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Vitamin A inhibits lipoperoxidation ascorbate-Fe^{++} dependent of rat kidney microsomes and mitochondria

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Abstract

In the present study it was investigated if Vitamin A supplementation could protect rat kidney microsomes and mitochondria from *in vitro* lipoperoxidation. After incubation of rat kidney microsomes and mitochondria in an ascorbate-Fe^{++} system, at 37°C during 60 min, it was observed that the total cpm/mg protein originated from light emission (chemiluminescence) was lower in those organelles obtained from the control group when compared with the vitamin A supplemented group. The fatty acid composition of microsomes and mitochondria from control group was profoundly modified when subjected to non-enzymatic lipoperoxidation with a considerable decrease of arachidonic acid, C20:4 (n-6) and docosapentaenoic acid, C22:5 (n-3) in mitochondria and docosahexaenoic acid C22:6 (n-3) in microsomes. As a consequence the peroxidizability index, a parameter based on the maximal rate of oxidation of specific fatty acids was higher in the supplemented animals than in those used as control. These results indicate that Vitamin A may act as antioxidant protecting rat kidney microsomes and mitochondria from deleterious effect. (Mol Cell Biochem **165**: 121–125, 1996)

Key words: lipoperoxidation, microsomes, mitochondria, rat kidney

Introduction

The polyunsaturated fatty acids located in biological membranes are excellent targets for peroxidation with peroxides formation [1, 2]. The consequence of peroxidation of unsaturated fatty acids membranes is severe: damage of membrane function, enzymatic inactivation, toxic effects on the cellular division, etc. [3, 4, 5]. There are good reason to considerate microsomes and mitochondria as interesting systems for lipoperoxidation studies [6, 7, 8], these organelles are a convenient experimental model for detailed studies of kinetic reaction and peroxidation mechanism, in addition the damage of these membranes is the motive of tissues alterations in many pathological process [9, 10]. The present study was designed to clarify whether or not microsomes and mitochondria obtained from rat kidney could be targets for non-enzymatic lipoperoxidation and to determine the level of protection of such organelles isolated from vitamin A treated animals. The degradative process was followed simultaneously by determination of chemiluminescence [11, 12] and fatty acid composition of microsomes and mitochondria isolated from rat kidney. The peroxidizability index [13] was used to evaluate the fatty acid alterations observed during the process.

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Materials and methods

All trans retinol palmitate type IV and butylated hydroxytoluene was from Sigma Chemical Co. (St. Louis, MO.). BSA (Fraction V) were obtained from Wako Pure Chemical Industries Ltd., Japan. Standards of fatty acids methyl esters were generously supplied by NU Chek Prep, Inc., Elysian, MN. USA. All other reagents and chemicals were of analytical grade from Sigma.

Animals and membrane preparation

Female Wistar AH/HOK rats 7 weeks old, weighing 120–137 g were used. Two groups of three rats each were considered, and designed A (vitamin A-supplemented), and B (control). All rats were fed commercial rat chow and water *ad-libitum*. Group A received one daily 0.39 g/kg body wt intraperitoneal injection of retinol palmitate, dissolved in 0.15 M NaCl, for 7 days. On the eighth day all the rats were sacrificed by cervical dislocation and the kidney was rapidly removed, cut into small pieces and washed extensively with 0.15 M NaCl. An homogenate 30% (w/v) was prepared in solution 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), 10 mM EDTA, using the potter-Elvejhem homogenizer. The homogenate was spun at 10,000 g for 10 min, 3 ml of the resultant supernatant was applied to a Sepharose column (1.6 × 12 cm) equilibrated and eluted with 10 mM Tris-HCl (pH 7.4), 0.01% NaN_3. The microsomal fraction appearing in the void volume (10–16 ml) was brought to 0.25 M sucrose by addition of solid sucrose. All operations were performed at 4°C and under dim light. The quality of this microsomal preparation is similar in composition as regards concentrations and activities of certain microsomal enzymes to that obtained by ultracentrifugation [14]. Mitochondria was prepared as already described [15].

Peroxidation of microsomes and mitochondria

Chemiluminescence and lipid peroxidation were initiated by adding ascorbate to microsomes or mitochondria [16, 17, 18, 19]. Membranes at a concentration of 1–6 mg of protein were incubated at 37°C with 0.01 M phosphate buffer (pH 7.4), 0.4 mM ascorbate, final volume 2 ml. Phosphate buffer is contaminated with sufficient iron to provide the necessary ferrous or ferric iron for lipoperoxidation [19]. Membrane preparations which lacked ascorbate were carried out simultaneously. Membrane light emission was determined over a 60 min period, chemiluminescence was recorded as cpm every 10 min and the sum of the total chemiluminescence was used to calculate cpm/mg protein . A maximal response obtained between 20–30 min after the addition of ascorbate, is used as maximal induced chemiluminescence.

Measurement of fatty acid composition

Microsomal or mitochondrial lipids were extracted with chloroform/methanol (2:1 v/v) [20] from native and peroxidized membranes. Fatty acids were transmethylated with 5% HCl in methanol at 80°C for 60 min. Fatty acids methyl esters were analyzed with a GC-14A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a DB-225 megabore column (30 m × 0.53 mm id, J and V Scientific, Folson, CA., USA). Nitrogen was used as a carrier gas. The injector and detector temperatures were maintained at 250°C, the column temperature was held at 90°C for 1 min, 90–180°C at 15°C/min, 180–200°C at 3°C/min, 200–220°C at 3°C/min, 220°C for 7 min. Fatty acids methyl esters peaks were identified by comparison of retention times with those of standards.

Peroxidizability index

Peroxidizability index (PI) was calculated according to the formula [13, 21], PI = (percent of monoenoic acids × 0.025) + (percent of dienoic acids × 1) + (percent of trienoic acids × 2) + (percent of tetraenoic acids × 4) + (percent of pentaenoic acids × 6) + (percent of hexaenoic acids × 8).

Protein determination

Proteins were determined by the method of Lowry *et al.* [22] using BSA as standard.

Estimation of retinoids

For extraction of vitamin A compounds, fractions were gently mixed with 2 vol ethanol-BHT, followed by successive additions of hexane-BHT. After brief centrifugation the upper phase was withdrawn and concentrated under nitrogen and then dissolved in a final volume of 3 ml of hexane-BHT. Retinoid concentration was measured with spectrophotometer Shimadzu at 330 nm using retinyl palmitate as standard [23].

Statistical analysis

Data were expressed as means ± S.D. Analysis of variance (ANOVA) was used to test the difference among groups. Where differences were significant, statistical significance of the difference between two means was determined using an unpaired *t*-test with Tukey HSD adjustment. $P < 0.05$ was taken as significant.

Results

Vitamin A concentration in homogenate, microsomal and mitochondrial preparations was appreciably higher in vitamin A supplemented than in the control group (Table 1).

After incubation of microsomes and mitochondria in an ascorbate-Fe^{++} system (60 min at 37°C) it was observed that the total cpm/mg protein originated from light emission (chemiluminescence) was lower in the supplemented than in the control group. Thus the percentage inhibition of lipoperoxidation was 33% for microsomes and 32.4 % for mitochondria, (Table 2).

When the total chemiluminescence ratio control/vitamin A supplemented group corresponding to mitochondria and microsomes isolated from rat kidney was compared, similar values, in the range 1.48–1.49, were obtained, (Table 2). These results may indicate that the protection by vitamin A is similar in both kinds of membranes. To determine more precisely if administration of vitamin A produce changes in maximal induced chemiluminescence during ascorbate-Fe^{++} lipoperoxidation assayed *in vitro*, we compared maximal light emission as a function of protein concentration using rat kidney microsomes and mitochondria isolated from control and vitamin A treated group. Either using microsomes or mitochondria, maximal induced chemiluminescence was higher in organelles isolated from control than from vitamin A treated group. The fatty acid composition of total lipids from rat kidney mitochondria and microsomes, native and peroxidized, obtained from vitamin A supplemented and control group is presented in Tables 3 and 4 respectively. As compared with native mitochondria, in the peroxidized organelles,

Table 1. Vitamin A concentration in homogenate, mitochondria and microsomes from vitamin A supplemented and control group

	mg vitamin A/mg protein	
Fraction	Vit A supp group	control group
Homogenate	0.223 ± 0.004	0.039 ± 0.006*
Mitochondria	0.083 ± 0.003	0.011 ± 0.002*
Microsomes	0.049 ± 0.005	0.013 ± 0.002*

Data are given as the mean ± S.D. of three experiments. Statistically significant differences between control and supplemented group are indicated by *p < 0.005 using Student's *t* test.

Table 2. Total chemiluminescence of rat kidney microsomes and mitochondria induced by ascorbate-Fe^{++}

	cpm/mg protein		Control group
Fraction	Vit A supp group	Control group	Vit A supp group
Mitochondria	859.999 ± 160.223	1.270.233 ± 198.738	1.48
Microsomes	269.050 ± 9.331	402.279 ± 37.456*	1.49

Data are given as the mean ± S.D. of three experiments. Statistically significant differences between control and supplemented group are indicated by *p < 0.05 using Student's *t* test.

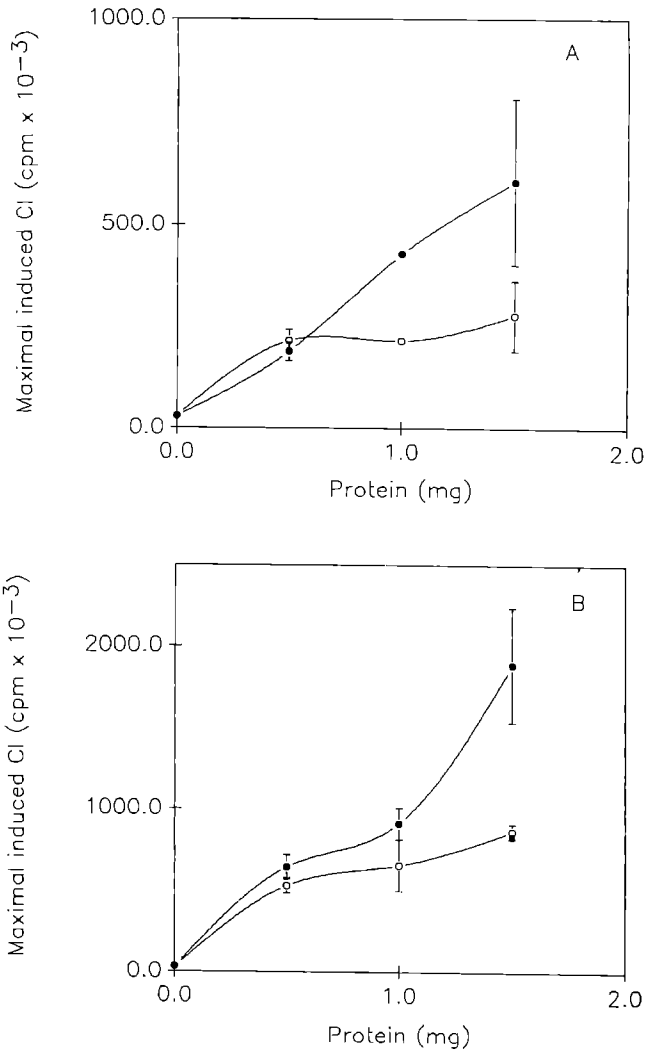

Fig. 1. Effect of treatment with vitamin A on ascorbate-Fe^{++} lipoperoxidation induced *in vitro*. Microsomal and mitochondrial membranes from control ●--● and vitamin A supplemented group O--O. A: microsomes, B: mitochondria. Each point is the mean value ± S.D. of three experiments.

the levels of C20:4 (n-6) and C22:5 (n-3) were lower. When fatty acid composition of microsomal lipids was analyzed only C22:5 (n-3) decreased significantly. There were marked differences when the peroxidizability index of native and peroxidized mitochondria and microsomes was compared. These changes were less pronounced in membranes derived from rats receiving vitamin A, Table 5. The changes in fatty acid composition of membranes subjected to lipoperoxidation in the presence of ascorbate-Fe^{++} produced an important decrease in the relative content of the more polyunsaturated fatty acids. As a result the peroxidizability index of peroxidized membranes in the vitamin A group was significantly higher than in the control group.

Table 3. Fatty acid composition (Area %) of rat kidney mitochondria native and peroxidized from vitamin A supplemented and control group

	Vitamin A supp group		Control group	
FattyAcid	Native	Peroxidized	Native	Peroxidized
C16:0	15.326 ± 4.439	15.487 ± 1.893	17.043 ± 1.939	19.018 ± 1.138
C16:1 (n-7)	tr	tr	tr	tr
C18:0	19.788 ± 0.913	20.920 ± 5.489	21.134 ± 2.479	27.286 ± 1.026
C18:1 (n-9)	4.334 ± 0.183	4.519 ± 1.089	4.639 ± 0.545	5.989 ± 0.225
C18:2 (n-6)	22.357 ± 6.503	29.314 ± 5.165	26.140 ± 3.947	30.181 ± 5.191
C18:3 (n-3)	0.715 ± 0.918	1.123 ± 0.586	1.669 ± 1.105	2.445 ± 0.532
C20:4 (n-6)	24.566 ± 3.531	14.690 ± 5.077*	16.319 ± 2.552	10.469 ± 3.987*
C22:5 (n-3)	2.766 ± 2.488	0.858 ± 1.247*	4.310 ± 2.750	0.580 ± 0.316*
C22:6 (n-3)	0.850 ± 1.472	1.846 ± 2.028	0.442 ± 0.765	tr

Data are given as the mean ± S.D. of three experiments. Statistically significant differences between native and peroxidized mitochondria are indicated by *$p < 0.05$ using Student's *t* test.

Table 4. Fatty acid composition (Area %) of rat kidney microsomes native and peroxidized from vitamin A supplemented and control group

	Vitamin A supp group		Control group	
Fatty Acid	Native	Peroxidized	Native	Peroxidized
C16:0	15.519 ± 2.200	12.832 ± 4.181	17.911 ± 2.427	8.236 ± 3.872
C16:1 (n-7)	tr	tr	tr	tr
C18:0	14.473 ± 1.924	9.033 ± 1.458	11.957 ± 2.535	7.830 ± 1.613
C18:1 (n-9)	8.874 ± 1.174	5.988 ± 1.962	7.336 ± 1.552	4.784 ± 0.966
C18:2 (n-6)	22.680 ± 3.019	10.973 ± 2.526	18.843 ± 4.741	10.015 ± 1.818
C18:3 (n-3)	6.934 ± 7.617	2.593 ± 0.633	2.647 ± 0.702	2.960 ± 0.645
C20:4 (n-6)	12.484 ± 3.373	13.352 ± 2.953	12.189 ± 0.640	13.532 ± 2.858
C22:5 (n-3)	0.840 ± 0.401	0.949 ± 0.584	1.249 ± 0.640	1.293 ± 0.000
C22:6 (n-3)	1.262 ± 0.534	0.691 ± 0.122*	0.419 ± 0.098	tr*

Data are given as the mean ± S.D. of three experiments. Statistically significant differences between native and peroxidized microsomes are indicated by *$p < 0.05$ using Student's *t* test.

Table 5. Peroxidizability index of native and peroxidized mitochondria and microsomes from vitamin A supplemented and control group

	Vitamin A supp group		Control group	
Fraction	Native	Peroxidized	Native	Peroxidized
Mitochondria	144.812 ± 5.827	110.380 ± 19.015	124.267 ± 17.689	80.546 ± 10.611
Microsomes	95.110 ± 7.121	79.102 ± 8.225	81.744 ± 4.644	72.139 ± 7.489

Data are given as the mean ± S.D. of three experiments. Statistically significant differences between native and peroxidized groups are indicated by *$p < 0.05$ using Student's *t* test.

Discussion

The retinoids play an important role in several biological activities, including: modulation of cellular differentiation, immune function, gap junction modifications [24] and interaction with growth factors [25], but unfortunately the action mechanism remain up to now unknown [25].

Vitamin A has a low capacity to scavenge free radicals but it cannot quench singlet oxygen [26]. The anticancerigen activity of vitamin A may be due, at least in part to its antioxidant activity [27]. However the antioxidant activity of retinoids does not correlate with the capacity to inhibit neoplasia [24] or cellular transformation [28]. Many studies have been carried out in order to characterize the changes in structure and composition of membranes [29, 30, 31, 32], exposed to oxidation and to determine how antioxidants agents could protect membranes against deleterious effects produced by free radicals, but only few studies have been directed to study those effects in kidney. By virtue of its liposolubility, vitamin A can partition into lipid membranes where it plays an important antioxidant role [33, 34, 35]. Many studies about oxidative stress carried out with membranes enriched in retinoids have demonstrated the protection by these compounds against *in vivo* and *in vitro* lipoperoxidation [36, 37]. Previous works in our laboratory have shown that the fatty acid composition of rat liver microsomes and mitochondria mem-

branes was modified after peroxidation in an ascorbate-Fe^{++} system [38]. In the present work we observed that as a consequence of the non-enzymatic lipoperoxidation, the polyunsaturated fatty acids present in microsomes and mitochondria, isolated from rat kidney were more damaged in the control than in the supplemented vitamin A group as indicated by the peroxidizability index and the values of chemiluminescence. Fatty acid analysis showed a considerable decrease of arachidonic acid, C20:4 (n-6) and docosapentaenoic acid, C22:5 (n-3) in mitochondria and docosahexaenoic acid, C22:6 (n-3) in microsomes. These changes may produce marked alterations in structure and function of rat kidney mitochondrial and microsomal membranes with the corresponding perturbations in several biological activities .These observations are in agreement with previous results from our laboratory [38] and those of Ciaccio *et al.* [37] and indicate a possible role for vitamin A as a physiological antioxidant in membranes.

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