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EFFECTS OF VITAMIN E AND SELENIUM DEFICIENCY ON THE FATTY ACID COMPOSITION OF RAT RETINAL TISSUES

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Summary

The fatty acid composition of retinal tissues was measured in rats maintained for 26—32 weeks on each of the following diets: a purified basal diet deficient in α-tocopherol and selenium, an identical control diet supplemented with α-tocopherol and selenium, and a commercial laboratory rat chow. Dietary deficiencies of antioxidant nutrients were found to cause a large decrease in total polyunsaturated fatty acids in the retinal pigment epithelium, a small decrease in the retinal rod outer segments, but no change in the whole retina or liver when compared to tissues from animals fed the vitamin E- and selenium-supplemented control diet. The polyunsaturated fatty acid content which we have observed for the retinal pigment epithelium from rats fed commercial lab chow is similar to that which we observed for bovine retinal pigment epithelium.

Our results indicate that changes in fatty acid composition are not generalized to all tissues in severely antioxidant-deficient animals, but that changes do occur in some tissues, such as the retinal pigment epithelium, which appears to be particularly sensitive to in vivo lipid peroxidation.

Introduction

α-Tocopherol and selenium are nutrients thought to be important in maintaining the antioxidant status of tissues in vivo. Tocopherol is believed to act as a free radical scavenger [1]. Selenium is an essential component of the enzyme glutathione peroxidase [2,3], which inhibits the accumulation of toxic

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peroxides [4]. Several workers have found decreases of polyunsaturated fatty acids in a number of organs from antioxidant-deficient animals [5—7]. These observations have been widely taken to indicate in vivo peroxidative loss of polyunsaturated fatty acids. This view is not, however, universal and a number of other authors have come to quite different conclusions, suggesting that the polyunsaturated fatty acid changes observed are the result of altered metabolism [8] or inadequate analytical methods [9,10]. Recently, Hafeman and Hoekstra [11] have demonstrated that ethane evolution is markedly increased in living rats which are deficient in selenium and vitamin E. Ethane is a by-product of the peroxidation of linolenate (18 : 3ω3) and other ω3 fatty acids [12] and its evolution by living animals is strong evidence that lipid peroxidation does occur in vivo in antioxidant-deficient animals. Ultrastructural changes have also been found in tissues from antioxidant-deficient animals [7]. Our interest in these phenomena developed because of our observations that retinal rod outer segments are highly susceptible to peroxidation in vitro [13] and that the retinas of vitamin E-deficient monkeys were reported to develop ultrastructural degeneration [14].

Changes in the polyunsaturated fatty acid composition of tissues in vivo as a result of antioxidant deficiency might be expected to be particularly dramatic in retinal tissues due to several factors. Retinal tissues are very high in polyunsaturated fatty acids [15—17]. Large amounts of oxygen are consumed by the abundant retinal mitochondria [18], and in addition, there is the potential for photodynamic oxidation due to photosensitive retinal pigments [19,20]. The combination of these elements would appear to create an adverse oxidative environment especially in antioxidant-deficient animals.

We have investigated the fatty acid composition of retinal tissues as a function of dietary antioxidant deficiencies using methods designed to minimize in vitro loss of polyunsaturated fatty acids. The retinal pigment epithelium, a thin layer of phagocytic cells which abuts the retinal photoreceptors and serves to aid in their maintenance [21], was found to be particularly sensitive to vitamin E and selenium deficiency; in marked contrast, the rod outer segments and whole retina were surprisingly insensitive.

Materials and Methods

Diets and animals. Male, weanling, inbred Agouti rats were divided into three dietary groups and maintained for 26—32 weeks under a 12 h light/dark cycle, on diets and under conditions described elsewhere [22]. Briefly, the deficient animals received a basal diet described in Table I. Supplemented animals were fed the same basal diet with 500 mg of DL-α-tocopherol and 8 mg of selenomethionine added per kg diet. The lab chow diet was an unpurified commercial rat chow (Feedstuffs Processing Co.; Berkeley, CA). All diets were analyzed for total fatty acid composition. The lab chow diet contained 4% fat composed of the following fatty acids: palmitic, 0.81%, stearic, 0.13%, oleic, 0.45%, linoleic, 1.66%, and linolenic, 0.14%. The remaining 0.81% was composed of short-chained saturated fatty acids.

Tissue preparation. Dark-adapted animals from each dietary group were killed 3—4 h into their normal light cycle after being anaesthetized with diethyl

TABLE I

COMPOSITION OF THE BASAL ANTIOXIDANT-DEFICIENT DIET

The weight percent of all dietary components including the major fatty acids from tocopherol-stripped corn oil are shown. Comparisons of the fatty acid content of the basal diet as a whole with that of the corn oil indicated that the corn oil was the only significant source of dietary fat. The basal diet contained less than 3 mg α-tocopherol per kg diet and less than 0.03 ppm selenium.

Ingredient	Weight percent of diet
Torula yeast	36.0
Sucrose	42.55
Corn oil, tocopherol stripped	
Contribution of major fatty acids	
Palmitic (16 : 0)	1.50
Oleic (18 : 1ω9)	3.90
Linoleic (18 : 2ω6)	9.20
Linoleic (18 : 3ω3)	0.03
Arachidic (20 : 0)	0.37
Vitamin mix *	2.2
Mineral mix **	4.0
L-Methionine	0.25

* Values given below are mg vitamin/kg diet. Ascorbic acid, 990; inositol, 110; choline chloride, 1650; *p*-aminobenzoic acid, 110; niacin, 99; riboflavin, 22; pyridoxine-HCl, 22; thiamin-HCl, 22; calcium pantothenate, 66; biotin, 0.5; folic acid, 2; vitamin B-12, 30; vitamin A palmitate in corn oil (200 I.U./mg), 99; vitamin D-2 in corn oil (400 I.U./mg), 5.5; menadione, 50; dextrose hydrate, 18 722.

** Values given below are g mineral/kg diet. $CaCO_3$, 6.54; $CaSO_4 \cdot 5H_2O$, 0.0072; $Ca_3(PO_4)_2 \cdot 2H_2O$, 14.22; ferric citrate $\cdot 3H_2O$, 0.64; $MnSO_4 \cdot H_2O$, 0.055; potassium citrate $\cdot H_2O$, 9.46; KI, 0.0016; K_2HPO_4, 3.094; NaCl, 4.324; $ZnCO_3$, 0.018; and $MgCO_3$, 1.64.

ether, unless otherwise indicated. The eyes were immediately enucleated and dissected under red light (Kodak no. 2 filters with 60 W bulbs). The retinas were collected into 1.0 ml of 60% buffered sucrose (for 12—24 retinas), under argon on ice. The buffer used throughout these experiments was 0.1 mM Na_2EDTA, 0.15 mM $CaCl_2$, 150 mM NaCl, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES) (pH 7.4), unless otherwise indicated.

The pooled retinas from a given dietary group were homogenized in the 60% sucrose buffer using a motor driven teflon pestle in a 5 ml glass tissue grinding sleeve under constant argon purging on ice. Rod outer segment membranes were purified by centrifugation after underlaying the homogenate on a 28—38% continuous sucrose gradient as described by Stone et al. [23]. The uppermost band, containing highly purified rod outer segments, was collected for analysis. Rod outer segment purity was determined by the ratio of absorbance of 280 nm (proportional to total protein) to Δ500 nm (total rhodopsin content) as previously described [24]. Purity was also monitored by polyacrylamide gel electrophoresis and assays of organic soluble phosphate (primarily phospholipid) per mg rhodopsin [23].

Analysis of whole-retina fatty acids were performed upon individual animals. Each animal was given a lethal, intraperitoneal injection of sodium pentobarbital (Nembutal, Abbot Laboratories, Chicago, IL). Anesthetized animals were perfused by cardiac puncture with a solution of 150 mM NaCl, 7 mM $NaNO_2$, 5 mM Na_2EDTA, 5 mM glucose, 10 mM HEPES (pH 7.4) for 5 min or until the effluent was clear. After enucleation from the perfused animals, the

eyes were dissected and the intact retinas carefully removed under normal laboratory lighting. The right and the left retina were combined in 1 ml of buffer and homogenized as described above. Light microscopy confirmed that the retinal homogenate was free of red blood cells. The homogenate was divided into two aliquots for fatty acid and vitamin E analysis.

The retinal pigment epithelium from bovine or rat was prepared by brushing the eye cups (retinas had been removed under dim red light) with a camel hair brush using a modification of the method of Berman et al. [25]. The pigment epithelial cell suspension was collected in 2.0 ml of 0.4 M sucrose buffer with a plastic catheter and syringe and then filtered through a cotton, gauze cloth. The pigment epithelial cell suspension was overlayed on 5 ml of 31% sucrose buffer and centrifuged at 13 000 rev./min in a Sorvall HB-4 rotor for 20 min at 4°C. A majority of the rod outer segment membrane population have an equilibrium density of about 1.13 (31% sucrose) or less [23] and therefore float above the retinal pigment epithelium pellet. Although the remaining outer segment membranes have a density of about 1.14 (33% sucrose) [23], the substitution of 35% sucrose prior to the centrifugation of crude retinal pigment epithelium does not yield cells with a significantly different fatty acid profile than obtained from cells purified through 31% sucrose. The sedimentation rate of the outer segments in 31% sucrose is too slow to allow outer segment contamination of the pigment epithelium pellet after a 20 min centrifugation. It is likely that these retinal pigment epithelial cells have lost much of their cytoplasm but little of their membranous components [26]. Our purification methods yield retinal pigment epithelial cells from which all major impurities have been removed but which may not as yet be completely pure.

Liver tissues were prepared for fatty acid analysis as described by Nelson [27].

Tocopherol determination and fatty acid analysis. Vitamin E content of plasma and whole retina was determined using a modification of the fluorimetric assay of Duggan [28]. 100 µl of plasma or 500 µl of retinal homogenate (2 retinas/ml) were added to an equal volume of buffer in a teflon capped, 1 dram glass vial. An aliquot of 200 proof redistilled ethanol (Gold Shield, IMC Chemical Group, Inc., Agnew, CA), equivalent to the volume of the buffer-sample mixture, and 1 ml of redistilled hexanes were then added. This mixture was vortexed for 1.5 min at a moderate speed on a Vari-Whirl mixer and was centrifuged 10 min at 1500 × *g*. Fluorescence of the top phase was measured at 330 nm emission using a 9 nm band width, and excitation at 294 nm with a 6 nm band width. External standards of α-tocopherol and blanks were run with each group of samples. In some experiments, internal standards of α-tocopherol (Sigma, St. Louis, MO) were included and a recovery of 95 ± 5% was found. Fatty acid analyses were performed using a modification of the method of Morrison and Smith [29] as previously described [23].

Results

The antioxidant status of the experimental animals was determined by measuring tissue levels of vitamin E and glutathione peroxidase and indirectly by monitoring body weight changes. Plasma tocopherol content was monitored as

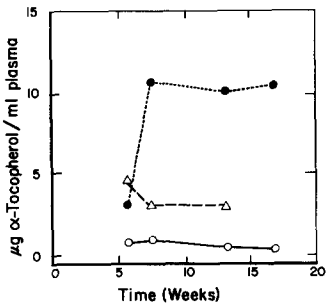

Fig. 1. Tocopherol content of plasma is plotted as a function of time, for rats on three different diets. The three dietary regimens were: a basal diet deficient in vitamin E and selenium (○———○); the same basal diet supplemented with vitamin E and selenium (●------●); and a commercial lab chow (△— — —△). Each point represents a determination on the pooled plasma samples from four animals. Samples (about 0.5 ml/animal) were collected from anesthetized animals by tail-bleeding into tubes containing 2 mg Na_2EDTA per ml blood. Blood samples were not collected from the lab chow group at 17 weeks.

a function of time on the diet as shown in Fig. 1 and was found to be low in the animals on the deficient diet relative to both supplemented and lab chow dietary groups. After 7.5 weeks on the respective diets, the plasma tocopherol content of the supplemented group was 3.5 times higher than the lab chow group and about 7.5 times higher than the deficient animals. These plasma tocopherol levels remained approximately the same for the duration of the experiment. The vitamin E content of whole retinas, from animals perfused with buffer to eliminate blood contamination, was assayed after 16 weeks and found to reflect the dietary tocopherol content. The μg tocopherol per g phospholipid in retinas was found to be 120 ± 30, 560 ± 30, 760 ± 30 for the deficient, lab chow, and supplemented dietary groups, respectively. These values are comparable to those reported by Nishiyama et al. [30]. Similarly, glutathione peroxidase was assayed in liver homogenates and red blood cells and found to be dramatically depressed in the tissues from animals on deficient diets compared with supplemented controls or lab chow diet animals [22].

The body weights of antioxidant-deficient animals paralleled those of animals on supplemented and lab chow diets until the 16th week, at which time the weight of the deficient animals no longer increased although the supplemented control animals continued to gain weight. The deficient animals maintained an average body weight similar to that of the lab chow group until the 26th week and then began to lose weight for the remainder of the experiment.

The fatty acid composition of whole retina is reported for the three dietary groups in Table II. The total polyunsaturated fatty acid content of the whole retina is equivalent for all three dietary groups. No significant differences in the profile of the individual fatty acids are seen between the antioxidant-deficient and -supplemented animals. The differences observed between the lab chow and experimental dietary groups, with regard to whole retinal fatty acid profiles, appear to be due to a mild deficiency of linolenate (18 : 3ω3) in the experimental diets which leads to decreases in the longer chain ω3 unsaturated fatty acids as reported by Tinoco et al. [31]. Our whole retina fatty acid values for rats fed commercial lab chow are in agreement with those reported by others [16,32].

TABLE II

MAJOR FATTY ACIDS OF WHOLE RETINA FOR ANTIOXIDANT-DEFICIENT, ANTIOXIDANT-SUPPLEMENTED AND LAB CHOW FED RATS

The content of major fatty acids and total polyunsaturated fatty acids from whole retina is reported as weight percent of the total fatty acids for three dietary groups maintained on their respective diets for 26 weeks. Minor fatty acids, not reported, make up 2.3, 3.5 and 3% of the total for the deficient, supplemented and lab chow diet groups, respectively. The standard error was calculated separately for each table entry using the number of determinations indicated (n). Each determination was performed on the tissues of individual animals.

Fatty acid	Deficient (n = 5)	Supplemented (n = 3)	Lab chow (n = 3)
16 : 0	14.2 ± 0.7	14.2 ± 0.9	15.4 ± 1.0
16 : 1	0.6 ± 0.1	0.5 ± 0.2	0.9 ± 0.3
18 : 0	23.6 ± 1.2	22.7 ± 1.6	22.2 ± 0.3
18 : 1	7.8 ± 0.4	7.5 ± 0.2	8.1 ± 0.7
18 : 2ω6	1.0 ± 0.1	0.9 ± 0.1	0.6 ± 0.2
20 : 4ω6	8.9 ± 0.6	8.9 ± 0.3	8.2 ± 0.4
22 : 4ω6	3.0 ± 0.2	2.9 ± 0.1	2.1 ± 0.2
22 : 5ω6	5.9 ± 1.0	5.5 ± 0.1	2.0 ± 0.5
22 : 5ω3	0.7 ± 0.3	0.6 ± 0.1	0.8 ± 0.1
22 : 6ω3	27.4 ± 1.3	28.6 ± 1.7	34.1 ± 1.8
24 : 4	4.1 ± 2.6	3.4 ± 1.3	1.9 ± 1.3
24 : 5	0.5 ± 0.4	0.8 ± 0.2	0.7 ± 0.3
Total polyunsaturated fatty acids	51.5 ± 2.0	51.6 ± 2.9	50.4 ± 0.4

TABLE III

MAJOR FATTY ACIDS OF ROD OUTER SEGMENT MEMBRANES FOR ANTIOXIDANT-DEFICIENT, ANTIOXIDANT-SUPPLEMENTED AND LAB CHOW FED RATS

The content of major fatty acids and total polyunsaturated fatty acids from rod outer segment membranes is reported as weight percent of the total fatty acids for three dietary groups maintained on their respective diets for 32 weeks. Minor fatty acids, not reported, represent 0.8, 2.5, and 2.5% of the total for the deficient, supplemented and lab chow diets, respectively. Rod outer segment membrane purity, as determined using the $A_{280nm}/A\Delta_{500nm}$ absorbance ratio as described in Materials and Methods, was 2.27 ± 0.0, 2.27 ± 0.03 and 2.25 ± 0.11 for the deficient, supplemented and lab chow animals, respectively. The standard error was calculated separately for each table entry from the number of determinations indicated (n). Each determination was performed on the pooled tissues of a minimum of six animals.

Fatty acid	Deficient (n = 2)	Supplemented (n = 2)	Lab chow (n = 2)
16 : 0	9.4 ± 0.4	8.6 ± 0.1	8.2 ± 0.2
16 : 1	0.4 ± 0.1	0.2 + 0.1	0.3 ± 0.1
18 : 0	24.1 ± 0.5	22.7 ± 0.7	22.5 ± 0.7
18 : 1	4.6 ± 0.1	3.6 ± 0.1	3.5 ± 0.2
18 : 2	0.7 ± 0.0	0.6 ± 0.1	0.4 ± 0.1
20 : 4ω6	5.9 ± 0.5	5.1 ± 0.6	5.2 ± 0.4
22 : 4ω6	4.1 ± 2.7	1.8 ± 0.6	2.2 ± 0.6
22 : 5ω6	7.5 ± 0.9	7.5 ± 0.3	3.6 ± 0.2
22 : 5ω3	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.1
22 : 6ω3	40.4 ± 2.0	43.1 ± 3.1	46.2 ± 3.6
24 : 4	1.3 ± 0.7	3.5 ± 1.9	3.7 ± 1.4
24 : 5	0.6 ± 0.1	0.5 ± 0.1	1.4 ± 0.0
Total polyunsaturated fatty acids	60.7 ± 0.3	62.4 ± 0.4	63.0 ± 1.3

The fatty acid composition of rod outer segment membranes purified from the retinas of animals maintained on the three dietary regimens is reported in Table III. The total polyunsaturated fatty acid values from each determination were averaged and found to show a lower standard error than many of the individual fatty acids. The outer segments from deficient animals show a significantly lower ($P < 0.025$) total polyunsaturated fatty acid content than the outer segments from supplemented animals. Decreases are also observed in the 22 : 6ω3 and 24 : 4 fatty acids of the deficient outer segments but these changes in individual fatty acids are not highly significant statistically. Once again the effects of the mild 18 : 3ω3 deficiency is seen when comparing the highly unsaturated ω3 and ω6 fatty acids of the purified diet groups with values reported for the lab chow-fed animals.

Plasma and whole liver were analyzed from deficient and supplemented dietary groups after 28 weeks of dietary regimen. No decrease in total polyunsaturated fatty acid content was observed in the antioxidant-deficient liver or plasma lipids when compared with supplemented controls. In fact, a slight increase of total polyunsaturated fatty acids was seen in deficient livers due to an elevation of arachidonate (20 : 4ω6).

In contrast to the other tissues investigated, the retinal pigment epithelium showed large fatty acid changes in the antioxidant-deficient animals when compared with supplemented controls as seen in Table IV. The content of 22 : 6ω3 in the deficient pigment epithelium is only about half the value for the supplemented control. The content of linoleic (18 : 2ω6), on the other hand, is elevated in the deficient samples over the supplemented value. The total polyunsaturated fatty acid content, however, is lower in the retinal pigment epithelium of the antioxidant-deficient animals than in supplemented tissues. Valid comparisons between the pigment epithelium of the deficient and supple-

TABLE IV

MAJOR FATTY ACIDS OF RETINAL PIGMENT EPITHELIUM FOR ANTIOXIDANT-DEFICIENT, ANTIOXIDANT-SUPPLEMENTED AND LAB CHOW FED RATS

The content of major fatty acids and total polyunsaturated fatty acids from retinal pigment epithelium is reported as weight percent of the total fatty acids for three dietary groups maintained on their respective diets for 32 weeks. Minor fatty acids, not reported, constitute 4.8, 4.3, and 3.4% of the total for deficient, supplemented and lab chow groups, respectively. The standard error was calculated separately for each table entry from the number of determinations indicated (n). Each determination was made on the pooled tissues of a minimum of six animals.

Fatty acid	Deficient (n = 2)	Supplemented (n = 1)	Lab chow (n = 2)
16 : 0	19.6 ± 0.0	16.9	17.2 ± 0.3
16 : 1	1.8 ± 0.7	0.3	1.2 ± 0.2
18 : 0	21.9 ± 0.9	22.6	21.9 ± 1.8
18 : 1	12.0 ± 0.1	10.1	10.0 ± 0.9
18 : 2ω6	9.3 ± 0.1	4.8	3.6 ± 0.5
20 : 4ω6	15.5 ± 0.5	13.9	13.0 ± 0.7
22 : 4ω6	3.4 ± 0.0	4.0	3.9 ± 0.3
22 : 5ω6	3.1 ± 0.4	4.1	2.4 ± 0.1
22 : 5ω3	—	0.3	0.8 ± 0.1
22 : 6ω3	9.7 ± 0.5	18.4	22.2 ± 1.0
Total polyunsaturated fatty acids	41.0 ± 1.3	45.5	45.9 ± 0.3

mented groups can be made even though it was possible to make only a single determination on pooled tissues from the supplemented group. Because the standard deviations are so small for both the deficient and lab chow pigment epithelium fatty acid values, each single determination in these experiments is therefore, a very close approximation to the sample mean. Furthermore, it is possible to statistically evaluate the difference in the 22 : 6ω3 values of the deficient and supplemented retinal pigment epithelium. This difference is significant, $P < 0.025$, when the supplemented value is compared to a confidence interval constructed about the mean of the deficient 22 : 6 values, i.e. $\overline{X}_{22:6} \pm [(\widehat{S.D.}) \times (t)]$ where the t value is determined at the 0.025 confidence level with one degree of freedom. In addition, the fatty acid profile of the supplemented group is not very different from that seen in the lab chow group as might be expected since minimal differences are observed in the other retinal tissues examined from these two groups and there is little difference in fluorescent pigment accumulation in the pigment epithelium of these two groups [22]. In contrast, we observed a build up of fluorescent pigment in the deficient pigment epithelium, where we also measured a lower polyunsaturated fatty acid content. Finally, those differences which are seen between the fatty acid profiles of the lab chow and supplemented pigment epithelium are the very kind of differences which would be expected as a consequence of the mild linolenate deficiency noted above and reported (vide supra) for the other retinal tissues examined.

The question arises; do the fatty acid profile changes observed accurately reflect the in vivo tissue composition, or are they instead changes resulting from the loss of polyunsaturated fatty acids from antioxidant-deficient tissues incurred during their isolation and manipulation in vitro. Some possible ways in which a loss of unsaturated fatty acids could occur in vitro are: lipid autoxidation, the selective loss of membranes rich in unsaturated fatty acids during cell purification, or the degradation of fatty acids arising from the release of lysosomal and other degradative enzymes. To test the effectiveness of our in vitro methods in minimizing lipid autoxidation in membranes high in polyunsaturated fatty acids, rod outer segments which were purified from antioxidant-deficient rats were air challenged at room temperature for 18 h in the presence of CaEDTA, an in vitro antioxidant, using methods previously described [13]. No changes in total polyunsaturated fatty acid content nor in the fatty acid profile were observed in the sample after incubation. The CaEDTA serves to chelate heavy metals known to be catalysts of lipid autoxidation, and release, in exchange, Ca^{2+} [33]. Omission of CaEDTA in parallel samples leads to large losses in polyunsaturated fatty acids consistent with our previous findings in the bovine system [13]. These results indicate that buffers with CaEDTA protect even the rod outer segments of vitamin E- and selenium-deficient rats from autoxidative loss of unsaturated fatty acids. Since outer segments are more highly polyunsaturated than pigment epithelium, this experiment suggests that in the presence of CaEDTA, the retinal pigment epithelium of deficient animals would also be protected from nonenzymatic lipid peroxidation.

Cell and organelle lysis could lead to the loss of polyunsaturated fatty acid-rich membrane vesicles into the preparation medium. Antioxidant-deficient cells might be expected to be more fragile and consequently more easily lysed

even under our relatively gentle purification conditions. No differences were, however, seen in the polyunsaturated fatty acid composition between bovine pigment epithelial cells which were purified after being hypotonically disrupted and those which were prepared isotonically. Membrane lysis could also result in the release of lysosomal enzymes and toxic by-products of digestion. When bovine pigment epithelial cells, which were hypotonically shocked after purification, were incubated at 0°C for 18 h, no loss of polyunsaturated fatty acids nor changes in fatty acid profile were observed. Samples stored at room temperature for 18 h, however, showed dramatic decreases in polyunsaturated fatty acids, even in the presence of CaEDTA, used to inhibit nonenzymatic lipid autoxidation. This suggests that enzymatic degradation could cause significant changes in the apparent fatty acid profile even over much shorter time periods if samples are not carefully handled in vitro.

The content of polyunsaturated fatty acids which we have observed in the retinal pigment epithelium of rats fed the commercial lab chow diet is much higher than previously reported for bovine pigment epithelium [25,34]. For comparison, bovine retinal pigment epithelium were prepared by the same method and analyzed. These bovine preparations were found to contain equally high levels of polyunsaturated fatty acids as found in rat (Farnsworth, C., Stone, W. and Dratz, E., unpublished). The major potential contaminant which could elevate the apparent polyunsaturated fatty acid content of the retinal pigment epithelium is the rod outer segment membrane, since almost half of its fatty acids is 22 : 6ω3. The possible contribution of outer segment lipids to the apparent fatty acid composition of the pigment epithelium was determined as follows. Pigment epithelium cells were isolated under red light from dark-adapted bovine eyes. The purified cells were then treated with 3% Ammonyx detergent to solubilize any rhodopsin present. Using light-dark difference spectra, no rhodopsin was detected. The fatty acid composition of pigment epithelium prepared under red light was not found to be significantly different from the composition of cells prepared under normal laboratory lighting conditions. Furthermore, the sensitivity of the fatty acids of the retinal pigment epithelium to dietary variables as compared to the insensitivity of the rod outer segments to the same dietary conditions argues that the content of fatty acids from the pigment epithelium is not strongly influenced by outer segment contamination. The absence of rod fragments was noted using phase contrast light microscopy which also indicated the presence of very few red blood cells. Although red blood cells would not elevate the apparent polyunsaturated fatty acid content of the retinal pigment epithelium, they have been noted as major contaminants [25] and therefore their possible contribution was further assessed. Crude bovine pigment epithelial cell suspensions were purified using a glass bead affinity binding method similar to that of Siakotos et al. [35]. This technique is used to separate phagocytic from non-phagocytic cells; alternatively pigment epithelial cells were centrifuged through 31% sucrose according to our method as described. Both preparations yielded comparable fatty acid profiles. Measurements of heme absorbance, at 420 nm, in the supernatant of purified retinal pigment epithelium, which had been hypotonically shocked and pelleted, indicated a negligible membrane contamination from red blood cells. Even though all major contaminants have been ruled out, the purification

methods presently available for retinal pigment epithelium still do not appear to yield completely pure cells. The fatty acid results might differ in detail but not in substance if remaining minor contaminants were eliminated.

Discussion

The changes we observed in the polyunsaturated fatty acid composition of retinal pigment epithelium from antioxidant-deficient rats are similar to the findings in testes of vitamin E-deficient rats reported by Bieri and Prival [6] and Carpenter [36]. In addition, Hayes [14] has reported a build-up of lipofuscin-type granules in the retinal pigment epithelium of vitamin E-deficient monkeys and Katz et al. [22] have found pronounced elevation of fluorescent material in antioxidant-deficient rat pigment epithelium, but not in the rod outer segments or other areas of the retina. The presence of fluorescent, lipofuscin-like material is widely thought to indicate the build-up of lipid peroxidation by-products [37]. All of these results, taken together, suggest that the retinal pigment epithelium is sensitive to lipid autoxidation in vivo.

Two possible factors which could contribute to the susceptibility of the retinal pigment epithelium to the effects of antioxidant deficiencies are noteworthy. Due to its role in rod outer segment renewal, the retinal pigment epithelium daily phagocytizes the apical tips from about one third of the rod cells of retina [38]. This results in the daily consumption of large amounts of polyunsaturated, fatty acid-rich membrane. In the antioxidant-deficient animal, these freshly phagocytized membranes might be extremely susceptible to autoxidation. Secondly, the containment of possibly toxic catabolites from the digestive vacuoles of the pigment epithelium, as well as the maintenance of intact primary lysosomes and microperoxisomes, might be expected to be more difficult in severely antioxidant-deficient cells. Serfass and Ganther [39] have shown that other types of phagocytic cells from selenium-deficient animals become inactivated due to the unchecked production of lipid peroxides during phagocytosis.

Contrary to our expectations, the fatty acid profiles of the whole retina and rod outer segment were not dramatically affected by vitamin E and selenium deficiency. Although Hayes [14] reports outer segment deterioration with vitamin E defieciency, the lesions he observed were confined to the central region of the retina. The modest decrease in total polyunsaturated fatty acids which we find in the outer segment component, coupled with the absence of detectable changes in the bulk of the retina as a whole, could be consistent with a central retinal lesion that comprises a small fraction of the total retinal area as observed by Hayes [14]. In addition, no loss of polyunsaturated fatty acids was observed in deficient liver, in agreement with Lee and Barnes [40]. Liver arachidonate was, however, elevated above the values of the supplemented control animals as previously reported by Witting [5].

The fatty acids of the whole retina and outer segment membrane seem to be spared from some of the more severe effects of antioxidant deficiency compared to the pigment epithelium. Several different factors may be responsible for this effect. One of these is the relatively rapid, new membrane synthesis at the base of the rod outer segment and the frequent shedding of the apical tips

of the outer segment. This ensures the systematic removal of the older membranes. This renewal mechanism would tend to minimize the contribution of membranes depleted of polyunsaturated fatty acids to the fatty acid profile of pooled tissues. This mechanism would also tend to inhibit the autocatalytic oxidation process itself, by limiting the accumulation of oxidation by-products. We observed no differences in the rate of synthesis and turnover of outer segment membranes between deficient and supplemented rats (at 20 weeks on defined diets) using radioautography (Bok, D., Farnsworth, C. and Dratz, E., unpublished), although under certain types of stress in vivo, the rate of membrane turnover increases [41]. Another possible factor contributing to the apparent insensitivity of the whole retina and outer segment fatty acids to peroxidative degradation is the enzyme superoxide dismutase. Substantial levels of this enzyme have been found in the retina and outer segments of rats [42] and it is known to possess antioxidant properties in vitro [43]. In addition, the concentrations of ascorbic acid [44]and glutathione [45]found in ocular tissues are also substantial. Both ascorbic acid and glutathione are thought to be effective inhibitors of lipid oxidation in vivo [1]. Other, as yet undescribed agents and nutrients may also be involved.

When a decrease in the polyunsaturated fatty acid composition of tissues from antioxidant-deficient animals is observed relative to control animals an in vivo loss of polyunsaturated fatty acids is not necessarily indicated. Inadequate antioxidant conditions in vitro can contribute to the apparent loss of polyunsaturated fatty acids [13]. With regard to the polyunsaturated fatty acid-rich rod outer segment membrane and whole retina, as well as experiments of this type on other tissues, the importance of maintaining stringent in vitro antioxidant conditions in order to obtain meaningful data becomes obvious. In vitro changes in normal fatty acid profiles can also appear rapidly in tissues with high levels of endogenous digestive enzymes if proper precautions are not taken. By using the in vitro precautions described above, it seems likely that the changes we have seen accurately reflect the in vivo status of the tissues analyzed.

Several different mechanisms have been suggested [1,10] to explain in vivo changes in the composition of tissue fatty acids in severely antioxidant-deficient animals. Direct in vivo peroxidation of tissue lipids is one possible mechanism [46]and could account for the actual decrease in total polyunsaturated fatty acid content observed in testes phospholipid by Bieri and Prival [6]and retinal pigment epithelium, in the present work. Antioxidant deficiences may, however, act more subtly by interfering with the metabolism of linoleate-derived polyenoic fatty acids as suggested by Bernhard et al. [8]or alternatively by disrupting normal tissue maturation, thereby causing changes in the fatty acid profiles indirectly. With regard to the effects of antioxidant deficiencies on maturation and development, it is known that the fatty acid composition of certain tissues change as part of normal maturation [36,47,48] and that the content of polyunsaturated fatty acids in the juvenile tissue is generally lower than in the mature adult, as reported for rat liver [49] and testes [36]. Since most nutritional investigations use weanling animals, changes seen in certain adult tissues may result, at least partially, from effects on development.

With regard to in vivo changes in fatty acid composition, a number of different responses to antioxidant deficiency are observed in different tissues. Some tissues are not affected, as seen in the whole retina, while others are only slightly affected, as seen in outer segment total polyunsaturated fatty acid composition. Other tissues such as the liver exhibit a slight elevation in polyunsaturated fatty acids. In the retinal pigment epithelium, we have observed a dramatic decrease in total polyunsaturated fatty acids and changes in the fatty acid profile. These results indicate that changes in fatty acid composition are not generalized to all tissues in severely antioxidant-deficient animals. The retinal pigment epithelium, however, appears to be extremely sensitive to antioxidant deficiencies.

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