Short-term vitamin A supplementation at therapeutic doses induces a pro-oxidative state in the hepatic environment and facilitates calcium-ion-induced oxidative stress in rat liver mitochondria independently from permeability transition pore formation

Detrimental effects of vitamin A supplementation on rat liver redox and bioenergetic states homeostasis

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Abstract There is a growing body of evidence showing that vitamin A induces toxic effects in several experimental models and in human beings. In the present work, we have investigated the effects of short-term vitamin A supplementation on the adult rat liver redox status. We have found that vitamin A at therapeutic doses induces a hepatic oxidative insult. Furthermore, we have observed increased antioxidant enzyme activity in the liver of vitamin-A-treated rats. Additionally, some mitochondrial dysfunction was found since superoxide anion production was increased in vitamin-A-treated rat liver submitochondrial particles, which may be the result of impaired mitochondrial electron transfer chain activity, as assessed here. We have also isolated rat liver mitochondria and challenged it with 75 μM $CaCl_2$, a non-oxidant agent that is able to induce mitochondrial oxidative stress indirectly. We have found that mitochondria isolated from vitamin-A-treated rat liver are more sensitive to $CaCl_2$ than control mitochondria regarding the redox status. Importantly, vitamin A seems to alter mitochondrial redox status independently of the participation of the mitochondrial permeability transition pore, which is activated by Ca^{2+} ions since cyclosporin A did not prevent the oxidative insult elicited by Ca^{2+} addition. Overall, we show here that mitochondria are a target of vitamin-A-associated toxicity also in vivo.

Keywords Vitamin A · Oxidative stress · Rat liver · Mitochondria · Calcium ions

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Abbreviations

CsA	Cyclosporin A
METC	Mitochondrial electron transfer chain
MPTP	Mitochondrial permeability transition pore
SMP	Submitochondrial particles

Introduction

Vitamin A is a micronutrient that is necessary in all stages of mammalian development. During adulthood, either vitamin A (retinol) or its derivatives (retinoids) also participate in the maintenance of mammalian organism homeostasis (Napoli 1999). The liver is the site where vitamin A is stored in an esterified form, namely retinyl palmitate (Olson and Gunning 1983; Napoli 1996). Vitamin A esters are more stable than free retinol and will be metabolized when retinol serum levels fall (Napoli 1996). In spite of this, there is a growing body of evidence suggesting that vitamin A may induce or facilitate liver toxicity. Low vitamin A doses might protect liver damage induced through CCl_4 administration (Rosengren et al. 1995). However, chronic treatment with vitamin A alone induces liver damage (Russell et al. 1974) and fails in protecting liver against CCl_4-induced toxicity (Elsisi et al. 1993a, b; Badger et al. 1996).

Therapeutically, vitamin A and retinoids are administrated in some pathological conditions at high doses, for instance leukemia and dermatological diseases (Norum 1993; Fenaux et al. 2001; Myhre et al. 2003). Short-term vitamin A exposition (i.e., days to weeks) at high doses is known to induce a myriad of side effects, including diarrhea and cognitive impairments (Geelen 1979; Myhre et al. 2003; O'Reilly et al. 2006). Additionally, vitamin A exposition as a food supplement or fortification may lead to intoxication, as recently published (Lam et al. 2006). However, the exact mechanism of such toxicity remains to be established. It is well established that retinoids are potent inducers of mitochondrial dysfunction, for instance mitochondrial swelling and leakage of cytochrome c (Rigobello et al. 1999; Klamt et al. 2005), with such an event culminating in increased reactive oxygen species (ROS) production (Klamt et al. 2005). Cytochrome c release to cytosol is a pro-apoptotic event, and ROS production is thought to participate positively in such event (Hengartner 2000; Ott et al. 2002; Kagan et al. 2004). Indeed, it has been shown that vitamin A and retinoids induce apoptosis in different cell types (Pfahl and Piedrafita 2003). However, excessive ROS or reactive nitrogen species production inhibit apoptosis progression since the caspases are dependent on a reduced thiol group in their active site to cleave their substrates (Hengartner 2000).

It has been demonstrated that vitamin A is a redox-active molecule whose behavior is pro- or antioxidant depending on its concentration. Recently, we have found that both short- and long-term vitamin A supplementation increased superoxide anion ($O_2^{-\bullet}$) production in submitochondrial particles (SMP) isolated from rat cerebral cortex and cerebellum (De Oliveira and Moreira 2007). Interestingly, we did not observe any adaptation to the insult promoted by vitamin A supplementation in the long-term exposition, showing that it is an important toxic effect of this vitamin A in those brain regions. Previously reported data also confirm the capacity of vitamin A (retinol) and its derivatives in inducing alterations in the cellular redox environment, including protein carbonylation, lipid peroxidation, and DNA damage induced by free radicals (Moreira et al. 1997; Dal-Pizzol et al. 2000; Klamt et al. 2000; Dal-Pizzol et al. 2001; Frota et al. 2004; De Oliveira et al. 2007a, b).

Therefore, we decided to investigate the effects of short-term vitamin A supplementation on rat liver regarding the redox effects of such treatment. Moreover, we have assessed mitochondrial electron transfer chain (METC) activity in the liver of vitamin-A-treated rats. We have also isolated liver mitochondria to analyze similar parameters. In addition, we have challenged isolated mitochondria with calcium, a non-oxidant agent, that is able to induce mitochondrial swelling and, indirectly, increases free radical production in mitochondria. The main objective of this work was to investigate whether a non-oxidant agent such as calcium ion was able to induce mitochondrial dysfunction in mitochondria isolated from vitamin-A-treated rats regarding redox environment parameters.

Materials and methods

Animals

We used adult male Wistar rats (90 days old; 250–320 g) obtained from our own breeding colony. They were caged in groups of five with free access to food and water and were maintained on a 12-h light–dark cycle (7:00–19:00 h) at $23\pm1°C$. All experimental procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals recommendations for animal care. The Ethical Committee for animal experimentation of

the Federal University of Rio Grande do Sul approved our research protocol.

Drugs and reagents

Arovit® (retinyl palmitate, a water soluble form of vitamin A) was purchased from Bayer, Sao Paulo, SP, Brazil. All other chemicals were purchased from Sigma, St. Louis, MO, USA. Vitamin A was prepared daily, and it occurred by protecting from light and by utilizing amber vials.

Drug administration

The animals were treated once a day for 3 or 7 days. All treatments were carried out at night (i.e., when the animals are more active and take a greater amount of food) in order to ensure maximum vitamin A absorption since this vitamin is better absorbed during or after a meal. The animals were randomly separated between groups and were treated with vehicle (0.15 M saline; control group), 1,000, 2,500, 4,500, or 9,000 IU/kg of retinyl palmitate (vitamin A) administered orally via a metallic gastric tube (gavage) in a maximum volume of 0.8 mL during each period of interest. We utilized six to seven animals in each experimental group. Vitamin A content in the diet of the animals was 12,500 IU/kg food. The daily vitamin A ingestion present only in the chow was calculated within the range of 350–400 UI/day. In the vitamin A supplementation groups, the rats were treated with vitamin A at doses ranging from 300 to 320 IU/day (1,000 IU/kg/day group), from 750 to 800 IU/day (2,500 IU/kg/day group), from 1,350 to 1,440 IU/day (4,500 IU/kg/day group), and from 2,700 to 2,880 IU/day (9,000 IU/kg/day group). Adequate measures were taken to minimize pain or discomfort.

Preparations of the samples

The animals were sacrificed by decapitation at 24 h after the last vitamin A administration. The liver was dissected out in ice immediately after the sacrifice and stored at −80°C for posterior biochemical analyses. The liver was homogenized in ice-cold 0.1 M phosphate buffer (pH 7.4). The homogenates were centrifuged ($700 \times g$, 5 min) to remove cellular debris. Supernatants were used for all biochemical assays described herein. SMP and intact mitochondria were isolated as described below. The results

regarding liver redox state were normalized by the protein content using bovine albumin as standard (Lowry et al. 1951).

Isolation of submitochondrial particles (SMP)

Briefly, to obtain SMP, liver was dissected and homogenized in 230 mM mannitol, 70 mM sucrose, 10 mM Tris–HCl, and 1 mM EDTA (pH 7.4). Freezing and thawing (three times) the mitochondrial solution gave rise to superoxide dismutase-free SMP. The SMP solution was also washed (twice) with 140 mM KCl, 20 mM Tris–HCl (pH 7.4) to ensure Mn–superoxide dismutase (SOD) release from mitochondria (Poderoso et al. 1996; De Oliveira and Moreira 2007).

Isolation of intact mitochondria

Mitochondria from fresh rat liver were isolated as described elsewhere (Klamt et al. 2005). Briefly, liver of Wistar rats suspended in ice-cold isolation buffer A (220 mM mannitol, 70 mM sucrose, 5 mM HEPES (pH 7.4), 1 mM EGTA, and 0.5 mg/mL fatty-acid free bovine serum albumin) was gently homogenized with a glass-homogenizer and centrifuged at $2000 \times g$ for 10 min at 4°C. Approximately three quarters of the supernatant was further centrifuged at $10,000 \times g$ for 10 min at 4°C in a new tube. The fluffy layer of the pellet was removed by gently shaking with buffer A, and the firmly packed sediment was resuspended in the same buffer without EGTA and centrifuged at $10,000 \times g$ for 10 min at 4°C. The mitochondrial pellet was resuspended in buffer B (210 mM mannitol, 70 mM sucrose, 10 mM HEPES–KOH (pH 7.4), 4.2 mM succinate, 0.5 mM KH_2PO_4, and 4 μg/mL rotenone). This procedure, which was designed to isolate intact mitochondria rather than to recover all of that present in the liver, yielded about 20 mg of mitochondrial protein per gram of liver.

Incubation of intact rat liver mitochondria

After the end of the in vivo treatments, the animals were killed as mentioned above. Liver mitochondria were isolated as described, and 1 mg/mL mitochondria were incubated (in the presence of O_2) for 10 min at 25°C with or without $CaCl_2$ and/or co-treatments. Co-treatments consisted of 1 mM dithiothreitol (DTT),

1 mM reduced glutathione (GSH), 200 U/mL native purified superoxide dismutase (SOD), 50 U/mL catalase (CAT), 850 nM cyclosporine A (CsA), 50 μM bongkrekic acid (BKA), or 10 μM Trolox™ (an analogue of vitamin E). Co-treatments, when used, were added to the incubation buffer with or without CaCl$_2$ in the beginning of the assay. After the ending of the incubation period, the samples were centrifuged at 10,000×g for 10 min at 4°C, and the mitochondrial pellet was resuspended in phosphate-buffered saline (pH 7.4) or in 140 mM KCl, 20 mM Tris–HCl (pH 7.4) buffer to assess oxidative stress markers or superoxide anion (O$_2^{-\bullet}$) production, respectively. To assess O$_2^{-\bullet}$ production in intact mitochondria, we froze and thawed mitochondria three times to obtain mitochondrial membranes without mitochondrial matrix so that mitochondrial SOD did not interfere with the assay as mentioned above.

Antioxidant enzyme activity estimations

SOD activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation in a spectrophotometer at 480 nm, as previously described (Misra and Fridovich 1972). Catalase (CAT) activity was assayed by measuring the rate of decrease in H$_2$O$_2$ absorbance in a spectrophotometer at 240 nm (Aebi 1984). A ratio between SOD activity and CAT activity (SOD/CAT ratio) was applied to better understand the effect of vitamin A supplementation upon these two oxidant-detoxifying enzymes that work sequentially converting the superoxide anion to water (Halliwell 2006). An imbalance between their activities is thought to facilitate oxidative-dependent alterations in the cellular environment, which may culminate in oxidative stress.

Thiobarbituric acid reactive species (TBARS)

As an index of lipid peroxidation, we detected hepatic and mitochondrial thiobarbituric acid reactive species (TBARS) formation through a hot and acidic reaction. This is widely adopted as a method for measurement of lipid redox state, as previously described (Draper and Hadley 1990). Briefly, the samples 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 absorbance in a spectrophotometer at

532 nm. We have obtained TBARS concentration in the samples from a calibration curve that was performed using 1,1,3,3-tetramethoxypropane as standard, which was subjected to the same treatment as that applied to the supernatants of the samples. Results are expressed as nanomoles TBARS per milligram protein.

Measurement of protein carbonyls

The oxidative damage to proteins was measured by the quantification of carbonyl groups based on the reaction with dinitrophenylhydrazine (DNPH) as previously described (Levine et al. 1990). Briefly, tissue homogenates or mitochondria were divided into two aliquots of 200 μl. Proteins were precipitated by the addition of 100 μl 20% TCA for 5 min on ice and centrifuged at 4,000×g for 5 min. The pellet was redissolved in 100 μl 0.2 M NaOH, and 100 μl of 2 M HCl or 10 mM 2,4-DNPH in 2 M HCl was added to duplicate aliquots for blanks or the derivatizing of carbonyl groups, respectively. Samples were left for 30 min at room temperature. Proteins were reprecipitated with 20% TCA and washed three times with 500 μl 1:1 ethanol/ethyl acetate with 15-min standing periods to remove excess DNPH. Samples were redissolved in 1 mL 8 M urea, pH 2.3, and the absorbance was read at 370 nm. The carbonyl content in nanomoles per milligram protein was calculated using a molar extinction coefficient of 22,000 M^{-1} cm^{-1} at 370 nm after subtraction of the blank absorbance according to the following equation: $\{[(Final\ Absorbance/ 22,000.22,000) \times V]/1,000\}/Q$, where V is the volume (in milliliters) of the sample utilized in the assay, and Q is the amount of protein (in milligrams) in the volume utilized to perform the carbonyl assay.

Measurement of protein and non-protein thiol content

Briefly, a supernatant aliquot of 20% TCA precipitated homogenates was diluted in SDS 0.1%, and 0.01 M 5,5′-dithionitrobis 2-nitrobenzoic acid (DTNB) in ethanol was added, and the intense yellow color was developed and read at 412 nm after 20 min (Ellman 1959). A blank sample was run simultaneously, except for the absence of DTNB. Protein thiol content was calculated after subtraction of the blank absorbance through the following equation utilizing the molar extinction coefficient of 13,600 M^{-1} cm^{-1} $\{[(Final$

$Absorbance/13,600) \times V]/1,000\}/Q$, where V is the volume (in milliliters) of the sample utilized in the assay, and Q is the amount of protein (in milligrams) in the volume utilized to perform the carbonyl assay. The free sulfhydryl (–SH) content (non-protein thiol content) was estimated in supernatants of 20% TCA precipitated homogenates by the same method and was calculated according to the same equation described above. Results are expressed as nano- or micromoles SH per milligram protein, depending on the sample analyzed (rat tissue or mitochondria).

Quantification of $O_2^{-\bullet}$ production

To quantify superoxide ($O_2^{-\bullet}$) production, SMP was incubated in reaction medium consisting of 230 mM mannitol, 70 mM sucrose, 10 mM HEPES–KOH (pH 7.4), 4.2 mM succinate, 0.5 mM KH_2PO_4, 0.1 μM catalase, and 1 mM epinephrine, and the increase in the absorbance (auto-oxidation of adrenaline to adrenochrome) was read in a spectrophotometer at 480 nm at 32°C, as previously described (Poderoso et al. 1996; De Oliveira and Moreira 2007).

Mitochondrial electron transfer chain (METC) enzyme activities

To obtain SMP from rat liver in order to assess METC activity, we homogenized the tissue in 250 mM sucrose, 2 mM EDTA, 10 mM Tris, pH 7.4, and 50 IU/mL heparin buffer. The samples were centrifuged 1,000×g, and the supernatants were colleted. Then, the samples were frozen and thawed three times, and METC activity detection was performed as described below.

Complex I–CoQ-III activity

Complex I–CoQ-III activity was determined by following the increase in absorbance due to reduction of cytochrome c at 550 nm with 580 nm as reference wavelength (ϵ=19.1 mM^{-1} cm^{-1}). The reaction buffer contained 20 mM potassium phosphate, pH 8.0, 2.0 mM KCN, 10 μM EDTA, 50 μM cytochrome c, and 20–45 μg supernatant protein. The reaction started by addition of 25 μM NADH and was monitored at 30°C for 3 min before the addition of 10 μM rotenone, after which the activity was monitored for an additional 3 min. Complex I–III activity was the rotenone-sensitive NADH/cytochrome c oxidoreductase activity (Shapira et al. 1990).

Complex II–CoQ-III activity

Complex II–CoQ-III activity was measured by following the increase in absorbance due to the reduction of cytochrome c at 550 nm with 580 nm as the reference wavelength (ϵ=21 mM^{-1} cm^{-1}). The reaction mixture consisting of 40 mM potassium phosphate, pH 7.4, and 16 mM succinate was preincubated with 50–100 μg supernatant protein at 30°C for 30 min. Subsequently, 4.0 mM sodium azide and 7.0 μM rotenone were added, and the reaction was started by the addition of 0.6 μg/mL cytochrome c and was monitored for 5 min at 30°C (Fisher et al. 1985).

Complex IV activity

Complex IV activity was measured by following the decrease in absorbance due to the oxidation of previously reduced cytochrome c at 550 nm with 580 nm as reference wavelength (ϵ=19.15 mM^{-1} cm^{-1}). The reaction buffer contained 10 mM potassium phosphate, pH 7.0, 0.6 mM n-dodecyl-β-D-maltoside, 2–4 μg supernatant protein, and the reaction was started with addition of 0.7 μg reduced cytochrome c. The activity of complex IV was measured at 25°C for 10 min (Rustin et al. 1994).

Statistical analyses

Biochemical data are expressed as means ± SEM. All analyses were performed using the Statistical Package for the Social Sciences (SPSS 12.0) software. p values were considered significant when $p<0.05$. Differences in experimental groups were determined by the Student's t test or the one-way ANOVA followed by the post hoc Duncan's test.

Results

Hepatic oxidative damage parameters

Lipid peroxidation

As depicted in Fig. 1a, we found increased lipid peroxidation in the liver of the rats that received vitamin

Fig. 1 Effects of short-term vitamin A supplementation on lipid peroxidation (**a**), protein carbonylation (**b**), protein (**c**), and non-protein (**d**) thiol content in the rat liver. Data are mean±SEM (n=7 per group). *p<0.01 (one-way ANOVA followed by the post hoc Duncan's test)
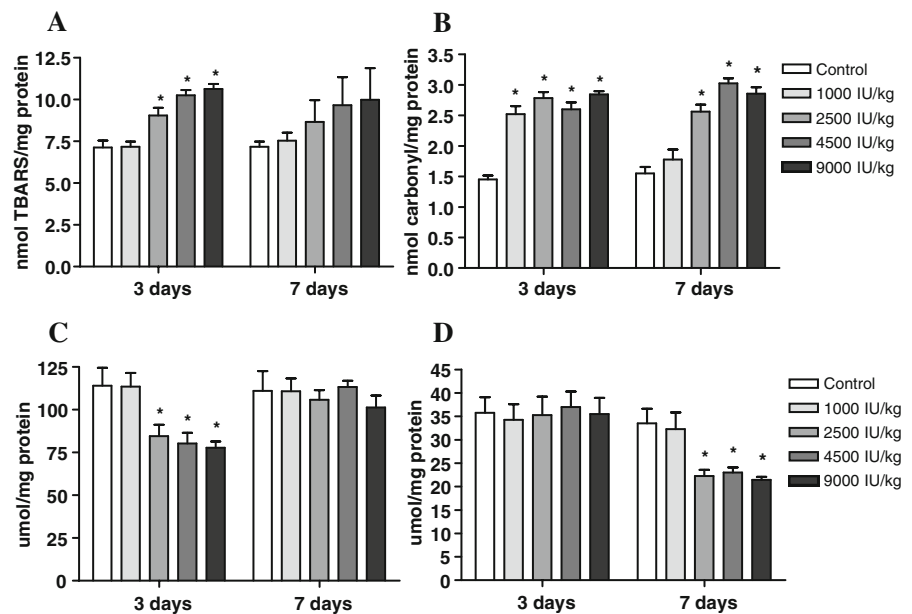
A supplementation at 2,500, 4,500, or 9,000 IU/kg/day for 3 days (1.4- to 1.7-fold; p<0.01). However, hepatic lipid peroxidation levels did not change after vitamin A supplementation for 7 days (Fig. 1a).

Protein carbonylation

We found a 1.3- to 1.7-fold increase of protein carbonylation levels in the liver of the rats that received vitamin A at any dose tested for 3 days (p<0.01; Fig. 1b). Vitamin A supplementation at 2,500, 4,500, or 9,000 IU/kg/day induced a 1.3- to 1.4-fold increase of hepatic protein carbonylation levels in the rats that were treated for 7 days (p<0.01; Fig. 1b).

Protein thiol content

As shown in Fig. 1c, we detected decreased protein thiol content (1.2- to 1.3-fold) in the liver of the rats that were treated with vitamin A at 2,500, 4,500, or 9,000 IU/kg/day for 3 days (p<0.01). However, protein thiol content did not change in the liver of the rats that were treated with vitamin A for 7 days (Fig. 1c).

Non-protein thiol content

Hepatic non-protein thiol content did not change in the rats that received vitamin A supplementation for 3 days (Fig. 1d). We found that vitamin A at 2,500, 4,500, or

9,000 IU/kg/day induced a 1.4- to 1.5-fold decrease of hepatic non-protein thiol content (p<0.01; Fig. 1d).

Antioxidant enzyme activities

SOD activity

Vitamin A supplementation at 9,000 IU/kg/day induced a 1.3-fold increase of SOD activity in the liver of the rats that were treated for 3 days (p<0.05; Fig. 2a). Sustained vitamin A supplementation at 2,500, 4,500, or 9,000 IU/kg/day for 7 days induced a 1.2- to 1.4-fold increase in hepatic SOD activity (p<0.05; Fig. 2a).

CAT activity

We found increased (1.4-fold) CAT activity in the liver of the rats that received vitamin A supplementation at 9,000 IU/kg/day for 3 days (p<0.05; Fig. 2b). CAT enzyme activity did not change in the liver of the rats that received vitamin A supplementation for 7 days (p<0.05; Fig. 2b).

SOD/CAT

Hepatic SOD/CAT increased in the animals that received vitamin A supplementation at 4,500 or 9,000 IU/kg/day for 7 days (p<0.05; Fig. 2c).

Fig. 2 Effects of short-term vitamin A supplementation on SOD (**a**), CAT (**b**), and SOD/CAT ratio (**c**) in the rat liver. Data are mean±SEM (*n*=7 per group). *$p<0.05$ (one-way ANOVA followed by the post hoc Duncan's test)
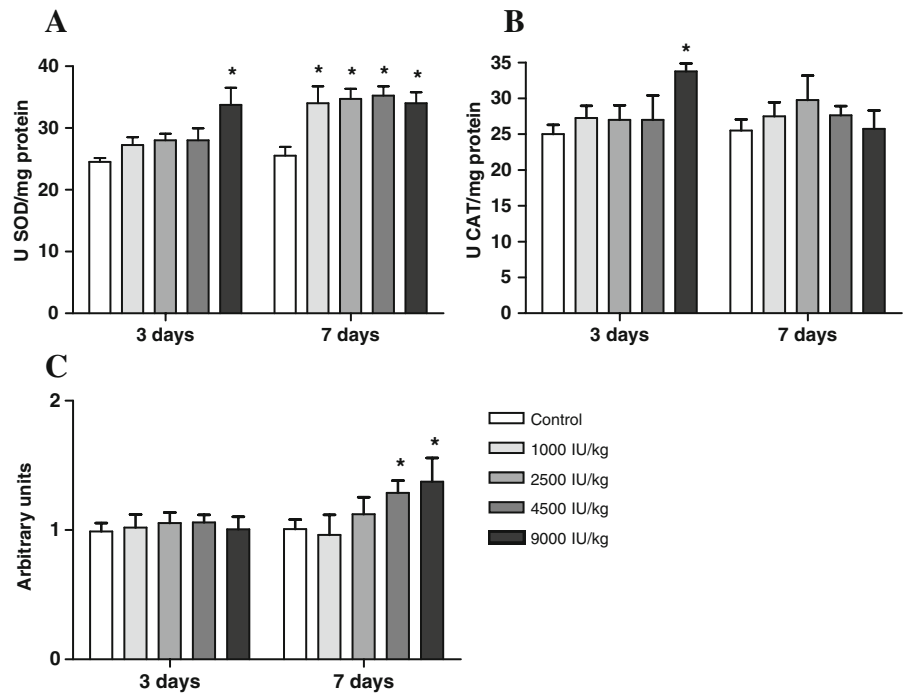
Hepatic submitochondrial particles (SMP) oxidative parameters

Superoxide anion ($O_2^{-•}$) production

We found increased (1.3- to 1.6-fold; $p<0.01$) $O_2^{-•}$ production in hepatic SMP of the rats that were treated with vitamin A at 2,500, 4,500, or 9,000 IU/kg/day for 3 days (Fig. 3a). Vitamin A supplementation at any dose tested induced a 1.3- to 1.7-fold increase of $O_2^{-•}$ production in hepatic SMP isolated from the rats that were treated for 7 days ($p<0.01$; Fig. 3a).

SMP lipid peroxidation

As depicted in Fig. 4b, we found increased (1.7- to 2.7-fold) lipid peroxidation levels in hepatic SMP of the rats that received vitamin A at 2,500, 4,500, or 9,000 IU/kg/day for 3 days ($p<0.01$). Vitamin A supplementation at 2,500, 4,500, or 9,000 IU/kg/day increased (1.6- to 2.7-fold) hepatic SMP lipid peroxidation levels in the rats that were treated for 7 days ($p<0.01$; Fig. 3b).

SMP protein carbonylation

Vitamin A supplementation at 9,000 IU/kg/day induced a 1.5-fold increase of SMP protein carbonylation levels in the rats that were treated for 3 days ($p<0.01$; Fig. 3c). Sustained vitamin A supplementation at 2,500, 4,500, or 9,000 IU/kg/day for 7 days induced a 1.5- to 1.8-fold increase of SMP protein carbonylation levels (Fig. 3c).

SMP protein thiol content

Hepatic SMP protein thiol content did not change in this experimental model (Fig. 3d).

Hepatic mitochondrial electron transfer chain (METC) enzyme activities

As depicted in Fig. 4a, vitamin A supplementation at 2,500, 4,500, or 9,000 IU/kg/day for 3 days induced an increase (1.3 to 1.5-fold) in hepatic complex I–III enzyme activity. We observed that vitamin A supplementation at any dose tested for 7 days induced an increase (1.3- to 1.6-fold) in hepatic complex I–III

Fig. 3 Effects of short-term vitamin A supplementation on superoxide anion production (**a**), lipid peroxidation (**b**), protein carbonylation (**c**), and protein thiol content (**d**) in SMP isolated form rat liver. Data are mean±SEM ($n=7$ per group). *$p<0.01$ (one-way ANOVA followed by the post hoc Duncan's test)
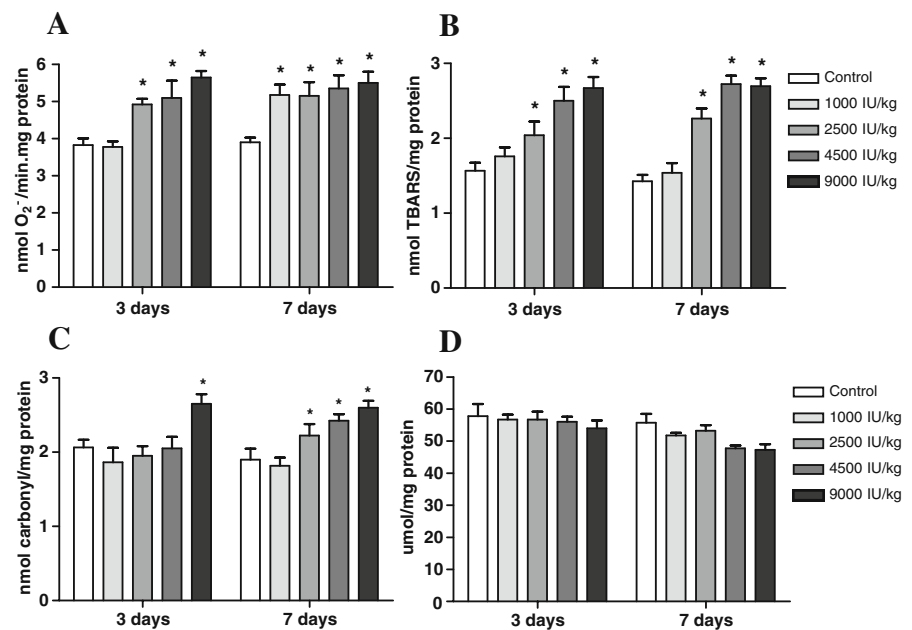
enzyme activity (Fig. 4a). In spite of this, we did not find any change in complex II–III and IV enzyme activities in the liver of vitamin-A-treated rats (Fig. 4b and c).

Incubated hepatic mitochondria

Lipid peroxidation

As shown in Table 1, mitochondria that were isolated from the liver of the rats that received vitamin A supplementation at 2,500, 4,500, or 9,000 IU/kg for 3 days present higher lipid peroxidation levels when incubated for 10 min with buffer C ($p<0.05$). Interestingly, 75 μM CaCl$_2$ induced a 2.5- to 2.9-fold increase of lipid peroxidation in liver mitochondria from the animals that received vitamin A supplementation at 2,500, 4,500, or 9,000 IU/kg for 3 days when compared with mitochondria isolated from the liver of the animals that received saline (vitamin A vehicle) for 3 days ($p<0.01$). A co-treatment with GSH, SOD, CAT, or Trolox™ decreased lipid peroxidation levels induced by CaCl$_2$ ($p<0.05$; Table 1).

A very similar effect was observed when mitochondria were isolated from the liver of the rats that received vitamin A supplementation at 2,500, 4,500, or 9,000 IU/kg for 7 days (Table 1). Co-treatment with mitochondrial permeability transition pore
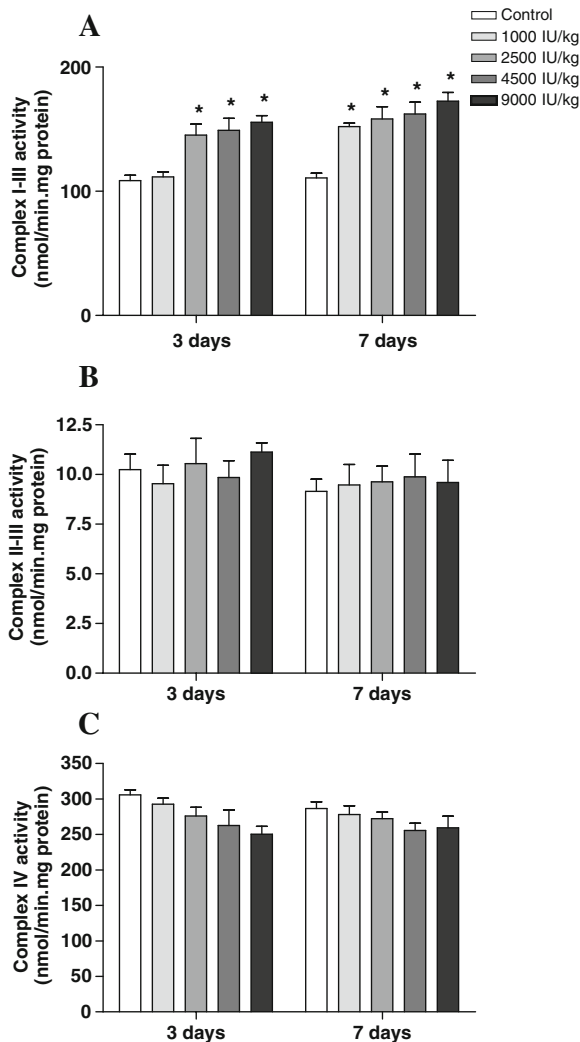
(MPTP) inhibitors did not prevent the oxidative damage induced by 75 μM CaCl$_2$ in mitochondria isolated from vitamin-A-treated rats (Table 2). The co-treatments alone did not alter mitochondrial lipid peroxidation levels (data not shown).

Protein carbonylation

We found increased protein carbonylation levels in incubated mitochondria (in the absence of CaCl$_2$) that were isolated from the liver of the rats that were treated with vitamin A at 9,000 IU/kg for 3 days ($p<0.05$; Table 3). CaCl$_2$ at 75 μM induced a 1.7- to 2.0-fold increase of protein carbonylation in the mitochondria that were isolated from the liver of the rats that received vitamin A supplementation at 4,500 or 9,000 IU/kg for 3 days ($p<0.01$; Table 2). CaCl$_2$-induced protein carbonylation was decreased by a co-treatment with GSH, SOD, or Trolox™ ($p<0.05$; Table 3).

Mitochondria that were isolated from the liver of the rats that were treated with vitamin A at 2,500, 4,500, or 9,000 IU/kg for 7 days showed increased (1.4- to 1.5-fold) protein carbonylation levels even when incubated in the absence of CaCl$_2$ ($p<0.05$; Table 2). However, the addition of CaCl$_2$ increased (1.7- to 2.0-fold) protein carbonylation levels in mitochondria that were isolated from the animals that received vitamin A at 2,500, 4,500, or 9,000 IU/kg

Fig. 4 Effects of short-term vitamin A supplementation on complex I–III (**a**), complex II–III (**b**), and complex IV (**c**) enzyme activities in rat liver mitochondria. Data are mean ± SEM ($n=7$ per group). *$p<0.05$ (one-way ANOVA followed by the post hoc Duncan's test)

when compared with mitochondria isolated from the animals that received saline ($p<0.01$; Table 2). Only the co-treatment with GSH, SOD, or Trolox™ prevented the increase in protein carbonylation induced by $CaCl_2$ ($p<0.05$; Table 3). Co-treatment with MPTP inhibitors did not prevent the oxidative damage induced by 75 μM $CaCl_2$ in mitochondria isolated from vitamin-A-treated rats (Table 4). The co-treatments alone did not interfere in the mitochondrial protein carbonylation levels (data not shown).

Protein thiol content

As depicted in Table 5, protein thiol content of mitochondria isolated from control or vitamin-A-treated rats did not change when incubated in the absence of $CaCl_2$. However, $CaCl_2$ induced a 1.3- to 1.4-fold decrease of protein thiol content in the mitochondria isolated from the animals that received vitamin A supplementation at 2,500, 4,500, or 9,000 IU/kg for 3 days when compared to mitochondria isolated from control animals ($p<0.05$). A co-treatment with GSH, SOD, or Trolox™ prevented the decrease in protein thiol content induced by $CaCl_2$ ($p<0.05$; Table 5).

We did not detect any change in liver mitochondria isolated from 7-day vitamin-A-treated rats when incubated without $CaCl_2$ regarding the redox state of protein thiol content (Table 5). However, $CaCl_2$ at 75 μM induced a decrease (1.5- to 1.6-fold) in the protein thiol content of the mitochondria that were isolated from the animals that received vitamin A supplementation at 2,500, 4,500, or 9,000 IU/kg for 7 days when compared to control animals ($p<0.05$; Table 5). A co-treatment with GSH, SOD, or Trolox™ prevented the decrease in protein thiol content induced by $CaCl_2$ ($p<0.05$; Table 5). Co-treatment with MPTP inhibitors did not prevent the oxidative damage induced by 75 μM $CaCl_2$ in mitochondria isolated from vitamin-A-treated rats (Table 6). The co-treatments alone did not alter mitochondrial protein thiol content (data not shown).

Superoxide anion ($O_2^{-\bullet}$) production

As shown in Table 7, mitochondria that were isolated from the liver of the rats that received vitamin A supplementation at 2,500, 4,500, or 9,000 IU/kg for 3 days produced more $O_2^{-\bullet}$ after incubation without $CaCl_2$ when compared to mitochondria isolated from the liver of control animals ($p<0.05$). The addition of $CaCl_2$ increased (2.0- to 2.45-fold) $O_2^{-\bullet}$ production in mitochondria isolated from vitamin-A-treated rats (2,500, 4,500, or 9,000 IU/kg vitamin A, 3 days; $p<0.01$). A co-treatment with GSH, SOD, or Trolox™ prevented the increase in $O_2^{-\bullet}$ production induced by $CaCl_2$ ($p<0.05$; Table 7).

We detected increased $O_2^{-\bullet}$ production in mitochondria isolated from the animals that received vitamin A supplementation at any dose tested for

Table 1 Effects of vitamin A supplementation and different antioxidant co-treatments on TBARS levels of isolated rat liver mitochondria incubated with 75 µM $CaCl_2$

Period (days)	Groups	Control	$CaCl_2$	$CaCl_2$ + DTT	$CaCl_2$ + GSH	$CaCl_2$ + SOD	$CaCl_2$ + CAT	$CaCl_2$ + Trolox
3	Saline	1.02±0.14	1.03±0.04	1.08±0.08	1.06±0.06	1.03±0.09	1.06±0.09	1.03±0.06
	1,000 IU/kg	1.12±0.05	1.21±0.11	1.23±0.07	1.24±0.04	1.12±0.09	1.15±0.1	1.14±0.04
	2,500 IU/kg	1.75±0.07*	2.62±0.2**·***	2.53±0.14	1.63±0.13****	1.64±0.12****	1.7±0.12****	1.77±0.2****
	4,500 IU/kg	1.85±0.08*	2.35±0.06**·***	2.23±0.11	1.79±0.16****	1.86±0.07****	1.73±0.09****	1.75±0.19****
	9,000 IU/kg	2.16±0.16*	2.94±0.2**·***	3.02±0.2	1.96±0.04****	1.91±0.12****	1.95±0.24****	1.96±0.07****
7	Saline	1.03±0.05	1±0.06	1.19±0.08	1.22±0.08	1.2±0.05	1.19±0.01	1.13±0.06
	1,000 IU/kg	1.05±0.05	1.22±0.13	1.16±0.13	1.23±0.11	1.11±0.04	1.1±0.06	1.06±0.04
	2,500 IU/kg	1.91±0.07*	2.79±0.09**·***	2.77±0.07	1.98±0.11****	1.93±0.03****	1.94±0.14****	1.82±0.1****
	4,500 IU/kg	1.9±0.08*	2.72±0.11**·***	2.75±0.22	1.84±0.14****	1.91±0.15****	1.96±0.04****	1.83±0.05****
	9,000 IU/kg	2.1±0.02*	2.84±0.12**·***	2.84±0.13	1.96±0.19****	1.97±0.13****	1.96±0.1****	1.84±0.19****

Values are mean ± SEM. $N=6$ per group, duplicate

*$p<0.05$;**$p<0.05$ different from respective control (i.e., without calcium); ***$p<0.01$ vitamin A vs. saline; ****$p<0.05$ different from the respective $CaCl_2$-treated group (one-way ANOVA followed by the post hoc Tukey's test, Student's t test)

7 days after incubation without $CaCl_2$ when compared to mitochondria isolated from the liver of control animals treated during the same period ($p<0.05$; Table 7). The addition of $CaCl_2$ increased $O_2^{-\bullet}$ production (2.0- to 2.7-fold) in mitochondria isolated from the rats that received vitamin A supplementation at any dose tested when compared to saline-treated animals (control animals; $p<0.01$; Table 7). A co-treatment with GSH, SOD, or Trolox™ prevented the increase in $O_2^{-\bullet}$ production induced by $CaCl_2$ ($p<0.05$; Table 7). Co-treatment with MPTP inhibitors did not prevent the oxidative damage induced by 75 µM $CaCl_2$ in mitochondria isolated from vitamin-A-treated rats (Table 8). The co-treatments alone did not alter mitochondrial $O_2^{-\bullet}$ production levels (data not shown).

Discussion

In this work, we investigated the short-term effects of vitamin A supplementation on rat liver redox parameters. In addition, we have isolated SMP and intact mitochondria from the liver of the animals that

Table 2 Effects of vitamin A supplementation and MPTP inhibitors on TBARS levels of isolated rat liver mitochondria incubated with 75 µM $CaCl_2$

Period (days)	Groups	Control	$CaCl_2$	$CaCl_2$ + CsA	$CaCl_2$ + BKA
3	Saline	1.02±0.14	1.03±0.04	1.09±0.1	1.04±0.08
	1,000 IU/kg	1.12±0.05	1.21±0.11	1.2±0.11	1.21±0.05
	2,500 IU/kg	1.75±0.07*	2.62±0.2**·***	2.51±0.14	2.61±0.09
	4,500 IU/kg	1.85±0.08*	2.35±0.06**·***	2.39±0.18	2.37±0.16
	9,000 IU/kg	2.16±0.16*	2.94±0.2**·***	3.09±0.11	3.02±0.13
7	Saline	1.03±0.05	1±0.06	0.97±0.03	1.15±0.11
	1,000 IU/kg	1.05±0.05	1.22±0.13	1.15±0.07	1.17±0.08
	2,500 IU/kg	1.91±0.07*	2.79±0.09**·***	2.75±0.08	2.85±0.09
	4,500 IU/kg	1.9±0.08*	2.72±0.11**·***	2.84±0.08	2.88±0.16
	9,000 IU/kg	2.1±0.02*	2.84±0.12**·***	2.73±0.21	2.75±0.13

Values are mean ± SEM. $N=6$ per group, duplicate

*$p<0.05$; **$p<0.05$ different from respective control (i.e., without calcium; one-way ANOVA followed by the post hoc Tukey's test, Student's t test); ***$p<0.01$ vitamin A vs. saline

Table 3 Effects of vitamin A supplementation and different antioxidant co-treatments on protein carbonylation levels of isolated rat liver mitochondria incubated with 75 μM CaCl$_2$

Period (days)	Groups	Control	CaCl$_2$	CaCl$_2$ + DTT	CaCl$_2$ + GSH	CaCl$_2$ + SOD	CaCl$_2$ + CAT	CaCl$_2$ + Trolox
3	Saline	1.82±0.06	1.74±0.13	1.61±0.12	1.54±0.09	1.59±0.09	1.66±0.1	1.71±0.06
	1,000 IU/kg	1.92±0.07	2.12±0.06	2.02±0.1	1.98±0.09	1.91±0.12	1.93±0.08	2.05±0.07
	2,500 IU/kg	1.9±0.04	1.97±0.19	1.96±0.14	2.05±0.14	1.95±0.05	1.95±0.06	1.95±0.07
	4,500 IU/kg	1.92±0.07	3.02±0.17**·***	2.9±0.06	2.01±0.13****	2.10±0.15****	2.99±0.19	1.95±0.13****
	9,000 IU/kg	2.67±0.09*	3.53±0.11**·***	3.36±0.2	2.55±0.12****	2.26±0.14****	3.44±0.18	2.43±0.25****
7	Saline	1.86±0.06	1.9±0.07	1.91±0.08	1.93±0.12	1.93±0.11	2.06±0.05	1.92±0.07
	1,000 IU/kg	1.92±0.08	1.89±0.06	1.8±0.18	1.92±0.09	2.07±0.08	1.89±0.05	1.74±0.23
	2,500 IU/kg	2.52±0.22*	3.2±0.13**·***	3.14±0.07	2.44±0.13****	2.26±0.2****	3.27±0.17	2.35±0.11****
	4,500 IU/kg	2.77±0.06*	3.7±0.16**·***	3.88±0.11	2.61±0.16****	2.44±0.16****	3.56±0.08	2.6±0.16****
	9,000 IU/kg	2.67±0.18*	3.85±0.07**·***	3.75±0.1	2.6±0.15****	2.52±0.16****	3.77±0.14	2.54±0.14****

Values are mean ± SEM. $N=6$ per group, duplicate

*$p<0.05$; **$p<0.05$ different from respective control (i.e., without calcium); ***$p<0.01$ vitamin A vs. saline; ****$p<0.05$ different from the respective CaCl$_2$-treated group (one-way ANOVA followed by the post hoc Tukey's test, Student's t test)

received vitamin A at different doses and analyzed the effects of vitamin A supplementation on the mitochondrial redox state. We have found that vitamin A supplementation for 3 or 7 days exerts a pro-oxidant state on rat liver and on isolated hepatic mitochondria, as well as on mitochondria that were incubated with CaCl$_2$, a non-oxidant agent that is able to, indirectly, induce oxidative stress in mitochondria through mitochondrial dysfunction, for instance mitochondrial swelling (Cai and Jones 1998).

Vitamin A is, as previously mentioned, a redox-active molecule, which is able to induce—or facilitate—

oxidative stress in several experimental models. We have recently reported increased superoxide anion (O$_2^{•-}$) production in SMP isolated from rat cerebral cortex and cerebellum (De Oliveira and Moreira 2007). Furthermore, we have previously reported that retinol induces mitochondrial swelling in vitro and, consequently, induces cytochrome c release from mitochondria (Klamt et al. 2005). In the present work, we demonstrate, for the first time, that mitochondria isolated from the livers of rats that received vitamin A at therapeutic doses for 3 or 7 days is more sensitive to CaCl$_2$ regarding the redox effects of this challenge.

Table 4 Effects of vitamin A supplementation and MPTP inhibitors on protein carbonylation levels of isolated rat liver mitochondria incubated with 75 μM CaCl$_2$

Period (days)	Groups	Control	CaCl$_2$	CaCl$_2$ + CsA	CaCl$_2$ + BKA
3	Saline	1.82±0.06	1.74±0.13	1.78±0.13	1.71±0.08
	1,000 IU/kg	1.92±0.07	2.12±0.06	1.94±0.1	1.95±0.17
	2,500 IU/kg	1.9±0.04	1.97±0.19	2.21±0.14	2.24±0.12
	4,500 IU/kg	1.92±0.07	3.02±0.17**·***	3.05±0.13	2.95±0.19
	9,000 IU/kg	2.67±0.09*	3.53±0.11**·***	3.32±0.19	3.54±0.12
7	Saline	1.86±0.06	1.9±0.07	1.96±0.04	1.94±0.11
	1,000 IU/kg	1.92±0.08	1.89±0.06	1.99±0.11	1.87±0.08
	2,500 IU/kg	2.52±0.22*	3.2±0.13**·***	3.19±0.15	3.21±0.08
	4,500 IU/kg	2.77±0.06*	3.7±0.16**·***	3.66±0.17	3.7±0.11
	9,000 IU/kg	2.67±0.18*	3.85±0.07**·***	3.87±0.07	3.85±0.16

Values are mean ± SEM. $N=6$ per group, duplicate

*$p<0.05$; **$p<0.05$ different from respective control (i.e., without calcium; one-way ANOVA followed by the post hoc Tukey's test, Student's t test); ***$p<0.01$ vitamin A vs. saline

Table 5 Effects of vitamin A supplementation and different antioxidant co-treatments on protein sulfhydryl content of isolated rat liver mitochondria incubated with 75 μM $CaCl_2$

Period (days)	Groups	Control	$CaCl_2$	$CaCl_2$ + DTT	$CaCl_2$ + GSH	$CaCl_2$ + SOD	$CaCl_2$ + CAT	$CaCl_2$ + Trolox
3	Saline	56.2±2.31	57.4±1.8	58.6±2.2	56.5±1.2	56.3±0.82	57.3±0.9	57.5±1.78
	1,000 IU/kg	56.6±1.37	58.3±1.56	59.6±0.78	59.2±1.2	59.4±2.26	57.9±1.85	58.4±2.24
	2,500 IU/kg	55.7±2.25	41.7±2.7*,**	41±2.02	54.5±1.99***	53.3±1.37***	41.9±1.84	56±1.24***
	4,500 IU/kg	56.7±0.7	39.7±2.28*,**	37.6±2.73	58.4±1.28***	52.4±0.72***	39.7±3.53	58.6±1.54***
	9,000 IU/kg	58.5±0.77	39.9±1.32*,**	40±3.12	59.6±0.82***	52±1.06***	42.9±3.32	58.7±2.1***
7	Saline	58.7±0.7	60±1.13	58.8±1.85	58.7±1.37	59.8±1.31	58.8±1.08	57.6±1.75
	1,000 IU/kg	61±0.9	58.8±0.8	60.9±1.33	58.8±1.72	59.2±1.28	59.8±1.14	59.7±1.33
	2,500 IU/kg	60.2±1.42	40.6±1.07*,**	38.4±1.8	60.6±1.92***	63.2±2.3***	40.4±3.17	61.3±1.71***
	4,500 IU/kg	62.1±0.49	40.76±1.41*,**	41.1±1.57	62.1±0.96***	63.3±1.77***	44.3±3.13	60.2±2.14***
	9,000 IU/kg	60.4±2.88	38.1±0.78*,**	42.3±4.1	60.6±2.73***	61.1±3.3***	44.9±3.91	61.9±1.98***

Values are mean ± SEM. $N=6$ per group, duplicate

*$p<0.05$ vitamin A vs. saline; **$p<0.05$ different from respective control (i.e., without calcium); ***$p<0.05$ different from the respective $CaCl_2$-treated group (one-way ANOVA followed by the post hoc Tukey's test, Student's t test)

Vitamin A supplementation induced lipid peroxidation, protein carbonylation, and oxidation of protein thiol groups in rat liver in vivo (Fig. 1a,b,c, and d). Interestingly, we did not find any change in lipid peroxidation levels in the liver of the rats that received vitamin A supplementation for 7 days (Fig. 1a). Also, we did not find any change in CAT enzyme activity in the same period (Fig. 2b). At least in part, the non-altered lipid peroxidation levels may be due to decreased rates of hydrogen peroxide production in rat liver in such period since CAT enzyme is not altered. Hydrogen peroxide is a diffusible reactive oxygen species, which is capable of inducing oxidative damage mainly to lipids due to its capacity to cross biological membranes (Halliwell 2006). Non-protein thiol content was decreased only in the animals that receive vitamin A supplementation for 7 days (Fig. 1d), showing that this treatment is able to, at least in part, consume some liver glutathione (GSH), which might facilitate the prooxidant effects that were observed in both SMP and intact mitochondria isolated from rat liver (Fig. 3a,b,c, and d; Tables 1, 3, 5, and 7). We found increased SOD and CAT enzyme activities in the liver of the rats that received vitamin A (Fig. 2a, and b). These results indicate an increased $O_2^{\bullet-}$ production since this pro-

Table 6 Effects of vitamin A supplementation and MPTP inhibitors on protein sulfhydryl content of isolated rat liver mitochondria incubated with 75 μM $CaCl_2$

Period (days)	Groups	Control	$CaCl_2$	$CaCl_2$ + CsA	$CaCl_2$ + BKA
3	Saline	56.2±2.31	57.4±1.8	58.4±1.3	58.2±2.8
	1,000 IU/kg	56.6±1.37	58.3±1.56	58.7±1.45	58.5±1.5
	2,500 IU/kg	55.7±2.25	41.7±2.7*,**	42.7±1.19	39.5±2.27
	4,500 IU/kg	56.7±0.7	39.7±2.28*,**	42.6±1.18	39.4±1.74
	9,000 IU/kg	58.5±0.77	39.9±1.32*,**	39.7±1.9	40.4±2.7
7	Saline	58.7±0.7	60±1.13	59.1±1.13	59.3±2.25
	1,000 IU/kg	61±0.9	58.8±0.8	58.8±2.1	59±1.58
	2,500 IU/kg	60.2±1.42	40.6±1.07*,**	39.8±2.6	41±2.08
	4,500 IU/kg	62.1±0.49	40.76±1.41*,**	39.7±2.33	40.2±1.47
	9,000 IU/kg	60.4±2.88	38.1±0.78*,**	40.1±1.67	39.8±2.65

Values are mean ± SEM. $N=6$ per group, duplicate

*$p<0.05$ vitamin A vs. saline; **$p<0.05$ different from respective control (i.e., without calcium; one-way ANOVA followed by the post hoc Tukey's test, Student's t test)

Table 7 Effects of vitamin A supplementation and different antioxidant co-treatments on superoxide anion production in isolated rat liver mitochondria incubated with 75 μM $CaCl_2$

Period (days)	Groups	Control	$CaCl_2$	$CaCl_2$ + DTT	$CaCl_2$ + GSH	$CaCl_2$ + SOD	$CaCl_2$ + CAT	$CaCl_2$ + Trolox
3	Saline	1.05±0.1	1.03±0.1	1.07±0.05	1.05±0.07	1±0.02	1.06±0.05	1.09±0.08
	1,000 IU/kg	1.14±0.06	1.12±0.04	1.11±0.07	1.12±0.05	1.19±0.05	1.08±0.05	1.18±0.07
	2,500 IU/kg	1.44±0.06*	2.1±0.13**·***	2.05±0.04	1.33±0.17****	1.32±0.26****	2.19±0.11	1.12±0.05****
	4,500 IU/kg	1.54±0.14*	2.4±0.12**·***	2.12±0.07	1.43±0.07****	1.24±0.27****	1.91±0.13	1.29±0.1****
	9,000 IU/kg	1.66±0.1	2.53±0.11**·***	2.29±0.14	1.3±0.14****	1.23±0.13****	2.15±0.03	1.2±0.06****
7	Saline	1.09±0.04	1.06±0.05	1.03±0.02	1.04±0.04	1.32±0.03	1.1±0.08	1.04±0.09
	1,000 IU/kg	1.59±0.17*	2.16±0.05**·***	2.15±0.06	1.51±0.25****	1.16±0.04****	1.15±0.02	1.43±0.22****
	2,500 IU/kg	1.98±0.09*	2.65±0.09**·***	2.5±0.06	1.7±0.26****	1.67±0.14****	2.17±0.09	1.74±0.19****
	4,500 IU/kg	1.99±0.17*	2.53±0.08**·***	2.7±0.12	1.83±0.11****	1.89±0.05****	2.2±0.09	1.92±0.11****
	9,000 IU/kg	2.09±0.12*	2.84±0.08**·***	2.86±0.06	1.78±0.31****	1.8±0.09****	2.26±0.06	1.76±0.17****

Values are mean ± SEM. $N=6$ per group, duplicate

*$p<0.05$; **$p<0.05$ different from respective control (i.e., without calcium); ***$p<0.1$ vitamin A vs. saline; ****$p<0.05$ different from the respective $CaCl_2$-treated group (one-way ANOVA followed by the post hoc Tukey's test, Student's t test)

oxidant agent allosterically activates SOD, which in turn produces H_2O_2 by dismutation of $O_2^{\bullet-}$ (Halliwell 2006). Actually, we found increased $O_2^{\bullet-}$ production in SMP isolated from the livers of the animals that received vitamin A supplementation (Fig. 3a). According to Fig. 4, we observed increased complex I–III enzyme activity, but unaltered complexes II–III and IV enzyme activities, suggesting mitochondrial uncoupling and, consequently, gave rise to increased $O_2^{\bullet-}$ production, as mentioned above.

The effects seen here when mitochondria were challenged with $CaCl_2$ might be a consequence of increased levels of markers of oxidative stress that were found in vivo in SMP, which represent mitochondrial membranes without mitochondrial matrix content (Poderoso et al. 1996). We found increased lipid peroxidation and protein carbonylation and decreased protein thiol content in SMP isolated from rat liver (Fig. 3a–d). This in vivo oxidative insult might facilitate the in vitro observed impairment in the mitochondrial redox state that was triggered by $CaCl_2$ treatment. Indeed, it was previously reported that oxidation of thiol groups induces mitochondrial dysfunction in several experimental models (Kowaltowski

Table 8 Effects of vitamin A supplementation and MPTP inhibitors on superoxide anion production in isolated rat liver mitochondria incubated with 75 μM $CaCl_2$

Period (days)	Groups	Control	$CaCl_2$	$CaCl_2$ + CsA	$CaCl_2$ + BKA
3	Saline	1.05±0.1	1.03±0.1	1.03±0.02	1.23±0.07
	1,000 IU/kg	1.14±0.06	1.12±0.04	1.17±0.06	1.04±0.07
	2,500 IU/kg	1.44±0.06*	2.1±0.13**·***	2.02±0.03	2.19±0.16
	4,500 IU/kg	1.54±0.14*	2.4±0.12**·***	2.25±0.1	2.29±0.1
	9,000 IU/kg	1.66±0.1	2.53±0.11**·***	2.48±0.06	2.37±0.09
7	Saline	1.09±0.04	1.06±0.05	1.05±0.02	1.07±0.05
	1,000 IU/kg	1.59±0.17*	2.16±0.05**·***	1.24±0.06	1.08±0.03
	2,500 IU/kg	1.98±0.09*	2.65±0.09**·***	2.02±0.04	2.07±0.17
	4,500 IU/kg	1.99±0.17*	2.53±0.08**·***	2.3±0.07	2.24±0.11
	9,000 IU/kg	2.09±0.12*	2.84±0.08**·***	2.64±0.12	2.64±0.1

Values are mean ± SEM. $N=6$ per group, duplicate

*$p<0.05$; **$p<0.05$ different from respective control (i.e., without calcium; one-way ANOVA followed by the post hoc Tukey's test, Student's t test); ***$p<0.1$ vitamin A vs. saline

et al. 1998; Kim et al. 2003; Aon et al. 2007). It is important to mention that the CaCl$_2$ concentration used here is considered to be low to moderate when compared to other reports (Borutaite et al. 1999; Duchen 2000). Higher CaCl$_2$ concentrations, for instance 150–300 μM, could induce effects not associated with the ability of calcium ions to open the MPTP.

In Tables 1 and 2, we observed that a co-treatment with DTT, CsA, or BKA did not protect isolated mitochondria against the pro-oxidant state induced by CaCl$_2$ regarding lipid peroxidation. However, the absence of effect of DTT is expected since this thiol reducer agent does not pass through biological membranes. Interestingly, addition of reduced gluta-thione (GSH), SOD, CAT, or Trolox™ effectively promote mitochondrial protection against CaCl$_2$-induced redox impairment (Table 1, 3, 5, and 7). It is important to mention, again, that CaCl$_2$ induces a pro-oxidant state in mitochondria through indirect action since this molecule did not present an intrinsic capacity to induce oxidative stress directly. The redox effects induced by CaCl$_2$ is very likely to be consequence of CaCl$_2$-dependent MPTP induction, as previously reported (Zamzami and Kroemer 2001). In spite of this, our data indicate a potential toxic effect of vitamin A upon rat liver mitochondria that is independent of MPTP activation. It is noteworthy that neither CsA nor BKA, two inhibitors of the MPTP, did protect mitochondria against CaCl$_2$ pro-oxidant effects (Tables 2, 4, 6, and 8). Protein carbonylation, oxidation of protein thiol content, and O$_2^{\bullet-}$ production were prevented when GSH, SOD, or Trolox™ were added to the incubation buffer, but CAT was ineffective in preventing those oxidant insults induced by CaCl$_2$ (Tables 3, 5, and 7, respectively), suggesting that H$_2$O$_2$ did not participate in altering those redox parameters in this experimental model. Taken together, these data reinforce our hypothesis that vitamin A is able to induce a mitochondrial impairment that is, at least in part, dependent on the redox environment. However, it was also demonstrated that vitamin A is a membranolytic agent (Goodall et al. 1980). This effect might be responsible for all the other effects that were seen in the present work since the doses applied here are considered to be high, even though therapeutically used (Norum 1993; Fenaux et al. 2001). Mitochondrial membrane breakage in vivo

could facilitate, for example, O$_2^{\bullet-}$ production since increased mitochondrial cytochrome c leakage facilitates electron transfer chain uncoupling (Borutaite et al. 1999; Klamt et al. 2005). Consequently, it could induce increased oxidative stress parameters in mitochondrial membranes.

Other important research groups have demonstrated that short-term vitamin A supplementation attenuated CCl$_4$-induced liver toxicity through increased CYP4502E1 protein levels (Inder et al. 1999). However, in a previous study, it was demonstrated that vitamin A facilitates acetaminophen-induced hepato-toxicity (Rosengren and Sipes 1995). Indeed, we have recently demonstrated that both short- and long-term vitamin A supplementation have induced a pro-oxidant insult in some rat brain regions and also altered the behavior of the animals, for instance, inducing anxiety-like behavior and decreasing both locomotion in and exploration of an open-field apparatus (De Oliveira and Moreira 2007; De Oliveira et al. 2007b).

Vitamin A supplementation, either therapeutically or inadvertently, is a cause of concern among researchers since this vitamin is promptly delivered to several human tissues through its binding proteins (Napoli 1999). This feature favors the induction of early signs of vitamin A toxicity. However, in some cases, some side effects are very difficult to be diagnosed since it alters, for example, cognition (Myhre et al. 2003). Indeed, some reports have been published showing that vitamin A or some of its derivatives induces depression in humans (Jick et al. 2000; Hull and D'Arcy 2003).

Based on the results presented here and also in previously reported data, we conclude that vitamin A is able to induce a pro-oxidant state in rat liver by, at least in part, inducing an in vivo impairment in the mitochondrial redox state (as assessed through hepatic SMP isolation), which might be responsible for the effects seen when CaCl$_2$ was used as an in vitro challenging agent against mitochondrial integrity. The results shown here indicate that liver mitochondria isolated from vitamin-A-treated rats are more sensitive to a non-oxidant agent when compared to saline-treated rats, i.e., the in vivo vitamin-A-induced oxidative damage favors the in vitro observed mitochondrial redox impairment. Unfortunately, it is almost impossible to indicate the vitamin A metabolite responsible for the observed effects, given the vast number of vitamin A metabolites existing. Addition-

ally, some vitamin A derivatives are very unstable molecules and would exert its effects too fast to be detected due to its high reactivity (Napoli 1999). Also, case reports of vitamin A toxicity have shown serum retinol concentrations within normal limits (Ellis et al. 1986; Croquet et al. 2000; Mills and Tanumihardjo 2006), indicating that serum retinol is not a good measure of vitamin A status during toxicity. Our data could be useful to explain some previously reported studies showing that the liver of rats that received vitamin A supplementation are more sensitive to some chemically stressful conditions. In addition, the herein observed impairment in METC activity may culminate in increased rates of superoxide anion production in the liver of patients treated under high vitamin A doses even acutely. We have observed here increased superoxide anion production levels in the liver of the rats that received vitamin A. Increased rates of superoxide anion production would lead to decreased hepatic function if persistent. Then, the results presented here may be useful to elucidate, at least in part, the mechanism by which vitamin A interferes with tissue homeostasis even during short-term exposure to high levels of this vitamin.

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