Prostaglandins, Leukotrienes and Essential Fatty Acids (2001) **64**(2), 75–80

doi:10.1054/plef.2001.0244, available online at http://www.idealibrary.com on IDEAL®

Comparative studies on lipid peroxidation of microsomes and mitochondria obtained from different rat tissues: effect of retinyl palmitate

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Summary The effect of retinyl palmitate on the polyunsaturated fatty-acid composition, chemiluminescence and peroxidizability index of microsomes and mitochondria obtained from rat liver, kidney, brain, lung and heart, was studied. After incubation of microsomes and mitochondria in an ascorbate Fe^{++} system (120 min at 37°C) it was observed that the total cpm/mg protein originated from light emission: chemiluminescence was lower in liver microsomes, mitochondria and kidney microsomes in the vitamin A group than in the control group. In mitochondria obtained from control rats, the most sensitive fatty acids for peroxidation were arachidonic acid C20 : 4 n6 in liver and docosahexaenoic acid C22 : 6 n3 in kidney and brain. In microsomes obtained from control rats, the most sensitive fatty acids for peroxidation were linoleic acid C18 : 2 n6 and C20 : 4 n6 in liver and C22 : 6 n3 in kidney. Changes in the most polyunsaturated fatty acids were not observed in organelles obtained from lung and heart. As a consequence the peroxidizability index, a parameter based on the maximal rate of oxidation of fatty acids, showed significant changes in liver, kidney and brain mitochondria, while in microsomes changes were significant in liver and kidney. These changes were less pronounced in membranes derived from rats receiving vitamin A. Our results confirm and extend previous observations that indicated that vitamin A may act as an antioxidant protecting membranes from deleterious effects. © 2001 Harcourt Publishers Ltd

INTRODUCTION

The oxidative degeneration of polyunsaturated fatty acids in biomembranes can be initiated by free radicals and can proceed in a chain reaction causing considerable membrane damage or producing lipid peroxides.[1,2] Free radicals are known to play a role in chemical toxicity, cardiopulmonary complications, cancer, radiation injury and inflammation as well as in degenerative diseases such as aging, arthritis and immune deficiencies. Not only is the occurrence of free radicals species in biological systems now established, but their involvement in both health and disease is undisputed.[3] The retinoids have been found to act effectively in vitro as antioxidants and radical scavengers,[4] vitamin A in large doses increases the antioxygenic potential of the tissues, and it is suggested that retinol also might be considered as a potential antioxidant similar to tocopherol in animal nutrition.[5] Vitamin A (retinol) and some of its analogous exhibited varying degrees of inhibition on induced iron and ascorbic acid lipid peroxidation.[6,7,8,9] Retinol, retinol acetate and retinol palmitate are the most effective inhibitors. We have shown that vitamin A inhibits chemiluminescence and lipid peroxidation of microsomes and mitochondria obtained from different rat tissues. Thus, to avoid artifacts and misinterpretations, we followed the degradation process by determining chemiluminescence[10] and evaluating the loss of specific fatty acids by the peroxidizability index calculated from fatty-acid composition determined by gas liquid chromatography.[11] The present study aims to compare the

Received 21 August 2000
Accepted 16 October 2000

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polyunsaturated fatty-acid composition and non-enzymatic lipid peroxidation of mitochondria and microsomes obtained from different rat tissues.

MATERIALS AND METHODS

All trans retinol palmitate type IV, butylated hydroxytoluene (BHT) and phenylmethylsulfonyl fluoride (PMFS) were from Sigma Chemical Co. (St. Louis, MO, USA). BSA (Fraction V) was obtained from Wako Pure Chemical Industries Ltd, Japan. All other reagents and chemicals were of analytical grade from Sigma, USA. Fatty acid standards were from Nu Chek Prep, Inc. Elysian MN, USA.

Animals and membrane preparation

Female Wistar AH/HOK rats 7 weeks old, weighing 120 to 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 0.195 g/kg body weight intraperitoneal injection of retinol palmitate, dissolved in 0.15 M NaCl. After 24 h the rats were sacrificed by cervical dislocation and liver, kidney, lung, brain and heart were rapidly removed, cut into small pieces and washed extensively with 0.15 M NaCl. An homogenate 30% (w/v) was prepared in a 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 3,000 × g for 5 min, pellets were discarded, and the supernatant was spun at 10,000 × g for 10 min to obtain mitochondria. All operations were performed at 4°C under dim light. The microsomes were obtained by calcium precipitation (31 µl of $CaCl_2$ 0.8 M were added to 3.1 ml of post-mitochondrial supernatant) and centrifugated during 10 min at 15,000 rpm.[12] After separation of cytosol and microsomes the microsomal pellet was suspended in solution A (0.25 M sucrose solution, 10 mM Tris-HCl pH 7.4, 0.01 M PMFS) using a Potter-Elvejhem homogenizer.

Lipid peroxidation of microsomes and mitochondria

Chemiluminescence and lipid peroxidation were initiated by adding ascorbate to microsomes or mitochondria.[13] Organelles (1 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 lipid peroxidation.[13,14] Membrane preparations which lacked ascorbate were carried out simultaneously. Membrane light emission was determined over a 120 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 and 30 min after the addition of ascorbate, is used as maximal induced chemiluminescence. Chemiluminescence was measured as counts per min in a liquid scintillation analyzer Packard 1900 TR.

Protein determination

Proteins were determined by the method of Lowry et al.[15] using bovine serum albumin as standard.

Measurement of fatty-acid composition

Microsomal and mitochondrial lipids were extracted with chloroform/methanol (2 : 1 v/v)[16] from native or peroxidized membranes. Fatty acids were transmethylated with F_3B in methanol at 60°C for 3 h. Fatty-acids methyl esters were analyzed with a GC-14 A gas chromatograph (Shimadzu Kyoto, Japan) equipped with a packed column (1.80 m × 4 mm i.d.) GP 10% DEGS-PS on 80/100 Supelcoport. Nitrogen was used as a carrier gas. The injector and detector temperatures were maintained at 250°C, the column temperature was held at 200°C. Fatty-acid methyl ester peaks were identified by comparison of the retention times with those of standard.

Peroxidizability index

Peroxidizability index (PI) was calculated according to the formula PI = (per cent of monoenoic acids × 0.025)+(per cent of dienoic acids × 1)+(per cent of trienoic acids × 2)+(per cent of tetraenoic acids × 4)+(per cent of pentaenoic acids × 6)+(per cent of hexaenoic acids × 8).[17]

Statistical analysis

The data were subjected to the Student's *t*-test. Data were expressed as mean ± SD. Statistical criterion for significance was selected at different *P*-values and indicated in each case.

RESULTS

Light emission of microsomes and mitochondria during lipid peroxidation

After incubation of microsomes and mitochondria in an ascorbate-Fe^{++} system (120 min at 37°C) it was observed that the total cpm/mg of protein originated from light emission (chemiluminescence) was lower in liver microsomes and mitochondria and kidney microsomes in vitamin A treated animals, than in the control group (Fig. 1). Thus, the lipid peroxidation inhibition produced

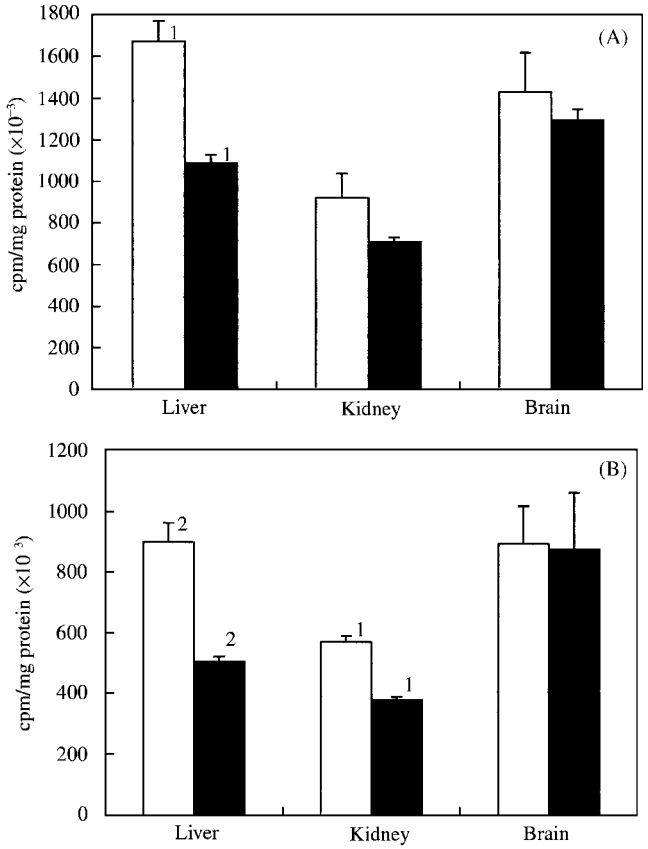

Fig. 1 Total chemiluminescence of mitochondria (A) and microsomes (B) induced by ascorbate Fe^{++} system from control □ and vitamin A treated rats ■. Data are given as the mean ± SD of three experiments. Statistically significant differences between control and supplemented group are indicated by [1]: $P < 0.001$; [2]: $P < 0.005$ using Student's *t*-test.

by vitamin A was 35% for liver mitochondria, 44% for liver microsomes and 34% for kidney microsomes.

Fatty acid composition of mitochondria and microsomes

Tables 1 and 2 show the polyunsaturated fatty-acid composition of total lipids of mitochondria and microsomes obtained from liver, kidney, brain, lung and heart of rats treated with vitamin A and from the control group. The lipid peroxidation of mitochondria obtained from those tissues showed significant changes in the polyunsaturated fatty-acid composition when both groups are compared. The changes in fatty-acid composition of organelles obtained from the control group were more pronounced than those from vitamin A group. A significant decrease of C20 : 4 n6 in liver and C22 : 6 n3 in kidney and brain was observed, whereas changes were not detected in lung and heart. When microsomes were analysed, a significant decrease of C18 : 2 n6 and C20 : 4 n6 in liver and C22 : 6 n3 in kidney was observed.

Table 1 Polyunsaturated fatty acids composition (area %) of mitochondria[a]

Fatty acids	Liver		Kidney		Brain		Lung		Heart	
Control mitochondria	Native	Peroxidized	Native	Peroxidized	Native	Peroxidized	Native	Peroxidized	Native	Peroxidized
C18:2 (n-6)	15.844 ± 1.459	8.694 ± 1.587	26.850 ± 0.948	7.657 ± 2.684	2.265 ± 0.195	1.082 ± 0.373	14.638 ± 0.145	8.875 ± 1.802	12.911 ± 5.160	5.936 ± 1.784
C20:4 (n-6)	20.986 ± 1.029	[1]2.962 ± 0.370	16.748 ± 1.460	6.092 ± 3.245	10.204 ± 0.281	3.987 ± 0.920	9.787 ± 0.353	6.454 ± 1.151	10.165 ± 2.607	9.618 ± 2.329
C22:6 (n-3)	14.450 ± 1.645	5.370 ± 2.625	2.756 ± 0.380	[2]0.619 ± 0.147	12.415 ± 0.311	[3]1.554 ± 0.177	1.979 ± 0.513	0.759 ± 0.354	6.833 ± 0.525	6.054 ± 0.667
Supplemented mitochondria										
Fatty acids	**Liver**		**Kidney**		**Brain**		**Lung**		**Heart**	
	Native	**Peroxidized**	**Native**	**Peroxidized**	**Native**	**Peroxidized**	**Native**	**Peroxidized**	**Native**	**Peroxidized**
C18:2 (n-6)	13.294 ± 0.704	6.756 ± 1.280	19.831 ± 1.947	4.618 ± 0.872	1.560 ± 0.296	0.941 ± 0.124	19.589 ± 2.370	7.846 ± 1.422	15.260 ± 4.683	10.476 ± 1.978
C20:4 (n-6)	23.303 ± 0.795	[1]8.051 ± 2.512	19.565 ± 3.102	9.321 ± 1.042	9.631 ± 0.184	3.300 ± 0.807	8.384 ± 2.563	4.564 ± 0.642	15.052 ± 3.256	10.259 ± 2.675
C22:6 (n-3)	13.709 ± 0.298	7.967 ± 0.855	3.090 ± 0.070	[2]2.706 ± 0.543	12.647 ± 0.462	[3]3.653 ± 0.424	1.925 ± 0.241	1.955 ± 0.464	8.473 ± 1.298	5.649 ± 1.644

[a]Data are given as the mean ± SD of three experiments. Statistically significant differences in fatty acids concentration of mitochondria peroxidized from control and supplemented group are indicated by [1]: $P < 0.05$; [2]: $P < 0.01$; [3]: $P < 0.005$ using Student's *t*-test.

Table 2 Polyunsaturated fatty acid composition (area %) of microsomes[a]

Fatty acids	Liver		Kidney		Brain		Lung		Heart	
Control microsomes	**Native**	**Peroxidized**	**Native**	**Peroxidized**	**Native**	**Peroxidized**	**Native**	**Peroxidized**	**Native**	**Peroxidized**
C18:2 (n-6)	9.734 ± 1.943	[1]2.549 ± 2.987	19.315 ± 3.722	11.223 ± 2.518	0.623 ± 0.540	0.678 ± 0.541	15.224 ± 3.967	14.945 ± 2.987	15.989 ± 3.876	14.432 ± 3.043
C20:4 (n-6)	21.216 ± 4.210	[2]3.218 ± 1.716	11.742 ± 4.215	10.725 ± 2.965	10.635 ± 4.802	9.340 ± 2.896	4.657 ± 1.768	4.068 ± 1.675	9.234 ± 1.985	8.934 ± 2.324
C22:6 (n-3)	0.534 ± 0.543	0.307 ± 0.367	1.420 ± 0.685	[3]tr	7.984 ± 3.546	5.086 ± 0.132	0.845 ± 1.946	tr	3.046 ± 0.786	2.846 ± 1.008
Supplemented microsomes										
Fatty acids	**Liver**		**Kidney**		**Brain**		**Lung**		**Heart**	
	Native	**Peroxidized**	**Native**	**Peroxidized**	**Native**	**Peroxidized**	**Native**	**Peroxidized**	**Native**	**Peroxidized**
C18:2 (n-6)	12.587 ± 0.366	[1]6.466 ± 1.098	21.324 ± 4.216	10.919 ± 3.215	0.756 ± 0.467	0.755 ± 0.552	14.746 ± 2.879	13.163 ± 3.231	16.007 ± 2.811	14.129 ± 4.023
C20:4 (n-6)	22.540 ± 4.939	[2]7.256 ± 1.345	13.647 ± 3.124	11.641 ± 2.946	10.334 ± 3.925	9.361 ± 4.076	5.647 ± 1.908	4.846 ± 2.091	9.006 ± 2.584	10.004 ± 1.987
C22:6 (n-3)	0.675 ± 0.465	0.542 ± 0.498	1.325 ± 0.876	[3]1.724 ± 0.591	8.943 ± 1.427	5.832 ± 1.803	tr	tr	3.253 ± 1.212	2.954 ± 0.998

[a]Data are given as the mean ± SD of three experiments. Statistically significant differences in fatty acids concentration of microsomes peroxidized from control and supplemented group are indicated by [1]: $P < 0.05$; [2]: $P < 0.003$; [3]: $P < 0.01$ using Student's *t*-test.

Peroxidizability index of microsomes and mitochondria

There were marked differences when the peroxidizability index of peroxidized mitochondria and microsomes from control and vitamin A group was compared (Tables 3 and 4). Significant changes in the peroxidizability index were observed in microsomes and mitochondria from liver and kidney, and brain mitochondria. These changes were less pronounced in membranes derived from rats receiving vitamin A. The changes in fatty-acid composition of membranes subject to lipid peroxidation 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 the peroxidized membranes in the vitamin A group was significantly higher than in the control group.

DISCUSSION

Although considerable research has already been performed to characterize the changes in structure, composition and physical properties of membranes subjected to oxidation,[18,19] it is important to know how biological compounds with antioxidant properties contribute to the protection of specialized membranes against deleterious effects produced by reactive oxygen species and other free radicals. Vitamin A has a low capacity to scavenge free radicals, but it cannot quench singlet oxygen.[20] By virtue of its liposolubility, vitamin A can partition into lipids membranes where it plays an important antioxidant role.[21,22,23] 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.[24,25] Lipid peroxidation studies in vitro are useful for the elucidation of possible mechanism of peroxide formation in vivo[26] since the high concentration of polyunsaturated fatty acids in membranes causes susceptibility to lipid peroxidative degradation.[27] The aim of our study was to evaluate the capacity of vitamin A to protect microsomes or mitochondria against lipid peroxidation in several tissues.[28,29] Previous investigations from our laboratory have shown that the fatty-acid composition of rat-liver microsomes is modified after non-enzymatic peroxidation in the presence of ascorbate Fe^{++}.[30,31] In the present study, we have determined that rat liver, kidney and brain mitochondria and liver, kidney microsomes obtained from supplemented vitamin A rats are protected against lipid peroxidation when compared with similar membranes obtained from control rats, as shown by the results obtained by chemiluminescence, peroxidizability index and polyunsaturated fatty acid composition. In our experimental conditions, before incubation in an ascorbate Fe^{++} system, we did not find any changes in the

Table 3 Peroxidability index of native and peroxidized mitochondria from vitamin A supplemented and control group[a]

		Liver	Kidney	Brain	Lung	Heart
Control group	Mitochondria native	215.549 ± 10.015	116.385 ± 8.689	143.030 ± 13.385	70.153 ± 19.325	108.859 ± 15.932
	Mitochondria peroxidized	[1]64.244 ± 5.644	[1]37.515 ± 4.272	[2]30.107 ± 3.573	41.342 ± 12.673	93.091 ± 8.375
Supplemented group	Mitochondria native	212.710 ± 13.215	123.261 ± 7.115	141.863 ± 10.347	68.958 ± 15.653	143.564 ± 17.345
	Mitochondria peroxidized	[1]102.860 ± 6.702	[1]64.010 ± 3.424	[2]43.800 ± 5.702	42.271 ± 11.072	98.123 ± 12.472

[a]Data are given as the mean ± SD of three experiments. Statistically significant differences in P.I. of mitochondria peroxidized from control and supplemented group are indicated by [1]: $P < 0.02$; [2]: $P < 0.008$ using Student's *t*-test.

Table 4 Peroxidability index of native and peroxidized microsomes from vitamin A supplemented and control group[a]

		Liver	Kidney	Brain	Lung	Heart
Control group	Microsomes native	103.945 ± 5.704	86.224 ± 5.931	111.542 ± 6.560	47.213 ± 4.540	85.166 ± 6.080
	Microsomes peroxidized	[1]24.232 ± 3.112	[2]59.487 ± 6.034	81.198 ± 6.560	38.943 ± 4.980	82.765 ± 4.870
Supplemented group	Microsomes native	113.143 ± 5.312	97.675 ± 6.879	119.467 ± 9.070	41.765 ± 5.465	85.845 ± 4.980
	Microsomes peroxidized	[1]43.758 ± 4.322	[2]67.776 ± 7.923	85.234 ± 5.400	37.183 ± 2.789	83.321 ± 5.450

[a]Data are given as the mean ± SD of three experiments. Statistically significant differences in P.I. of microsomes peroxidized from control and supplemented group are indicated by [1]: $P < 0.001$; [2]: $P < 0.01$ using Student's *t*-test.

polyunsaturated fatty-acid composition of mitochondria and microsomes from liver, kidney, brain, lung and heart of rats treated with vitamin A when compared with the control group. In a previous study it has been demonstrated that the fatty-acid composition, both of liver retinyl esters and the total lipids of liver can be significantly altered by high intake of vitamin A,[32] with an increase of the ratios of more unsaturated to less unsaturated fatty acids while those of elongated to unelongated fatty acids fell. One possible explanation for this discrepancy between fatty-acid composition may be the time and amount of vitamin A provided to the animals.[33] Also it has been demonstrated that brain and heart membrane preparations from rats receiving vitamin A, assayed in vitro in the presence of an ascorbate Fe^{++} system showed a delay at the beginning of the lipid peroxidation and generated lesser amounts of thiobarbituric acid (TBARS) with respect to membranes from control rats.[25] In conclusion our results are consistent with the hypothesis that vitamin A may act as a physiological antioxidant in the cell membranes where it is localized. However, further studies are needed to more adequately evaluate these observations.

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