochimica et Biophysica Acta 1726, 14–20.

LeBel, C.P., Ischiropoulos, H., Bondy, S.C., 1992. Evaluation of the probe 2′,7′-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. Chemical Research in Toxicology 5, 227–231.

Liao, Y.P., Ho, S.Y., Liou, J.C., 2004. Non-genomic regulation of transmitter release by retinoic acid at developing motoneurons in Xenopus cell culture. Journal of Cell Science 117, 2917–2924.

Lidén, M., Tryggvason, K., Erikkson, U., 2003. Structure and function of dehydrogenases of the short chain dehydrogenase/reductase family. Molecular Aspects of Medicine 24, 403–409.

Murata, M., Kawanishi, S., 2000. Oxidative DNA damage by vitamin A and its derivative via superoxide generation. Journal of Biological Chemistry 275, 2003–2008.

Njar, V.C., Gediya, L., Purushottamachar, P., Chopra, P., Vasaitis, T.S., Khandelwal, A., Mehta, J., Huynh, C., Belosay, A., Patel, J., 2006. Retinoic acid metabolism blocking agents (RAMBAs) for treatment of cancer and dermatological diseases. Bioorganic & Medicinal Chemistry 14, 4323–4340.

Obermuller-Jevic, U.C., Francz, P.I., Frank, J., Flaccus, A., Biesalski, H.K., 1999. Enhancement of the UVA induction of haem oxygenase-1 expression by beta-carotene in human skin fibroblasts. FEBS Letters 460, 212–216.

Ochoa, W.F., Torrecillas, A., Fita, I., Verdaquer, N., Corbalan-Garcia, S., Gomez-Fernandez, J.C., 2003. Retinoic acid binds to the C2-domain of Protein Kinase C(alpha). Biochemistry 42, 8774–8779.

Okuno, M., Kojima, S., Matsushima-Nishiwaki, R., Tsurumi, H., Muto, Y., Friedman, S.L., Moriwaki, H., 2004. Retinoids in cancer chemoprevention. Current Cancer Drug Targets 4, 285–298.

Omenn, G.S., 2007. Chemoprevention of lung cancers: lessons from CARET, the beta-carotene and retinol efficiency trial, and prospects for the future. European Journal of Cancer Prevention 16, 184–191.

Omenn, G.S., Goodman, G.E., Thornquist, M.D., Lund, B., Metch, B., Gylys-Colwell, I., 1994. The beta-carotene and retinol efficacy trial (CARET) for chemoprevention of lung cancer in high risk populations: smokers and asbestos-exposed workers. Cancer Research 54, 2038s–2043s.

Ono, K., Yamada, M., 2007. Vitamin A potently destabilizes preformed alpha-synuclein fibrils in vitro: implications for Lewy body diseases. Neurobiology of Diseases 25, 446–454.

Palozza, P., Serini, S., Di Nicuolo, F., Piccioni, E., Calviello, G., 2003. Prooxidant effects of beta-carotene in cultured cells. Molecular Aspects of Medicine 24, 353–362.

Penniston, K.L., Tanumihardjo, S.A., 2006. The acute and chronic toxic effects of vitamin A. American Journal of Clinical Nutrition 83, 191–201.

Ross, A.C., 1993. Cellular metabolism and activation of retinoids: roles of cellular retinoid-binding proteins. FASEB Journal 7, 317–327.

Terman, A., Brunk, U.M., 2006. Oxidative stress, accumulation of biological 'garbage', and aging. Antioxidant and Redox Signalling 8, 197–204.

Wang, H., Joseph, J.A., 1999. Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. Free Radicals Biology and Medicine 27, 612–616.

Zile, M.H., 2001. Function of vitamin A in vertebrate embryonic development. Journal of Nutrition 131, 705–708.