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Vitamin A deficiency induces prooxidant environment and inflammation in rat aorta

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Abstract

We evaluated whether nutritional vitamin A deficiency generates oxidative stress and inflammation in aorta. Wistar male rats (21 days old) were given free access to a control (8 mg retinol as retinyl palmitate/kg) or a vitamin A- deficient diet for three months. One group of deficient animals was fed with the control diet fifteen days before sacrifice. Thiobarbituric acid-reactive substances (TBARS) and nitrite concentration where both analyzed in serum and aorta. Aorta Copper–Zinc Superoxide dismutase (CuZnSOD), Glutathion peroxidase (GPx) and Catalase (CAT) activities were measured. In addition, binding activity of the nuclear factor- kB (NF-kB), inducible and endothelial Nitric Oxide synthase (iNOS and eNOS, respectively) and Ciclooxygenase-2 (COX-2) expressions were determinated in aorta. Rats fed the vitamin A- deficient diet were characterized by sub-clinical plasma retinol concentration and showed increased serum and aorta concentrations of TBARS compared to controls. Lower than control activities of CuZnSOD, GPx, and CAT were observed in aorta of the vitamin A- deficient group. The binding activity of NF- kB was higher in vitamin A- deficient animals than controls. In addition, NO production evaluated as nitrite concentration increased in aorta and serum, associated with a higher expression of iNOS, eNOS and COX-2 in aorta of vitamin A-deficient rats. The incorporation of vitamin A into the diet of vitamin A-deficient rats reverted the changes observed in TBARS level, CuZnSOD and GPx activities, nitrite concentration and also, iNOS, eNOS and COX-2 expression. Prooxidant environment and inflammation are induced by vitamin A deficiency in rat aorta.

Keywords: *Vitamin A, aorta, oxidative stress, lipid peroxidation, antioxidant enzymes, nitric oxide, inflammation*

Introduction

Vitamin A derivatives (retinoids), and in particular all-trans- retinoic acid (atRA), inhibit cellular proliferation and promote cellular differentiation. These actions have important effects on the development of the cardiovascular system. It is known that atRA regulates angiogenesis [1], may retard the development of atherosclerosis [2] and inhibits neointima formation. Also, atRA modulates the growth, differentiation, and morphology of the endothelial cells. Endothelial cells are exposed to high concentration of circulating atRA, express retinoid receptors, and play a significant role in atRA metabolism compared with other cell types [3,4].

Oxidative stress is closely linked to the progression of many diseases such as preeclampsia [5], diabetes [6], and atherosclerosis. [7] These conditions share widespread vascular abnormalities, which is probably the result of endothelial dysfunction.

The endothelium plays an important role in maintaining vascular tone, which is mediated in part by the enzymes nitric oxide synthase (NOS) and ciclooxygenase (COX). NOS catalyzes the reaction of L-arginine to nitric oxide (NO), whereas COX uses

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arachidonic acid as substrate, forming prostaglandin H_2 (PGH_2). PGH_2 is transformed into vasoactive molecules, such as prostacyclin and tromboxane. Both NOS and COX have inducible isoforms (iNOS and COX-2), which are oxidant sensitive through the activation of nuclear factor -KB (NF-kB) [8,9].

NF-kB is involved in the pathogenesis of proliferative disorders of the vasculature, including restenosis [10,11] and atherosclerosis [12]. Previous studies showed that retinoic acid (RA) may function as an inhibitor of NF-kB [13].

The involvement of NOS and COX in altering vascular function has been implicated in conditions characterized by oxidative stress [14,15]. Enhanced NOS activity in an environment of oxidative stress would result in NO scavenging by superoxide anion, forming the potent pro-oxidant peroxynitrite, thus reducing nitric oxide bioavailability as a vasodilator [14]. It is well known that endothelial NO synthase (eNOS) expression is altered in cardiovascular diseases characterized by vascular dysfunction. Endothelium-dependent relaxation mediated by eNOS declines with oxidative stress. The underlying cellular and molecular mechanism might involve: (a) Changes in expression and/or activity of eNOS [16], (b) Increased breakdown of NO due to an augmented production of superoxide anions [17], and (c) Loss of antioxidant capacity [18] which normally provides cellular protection against reactive oxygen species. COX-2 may also mediate vascular dysfunction in conditions characterized by oxidative stress. For example, in carotid arteries and macrophages from patients with atherosclerosis, COX-2 expression is elevated [19,15].

The aim of the present investigations is to test whether vitamin A deficiency is associated to oxidative stress and inflammation in aorta and also, to study the possible pathway that modulates vessel reactivity.

Materials and methods

Diet and experimental design

Male Wistar rats were weaned at 21 days of age and immediately assigned randomly (8 per group) to either the experimental diet, devoid of vitamin A (vitamin A-deficient group), or the same diet with 4000 IU of vitamin A (8 mg retinol as retinyl palmitate per kg of diet) (control group) for three months. Also, a group of eight deficient animals was fed with the control diet fifteen days before sacrifice (vitamin A-refed group) in order to supply these animals with vitamin A. Rats were housed in individual cages and kept in a 21–23°C controlled environment with a 12 h light: dark cycle. They were given free access to food and water throughout the entire 3 months of the experimental period. Diets were prepared according to AIN-93 for laboratory rodents [20]. Both diets had the following composition (g/kg): 397.5 cornstarch, 100 sucrose, 132 dextrinized cornstarch, 200 vitamin-free casein, 70 soybean oil, 50 cellulose fiber, 35 AIN-93 mineral mix, 10 AIN-93 vitamin mix (devoid of vitamin A for the vitamin A-deficient diet), 3 L-cystine, 2.5 choline bitartrate and 0.014 tert-butylhydroquinone. Body weight and food intake were registered daily.

Plasma retinol concentration analyses

Rats were killed by cervical dislocation at 09:00 h. Blood samples were collected in EDTA-coated tubes. To minimize photoisomerization of vitamin A, the plasma was taken under reduced yellow light and frozen in the dark at −70°C until determination of retinol concentrations. Analyses were carried out within 1–3 week of obtaining the samples. Plasma retinol concentration was determined by high performance liquid chromatography [21]. Retinoids were extracted from plasma (0.5 ml) into hexane containing 5 μg butylated hydroxytoluene/ml as antioxidant for analysis. Retinyl acetate was used as internal standard. Chromatography was performed on a Nucleosil 125 C-18 HPLC column with methanol: water (95:5, *v*/*v*) as the mobile phase. Retinol was detected by UV absorbance at 325 nm (Model 440, Waters Associates) and peak areas were calculated by integration (Spectra Physics Analytical).

Serum and aorta TBARs and nitrite determinations

TBARS were measured as described by Jentzsch et al. [22]. Absorption was read at 535 and 572 nm to correct baseline absorption. Malondialdehyde (MDA) equivalents were calculated using the difference in absorption at the two wavelengths and quantification was made with calibration curve.

NO formation was measured indirectly by assaying nitrite, one stable product of NO oxidation [23]. Nitrites were determined using the Griess reagents and absorbance was read at 540 nm.

Antioxidant enzyme activities

Samples to be processed for determination of the activity of antioxidant enzymes (30 mg of wet weight) were homogenized in 120 mM KCl, 30 mM phosphate buffer (pH 7.2) at 4°C. The suspension was centrifuged at 800*g* for 10 min at 4°C to remove nuclei and cell debris. The pellet was discarded and the supernatant was used as homogenate [24]. The enzyme determinations were performed immediately.

Catalase (CAT) activity was determined by measuring the decrease in absorption at 240 nm in a reaction medium containing 50 mM phosphate buffer (pH 7.3) and 3 mM H_2O_2 [25]. One catalase unit is defined as the amount of the enzyme required to decompose

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1 μM of H_2O_2/min. Glutathion peroxidase (GPx) activity was determined following NADPH oxidation at 340 nm in a reaction medium containing 0.2 mM GSH, 0.25 U/ml yeast glutathione reductase, 0.5 mM and Ter-butyl hydroperoxide (BHT) (Sigma Chemical Co. St. Louis, MO. U.S.A), and 50 mM phosphate buffer (pH 7.2) [26]. Copper–Zinc Superoxide dismutase (CuZnSOD) activity was determined on the basis of its inhibitory action on the rate of superoxide-dependent reduction of cytochrome c by xanthine–xanthine oxidase at 560 nm. The reaction medium contained 50 mM phosphate buffer (pH 7.8), 50 μM xanthine, 20 μM cytochrome c and xanthine oxidase (Sigma Chemical Co.), to detect 0.025 absorbance units/min [27]. One unit of SOD was defined as the amount of enzyme that inhibits cytochrome c reduction by 50%.

Electrophoretic mobility shif assay (EMSA)

The tissue was homogenized in 200 μl of 20 mM HEPES buffer (pH 7.9), containing NaCl (350 mM), glycerol (50%), Igepal (1%), $MgCl_2$ (1mM), EDTA (0.5 mM), DTT (5 mM), and protease inhibitors: PMSF (0.5 mM), leupeptin (1mg/l), pepstatin (1mg/l), aprotinin (1.5 mg/l), bestatin (2 mg/l) and sodium pervanadate (0.4 mM). Samples were centrifuged for 10 min at 10.000*g* at 4°C. The supernatant was transferred to a new tube and protein concentration was determined by the method of Bradford [28]. Samples were stored at −80°C. For the EMSA, the oligonucleotids containing the consensus sequence for NF-kB were end labelled with [γ^{-32} P] ATP using T4 polynucloetide kinase (Promega, Madison WI) and purified using Chroma Spin 10 (CLONTECH laboratories, Palo Alto, CA). The labelled oligonucleotides were incubated with 16 μg of aorta proteins for 20 min at room temperature in Tris–HCl buffer (50 mM, pH 7.5), containing glycerol (20%), $MgCl_2$ (5 mM), EDTA (2.5 mM), DTT (2.5 mM), NaCl (250 mM), and poly (dI-dC) (0.25 g/l). The products were separated by electrophoresis in 4% nondenaturing polyacrilamide gel, using TBE 0.5 X (Tris Borate 45 mM, EDTA 1mM) as the running buffer. The gels were dried and exposed to an X-ray film for 12 h and the bands were quantitated by densitometric analysis.

Western blot analysis for iNOS, eNOS and COX-2

Aortas were homogenized as described previously. Protein was measured by the method of Lowry et al. [29] using bovine serum albumin as standard. 40 μg of proteins were mixed with 10 μl of sample buffer (125 mM Tris–HCl, pH 6.8, 4% SDS, 3.5 mM DTT, 0.02% bromophenol blue and 20% glycerol), boiled for 2–3 min and loaded into an 8% SDS-PAGE gel. Protein molecular mass markers were always loaded on each gel. Separated proteins were transferred to PVDF membranes (Polyscreen NEF 1000 purchased from NEN Life Science Products) using a blot transfer system (BioRad Laboratories, Hercules, CA). After being blocked with 5% BSA-TBS solution (20 mM Tris, 500 mM NaCl, pH 7.5) overnight, at 4°C, membranes were incubated with a primary rabbit anti-iNOS, anti-eNOS and anti-COX-2 polyclonal antibody solution (Santa Cruz Biotechnology) (1:1000 dilution) respectively, for 1 h, at room temperature. β–Actin expression was measured as a control for protein loading using a rabbit polyclonal antibody. After washing three times with TTBS (0.1% Tween 20, 100 mM Tris–HCl, pH 7.5, 150 mM NaCl), membranes were incubated with an anti-rabbit IgG secondary antibody linked to peroxidase for 1 h at room temperature. Membranes were washed again and the color was developed using a Vectastain ABC-detection system.

Statistical analyses

Data are presented as means ± SEM. They were analyzed by one-way ANOVA. Statistical significance was accepted at $p < 0.05$.

Results

Body weight and plasma retinol concentration

The initial body weight (g) of the animals of the three dietary groups was 50 ± 2. At the time of killing, the body weight of rats fed the vitamin A-deficient diet was significantly lower than that of control (316.26 ± 10.91 vs. 361.86 ± 8.76, $p < 0.01$), while that of the vitamin A-refed group was near to control and significantly higher than that of vitamin A-deficient rats (359.56 ± 5.48 vs. 316.26 ± 10.91, $p < 0.01$).

Vitamin A deficiency was determined by the content of retinol in plasma. The plasma retinol concentrations (μmol/l) of rats fed the vitamin A-deficient diet were significantly lower (0.44 ± 0.01 vs. 1.39 ± 0.01, $p < 0.01$) than those of controls. Vitamin A refeeding considerably increased the plasma vitamin A concentration in relation to vitamin A-deficient rats (1.03 ± 0.06 vs. 0.44 ± 0.01, $p < 0.01$).

Lipid peroxidation and antioxidant enzymes

Serum and aorta TBARS were significantly higher in vitamin A-deficient rats ($p < 0.001$ and $p < 0.05$, respectively) in comparison with control animals, while in the vitamin A- refed group the serum and aorta TBARS levels reached control values (Table I).

Vitamin A deficiency affected the antioxidant enzyme activities in aorta. CuZnSOD, GPx and CAT activities were significantly decreased ($p < 0.01$, $p < 0.01$ and $p < 0.05$, respectively) in vitamin A- deficient

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Table I. TBARS in serum and aorta and antioxidant enzyme activities in aorta.

	Dietary group		
	Control	Vitamin A-deficient	Vitamin A-refed
TBARS			
Serum (μmol MDA/mg protein)	9.71 ± 0.89	13.41 ± 1.23*	10.09 ± 0.80
Aorta (pmol MDA/mg protein)	11.9 ± 0.01	14.1 ± 0.03†	11.7 ± 0.03
Aorta enzymes			
Catalase (U/mg protein)	9.01 ± 1.87	3.85 ± 0.41‡	3.50 ± 0.40
Glutathione peroxidase (μmol NADPH/min/mg protein)	0.23 ± 0.01	0.09 ± 0.025¶	0.20 ± 0.01
Superoxide dismutase (U/mg protein)	143.25 ± 16.62	43.90 ± 5.43§	81.06 ± 10.35

Data are presented as means ± SEM ($n = 8$).
*Different from control and vitamin A-refed rats ($p < 0.001$).
†Different from control and vitamin A-refed rats ($p < 0.05$).
‡Different from control ($p < 0.05$).
¶Different from control and vitamin A-refed rats ($p < 0.01$).
§Different from control ($p < 0.01$) and vitamin A-refed rats ($p < 0.05$).

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group in relation to control. Vitamin A-refed rats showed an important increase in SOD ($p < 0.05$) and GPx ($p < 0.05$) activities compared with vitamin A-deficient animals, with GPx almost reaching the control value. CAT activity was not modified by vitamin A refeeding (Table I).

Nitrite content

In rats fed the vitamin A-deficient diet the NO production increased in serum ($p < 0.001$) and aorta ($p < 0.05$) compared with control. These values were reverted by vitamin A refeeding reaching values similar to control (Table II).

NF-kB binding activity

The EMSA analysis for NF-kB of aorta obtained from rats of the three dietary groups is shown in Figure 1. The NF-kB binding activity was significantly higher in aorta from vitamin A- deficient rats ($p < 0.05$) than those from control and vitamin A- refed groups (Figure 1B).

iNOS, eNOS and COX-2 expression

Western blot analysis showed an increased expression of iNOS and eNOS in vitamin A- deficient rats ($p < 0.05$), in relation to controls. Vitamin A refeeding in deficient animals normalized the iNOS and eNOS expression to control values (Figures 2 and 3).

Knowing that large evidence implicates COX-2 in inflammation [30] and that cells expressing COX-2 also express iNOS [30,31], we analyzed COX-2 by Western Blot in aortas obtained from rats at the three dietary groups. The increased expression of COX-2 in aortas from vitamin A-deficient animals ($p < 0.05$) as compared to controls was restored by vitamin A refeeding (Figure 4).

Table II. Nitrite content in serum and aorta.

	Dietary group		
	Control	Vitamin A-deficient	Vitamin A-refed
	Nitrite μmol $NaNO_2$/mg protein		
Serum	6.41 ± 0.44	18.60 ± 1.87*	8.19 ± 1.19
Aorta	0.70 ± 0.10	1.02 ± 0.07†	0.57 ± 0.02

Data are presented as means ± SEM ($n = 8$).
*Different from control and vitamin A-refed rats ($p < 0.001$).
†Different from control and vitamin A-refed rats ($p < 0.05$).

Discussion

The present *in vivo* study was undertaken to investigate the effect of vitamin A deprivation on oxidant and inflammatory parameters in rat aorta.

The data presented here indicate that vitamin A has potentially beneficial effects on rat aorta, which are related to its antioxidant protection.

A significant degree of oxidative stress is produced by vitamin A deficiency in our experimental model as was indicated by the high circulating and aorta TBARS levels. We have previously demonstrated high TBARS concentration in heart of vitamin A- deficient rats [32]. It is known that vitamin A deficiency leads to a reduction in the essential fatty acids and increases lipid peroxidation in retinol deficient microsomal membranes of the liver, kidney, spleen and brain [33]. In addition, there is a strong positive correlation between lipid peroxidation and cardiovascular disease [34].

It has been observed that alpha- tocopherol [35] and beta- carotene [36] are protective on vascular cells from injury induced by oxidized low-density lipoprotein (oxLDL). Moreover, it has been demonstrated that enrichment of LDL with beta- carotene protected it from cell-mediated oxidation [37]. Thus, vitamin A is linked to a variety of factors that determine the susceptibility to oxidative stress in the vessels.

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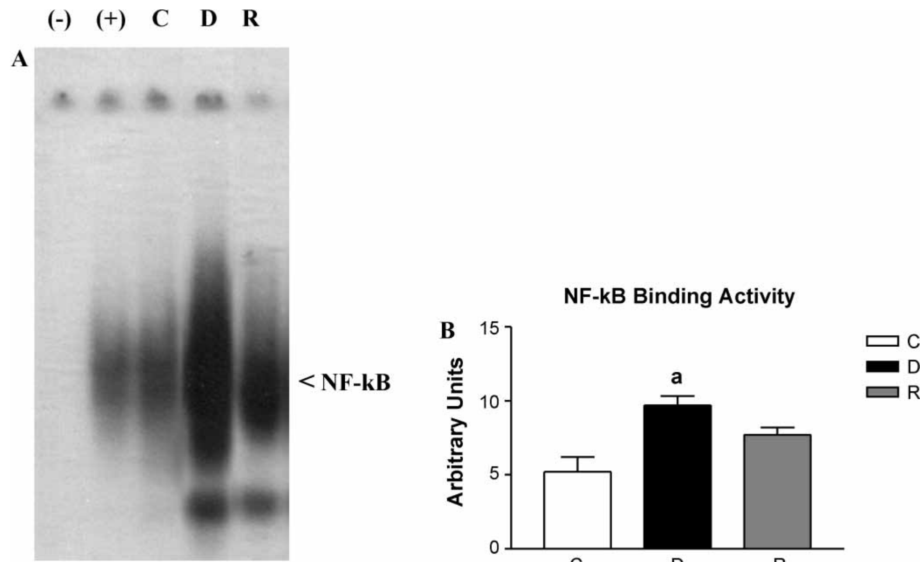

Figure 1. NF-kB binding activity of aorta. (A) Aorta fraction incubated with a 100- fold molar excess of unlabeled oligonucleotide containing the consensus sequence for NF-kB(−), or Oct-1 (+). (B) Quantitative analysis of aorta extracts from control, vitamin A- deficient and vitamin A- refed rats. Values are means ± SEM ($n = 4$ per dietary group); a indicates differences when vitamin A-deficient rats (D) were compared to control (C) and vitamin A-refed rats (R) ($p < 0.05$).

The antioxidant enzyme defenses were modified in aorta of vitamin A- deficient rats showing a significant decrease of CuZnSOD, GPx and CAT activities. It is well known that retinoids exert their effects on gene expression via the retinoic acid receptor (RAR). Although superoxide anion may be accumulated due to the decreased SOD activity, and that accumulation could contribute to oxidative damage, recent works suggest that vitamin A modulates the upregulation of several major scavenger enzyme genes at the mRNA level such as glutathione transferase, which detoxifies both lipid and DNA hydroperoxides and their derivatives [38]. Yoo et al. (1999) have suggested that RA participates in the induction of SOD gene by the peroxisoma proliferator-activated receptor (PPAR) [39]. In addition, Girnun et al. (2002) demonstrated that PPAR gamma and retinoic X receptor-alpha (RXR- alpha) are able to bind to the PPAR- response element (PPRE) in the CAT promoter, which probably leads to modulating its expression and hence down-regulating the oxidative response via scavenging of reactive oxygen species (ROS) [40].

To appreciate the precise contributions of NO to a pathological process induced by vitamin A deficiency on aorta, we determined whether the levels of stable metabolites of NO, measured as nitrite, were associated to iNOS and eNOS expression. The data obtained provide evidence that NO-related species are accumulated in serum and aorta during the course of vitamin A deficiency in rat. It has been demonstrated that the atRA produces concentration-dependent

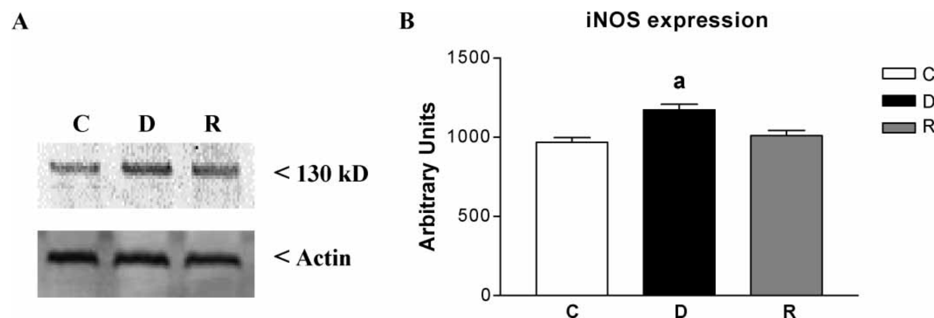

Figure 2. Effect of vitamin A deficiency on the expression of iNOS. (A) Inmunoblot analyses of iNOS expression in aorta of control, vitamin A- deficient and vitamin A- refed rats. β-Actin expression was used as a control for protein loading. (B) Quantitative analysis of iNOS. Values are means ± SEM ($n = 4$ per dietary group); a indicates differences when vitamin A-deficient rats (D) were compared to control (C) and vitamin A-refed rats (R) ($p < 0.05$).

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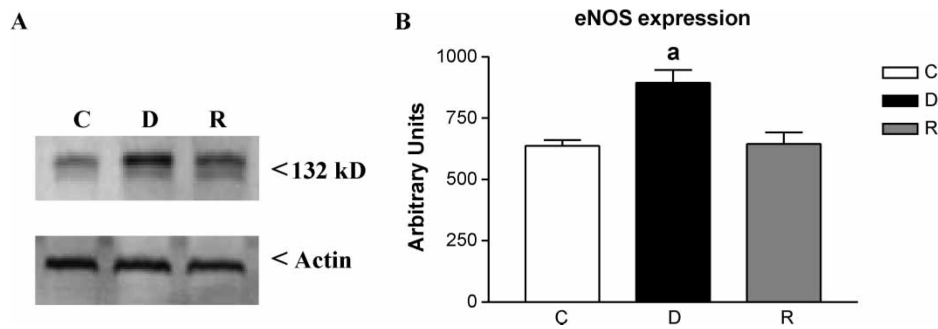

Figure 3. Effect of vitamin A deficiency on the expression of eNOS. (A) Inmunoblot analyses of eNOS expression in aorta of control, vitamin A- deficient and vitamin A- refed rats. β-Actin expression was used as a control for protein loading. (B) Quantitative analysis of eNOS. Values are means ± SEM ($n = 4$ per dietary group); a indicates differences when vitamin A-deficient rats (D) were compared to control (C) and vitamin A-refed rats (R) ($p < 0.05$).

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inhibition of IL-1 beta -induced nitrite production in cultured vascular smooth muscle cells (VSMC) [41]. This situation is interesting because under pathological conditions when NO and superoxide anion are both produced at high levels, these can react extremely rapidly to form the potent oxidant peroxynitrite. This molecule represents a relevant mediator of oxidative modifications in LDL [42]. It is known that oxidant modified LDL particles are up taken by macrophages, and the uncontrolled uptake of oxLDL leads to the formation of lipid-loaded foam cells, a hallmark of early stage atherosclerosis. In addition, it is quite possible that secondary metabolites of arachidonic acid and nitrites function as potent quimiotaxis for polymorphonuclears and macrophages. Their accumulation and activation would likely amplify the production of excessive ROS. This situation can lead to the subsequent vascular injury from inflammatory responses and possible release of growth factors and cytokines.

Our observations about the coordinated induction of both enzymes, iNOS and COX-2, can be a cellular effect resulting from redox changes induced by the vitamin A deficiency. The binding activity of the transcriptional activator NF-kB was increased in aorta of vitamin A-deficient rats. NF-kB, considered as a "sensor" of oxidative stress, could be responsible for the coordinated expressions of iNOS and COX-2 under the oxidative stress conditions found in our experimental model. In addition, Cooke et al. (2002) demonstrated that peroxynitrite increase levels of iNOS and COX-2 through activation of NF- kB [43].

In summary, we have found that inflammation and prooxidant environment are associated with vitamin A deficiency in aorta. Perhaps, when this dietary deficiency happens in patients with cardiovascular disease, it produces more intense pathogenesis, suggesting that the addition of both situations can lead to worse prognosis in patients at risk.

The interactions among the antioxidant nutrients are likely to be very important in protecting cells because the concentration of each antioxidant alone may not be adequate to effectively protect the vascular cells against lipid peroxidation. It is important to consider that differing interactions among variables of vitamin A metabolism and other antioxidants such as vitamin E, and also between vitamin A and lipid metabolism, have been demonstrated [44].

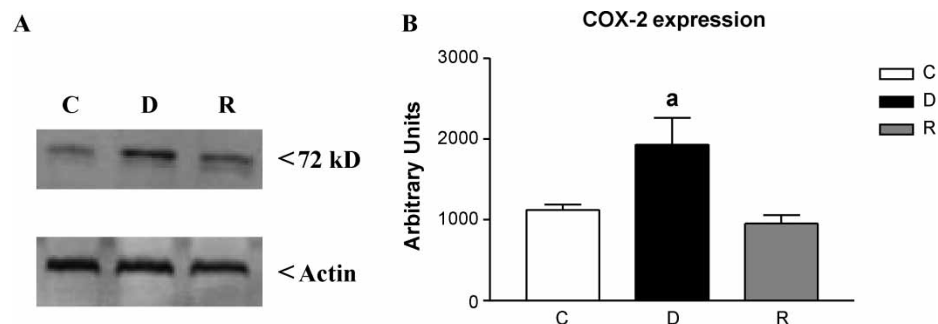

Figure 4. Effect of vitamin A deficiency on the expression of COX-2. (A) Inmunoblot analyses of COX-2 expression in aorta of control, vitamin A- deficient and vitamin A-refed rats. β-Actin expression was used as a control for protein loading. (B) Quantitative analysis of COX-2. Values are means ± SEM ($n = 4$ per dietary group); a indicates differences when vitamin A-deficient rats (D) were compared to control (C) and vitamin A-refed rats (R) ($p < 0.05$).

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Our results indicate that three months of feeding the vitamin A-deficient diet to the rats causes a significant degree of vitamin A deficiency. The incorporation of vitamin A to the diet of vitamin A-deficient rats considerably improves the redox and inflammatory changes. Further studies will be necessary to determine the significance of these changes on aorta physiology, and to explore the role of cytokines and other oxidized mediators on the development of inflammatory changes.

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