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Relative α-Tocopherol Deficiency in Cultured Cells: Free Radical-Mediated Lipid Peroxidation, Lipid Oxidizability, and Cellular Polyunsaturated Fatty Acid Content

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We propose that most cultured cells are deficient in vitamin E. Using our optimized assay for tocopherol, we find that L1210 lymphoblastic leukemia cells, cultured in standard growth media, contain only 2.3 ± 0.03 μg of tocopherol/10^8 cells, whereas when they are transplanted and grown for the same time in the ascites fluid of mice fed standard diets, this increases to 5.8 ± 0.6 μg of tocopherol/10^8 cells. This apparent tocopherol deficiency in cultured cells is likely due to the low concentrations of tocopherol contained in most tissue culture media, even with the addition of serum. To further study this apparent deficiency and the relationship of cellular tocopherol to membrane lipid *bis*-allylic hydrogen positions, we supplemented the growth media of L1210 lymphoblastic leukemia cells with α-tocopherol and compared the resultant cellular tocopherol content to the degree of unsaturation of cellular lipids. α-Tocopherol was incorporated by cells in a time- and concentration-dependent manner with plateaus at 24 h and 100 μM, respectively. A maximum 400% increase in cellular tocopherol was easily achieved. By experimentally modifying the fatty acid content of cellular lipids, we were able to determine that cellular tocopherol uptake and content is not a function of cellular lipid composition; cells enriched with polyunsaturated lipids incorporated tocopherol to the same extent as those enriched with more saturated lipids. Thus, as the cellular polyunsaturated fatty acid content increases, the tocopherol:*bis*-allylic position ratio in the cells decreases, resulting in less antioxidant protection for each lipid double bond. Consequently, when polyunsaturated fatty acid-enriched cells are exposed to an oxidative stress, such as Fe^{2+}, their tocopherol levels decline much faster than cells enriched with saturated fatty acids. This decline is consistent with their respective tocopherol:*bis*-allylic position ratio. These results provide a basis, at the cellular level, for investigators to consider vitamin E when studying cell response to oxidative stress.

Key Words: **α-tocopherol; vitamin E; oxidizability; polyunsaturated fatty acids; free radicals; lipid peroxidation; antioxidants.**

α-Tocopherol is the major cellular lipid-soluble, small-molecule antioxidant. It suppresses fatty acid oxidation by contributing a hydrogen atom from its phenolic hydroxyl group to lipid-derived peroxyl, and perhaps alkoxyl, radicals (1, 2). This action prevents the peroxyl radical from attacking the unsaturated fatty acids of adjacent membrane lipids, thereby inhibiting the propagation phase of lipid oxidation reactions. The membrane location of tocopherol would seem well suited for this purpose since lipid-derived free radicals are generated therein. However, the importance of the relationship of tocopherol content to the extent of lipid polyunsaturation and whether there is compensatory change in membrane tocopherol with polyunsaturation, are unknown.

The lowest carbon–hydrogen bond dissociation energies of a fatty acid are found at *bis*-allylic methylene positions, i.e., between adjacent double bonds (3–5). Thus, these positions are the thermodynamically favored sites for attack by lipid peroxyl radicals in polyunsaturated fatty acids (PUFA).[2] Cells with greater PUFA content, and hence a higher number of *bis*-allylic

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[2] Abbreviations used: DBI, double bond index, which is the mean number of double bonds per fatty acid molecule; FBS, fetal bovine serum; MBI, methylene bridge index, which is the mean number of *bis*-allylic methylene positions per fatty acid molecule; PUFA, polyunsaturated fatty acids.

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positions (a larger MBI[3]), are more oxidizable. It would seem reasonable that cells with higher PUFA content would benefit from higher tocopherol content, since they have the potential for greater radical formation (5). In fact, cells might even benefit from an ability to adjust the number of tocopherol molecules to the number of *bis*-allylic hydrogen positions in order to provide a balanced antioxidant protection.

There could be a preferential association of tocopherol with PUFA, suggesting an offsetting relationship between the number of molecules of antioxidant and susceptible sites. For example, in ethanol solution, the equilibrium constant for the formation of α-tocopherol complexes with fatty acids increases exponentially with an increase in the number of double bonds in the fatty acids (6).

The number of antioxidant molecules available per susceptible fatty acid *bis*-allylic position may be a major determinant in the response of an organism to an oxidative stress. In this regard, some investigators have found a direct correlation between cellular tocopherol levels and the degree of lipid polyunsaturation, suggesting that cells compensate by increasing their lipid soluble antioxidants. Following are some examples: (a) The muscle phospholipids of the striped bass, a commercial fish species, have a 5% greater peroxidizability index, an estimate based on weighted contribution of fatty acids with different number of double bonds. However, they also have 74% more total tocopherol than a hybrid of this species (7). (b) Normal human subjects consuming a high-fat diet for 5 days have greater plasma tocopherol (about fourfold) in response to the administration of oral tocopherol supplements than those consuming a diet with lower fat content (8). (c) In porcine skeletal muscle taken from two different anatomical locations, there was more tocopherol in the site with higher PUFA (9). These results suggest that cells could alter their tocopherol content to compensate for PUFA levels.

Most growth media currently in use contain limited amounts of tocopherol. Therefore, the tocopherol must come from the serum in the growth medium, which has limited amounts. Since most initial work with experimental drugs, especially those which function through an oxidative mechanism, begins with *in vitro* observations, examination of the tocopherol levels and the relationship of tocopherol to double bonds would seem to be extremely important in tissue culture systems.

We have developed a model that allows us to modify the polyunsaturation of cellular lipids, thereby providing a way to examine the relationship of polyunsaturation to tocopherol content. This modification is specific for the fatty acids and does not change the total phospholipid content, cholesterol content, or the phospholipid head group composition; thus, there is no change in the basic architecture of the membranes (10, 11). Using this model, we have made comparisons between cellular fatty acid composition and resulting tocopherol levels.

[3] MBI, methylene bridge index, is the average number of *bis*-allylic methylene positions per fatty acid (or fatty acyl chain) in a lipid ensemble. The MBI is the best measure to use to express the relative oxidizability of a lipid (5).

MATERIALS AND METHODS

Cells, chemicals, media, and fatty acid modification. L1210 murine lymphocytic leukemia cells were obtained from the American Type Culture Collection and maintained in continuous cell suspension at 37°C in RPMI 1640 medium and 10% fetal bovine serum. DL-α-tocopherol acetate, DL-α-tocopherol succinate, DL-α-tocopherol phosphate, (±) α-tocopherol, and chelating resin (iminodiacetic acid) were obtained from Sigma Chemical Co. (St. Louis, MO). L-Ascorbic acid, ferrous sulfate, HPLC grade methanol, and hexane were obtained from Fisher Chemical (Fairlawn, NJ). Phosphate-buffered saline (PBS) was made without Ca^{2+} and Mg^{2+} (2.68 mM KCl, 1.47 mM KH_2PO_4, 136.8 mM NaCl, 8.06 mM NaH_2PO_4, and 0.5 mg/ml chelating resin, pH 7.4). FBS was obtained from Sigma (for use in growth media) and HyClone (Logan, UT).

For lipid modification, 32 μM fatty acid was added to the growth media of the L1210 cells (5, 12–15). This concentration has no appreciable effect on cell viability (12). Fatty acid content of the cells was determined by a gas–liquid chromatography method (11, 12). Fatty acids are abbreviated as number of carbon atoms:number of double bonds; ω refers to the position of the first double bond from the methyl terminus. The MBI was calculated from the mole percent composition of each type of fatty acid molecule.

L1210 cells from ascites fluid. For the *ex vivo* experiments, 1×10^5 L1210 cells from tissue culture were injected into the peritoneum of DBA/2 mice. After 8 days of tumor growth, the ascitic fluid, containing the L1210 cells, was harvested and centrifuged at 750*g* for 10 min. The cells were washed three times with PBS (as above), and the tocopherol content determined. The mice were fed a standard commercial mouse chow (Formulab Diet) which contains vitamin E at 55 IU/kg.

Glassware preparation. All glassware used was treated by the method of Buettner (16). Briefly, it was cleaned and then soaked for 48 h in 1 M HCl and then another 48 h in deionized distilled H_2O containing chelating resin (5 g/liter).

Tocopherol determination. We optimized a technique for the quantification of tocopherol based on the natural fluorescence of free tocopherols (17). The oxidized form, tocopheryl quinone, does not fluoresce, therefore, for accurate tocopherol determinations, oxidation of tocopherol to the tocopheryl quinone must be prevented.[4] Other investigators have reported reliable techniques for the determination of cellular tocopherol with good sensitivity (18–21). However, to protect the tocopherol from oxidation, we added ascorbic acid at *every* analytic step—to the cell suspension after incubation, with each wash, and in the final extraction. The ascorbic acid used in this assay serves as an antioxidant. It protects the tocopherol from oxidation, thus preventing tocopheryl quinone formation. Others have used ascorbic acid to protect tocopherol; however, some reported HPLC procedures have not added ascorbate (21, 22) or have added it at fewer steps (18, 19, 23). The diacid form of ascorbate that exists in the ethanol/hexane solvent has an absorption maximum at 246 nm, $\epsilon_{246} \cong 11{,}000\ M^{-1}\ cm^{-1}$. At the 1 mM level, the tail of the ascorbic acid absorption will not interfere with the 292-nm fluorescence excitation of the tocopherol. In addition, we eliminated a saponification

[4] The tocopheryl quinone is a ring-breakage product. Thus, once formed, tocopherol is irreversibly lost.

step, similar to Pascoe *et al.* (21), in order to minimize tocopherol loss following the incubations and during the analysis. This allowed us to restrict our analysis to free tocopherol and not include tocopherol esters. We also rigorously removed the trace amounts of adventitious catalytic transition metals contained in the phosphate-buffered saline and water and thoroughly washed the glassware to remove these metals (16). These efforts further protect tocopherol from oxidation.

For tocopherol analysis, L1210 cells were harvested by centrifugation. They were resuspended in ice-cold PBS, pH 7.4, in a 50-ml plastic conical centrifuge tube containing 1.7×10^8 cells. To the cell suspension was added 20 μl of 200 mM ascorbic acid. The suspension was centrifuged at 750*g* for 10 min, the supernatant discarded, and the wash repeated with the addition of the same amount of ascorbic acid. To the final wash pellet was added 5 μl of 200 mM ascorbic acid. The pellet was finger-vortexed and placed on ice for 2 min and then transferred by a glass Pasteur pipet to a cold 15-ml glass-stoppered centrifuge tube. To the cell suspension was added 500 μl of 95% ethanol. The sample was vortexed and then sonicated for 10 min followed by the addition of 500 μl of ice-cold hexane. The sample was vortexed for 1 min and centrifuged at 300*g* for 3 min to separate the phases. An aliquot of the top (hexane) phase was placed into a small Teflon-capped glass vial containing 5 μl of 200 mM ascorbic acid. The head space air was replaced with N_2 and the vial stored at −70°C for no more than 24 h until HPLC analysis.

The extracted samples were analyzed by reverse-phase HPLC. The hexane extract samples were injected (100 μl injection volume), using a gas-tight, Teflon-plunger Hamilton syringe (24) into a Gilson Model 320 HPLC. An Alltech C18 ODS (3 μm) column was used with a mobile phase of hexane:methanol (99:1, v/v) at a flow rate of 2 ml/min. Tocopherol content was determined using a FD200 Spectrovision fluorescence detector with excitation at 292 nm and emission detection at 320 nm. The sensitivity of our method is 14 pmol.

Oxidative stress and tocopherol. The experiments to examine the effect of oxidative stress on cellular α-tocopherol were performed as follows. L1210 (5×10^7) cells were enriched with 22:6 or 18:1 by adding 32 μM of the fatty acid to the growth media for 48 h. During the final 24 h, 12.5 μM tocopherol acetate was added to the growth media to increase cellular tocopherol. The enriched cells were washed and then incubated in 0.9% NaCl in 95% air/5% CO_2 at 37°C with or without 100 μM Fe^{2+} and the cellular tocopherol determined at selected time points.

RESULTS AND DISCUSSION

Cellular Tocopherol Deficiency in Culture

We found the tocopherol content of L1210 cells grown in tissue culture for 8 days to be only 2.3 ± 0.03 μg/10^8 cells (n = 3). This is equivalent to 0.2 ng tocopherol/μg protein since L1210 cells contain 103.4 ± 5.8 μg protein/10^6 cells (n = 3). This is slightly lower than that reported in the hepatocyte, which is a much larger cell (25), and about 60% that of the freshly obtained normal lymphocyte, which is smaller and more differentiated (18). Injecting these tissue culture L1210 cells into the peritoneum of DBA/2 mice and allowing them to grow for 8 days in the ascites fluid raised the tocopherol level to 5.79 ± 0.55 μg/10^8 cells, an increase of more than twofold compared to cells grown in tissue culture.

The relative cellular tocopherol deficiency of cultured cells compared to those grown *in vivo* is due to the limited amount of tocopherol contained in the tissue culture growth media. In the list of 28 media available from a commercial source (Gibco BRL Catalog, 1993–1994), 22 contain no tocopherol. This includes commonly used media such as modified Eagle's medium (MEM)–Earle's salts, basal medium Eagle, Waymouth's media, RPMI 1640, RPMI 1630, McCoy's 5A media, Iscove's modified Dulbecco's media, Dulbecco's modified Eagle media, and CMRL media 1066. Some media contain very small amounts of tocopherol, e.g., basal media Eagle (BME) (1 mg/liter), medium 199 (0.01 mg/L), media NCTC-109 and -135 (0.025 mg/L), William's media E (0.01 mg/L), and medium 199 (0.01 mg/L). The greatest concentration of tocopherol was in basal media Eagle at 2 μM. The addition of 10–20% serum to a growth media cannot provide sufficient tocopherol since serum contains limited amounts. For example, we determined the tocopherol in FBS used in the present study (Sigma lot 33H0995) after heat inactivation (56°C for 30 min) to be 0.193 ± 0.046 μg/ml of α-tocopherol and 0.287 ± 0.05 μg/ml of γ-tocopherol (mean and SE of three replicates). Therefore, FBS from Sigma provides about 45 nM α-tocopherol when used at 10% concentration in a culture medium that contains no tocopherol. For comparison, the defined FBS from HyClone contains <1–3 μg/ml in three different lots according to the material included with the serum. Taken together this indicates a deficiency of tocopherol in the L1210 cells grown in usual media. It is likely that the majority of *in vitro* studies in the literature are performed on cells that are deficient in tocopherol.

Cell Tocopherol Uptake

Cells must obtain tocopherol from their environment. Therefore, we examined the characteristics of this uptake. L1210 cells were incubated 0–36 h at 37°C with 100 μM α-tocopherol acetate. It was taken up by the cells and deesterified to α-tocopherol in a linear, time-dependent manner which reached saturation at 24 h (Fig. 1). Uptake was also dependent upon the concentration of tocopherol in the media (Fig. 2). For a 24-h incubation, the maximum level was reached with a media content of 100 μM.

Other than time and concentration, the uptake also depended upon the form of tocopherol provided to the cell. When cells were incubated with identical concentrations of either tocopherol succinate or phosphate, the uptake of tocopherol from each was slower than from the acetate, but similar to one another up to 25 μM (Fig. 2, inset). Thereafter, we found that the lower uptake by succinate was due mostly to a cytotoxic effect of this ester on the L1210 cell. Fariss *et al.* have shown that the antiproliferative effect of α-tocopheryl hemisuccinate was due to the intact compound and not to release of succinate or tocopherol (26). Thus, for tissue culture work, tocopherol acetate appears to be the best form to use in media supplementation.

It has previously been demonstrated that nontrans-

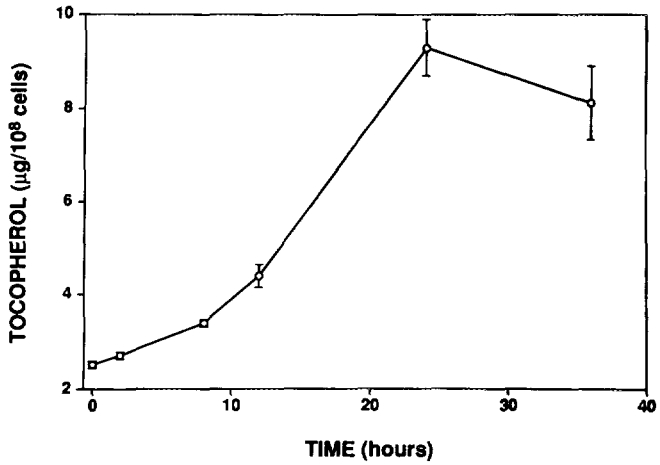

FIG. 1. Time course for accumulation of α-tocopherol in L1210 cells. Cells were incubated with 100 μM α-tocopherol acetate added to the growth medium for 36 h. At specified times the cells were collected by centrifugation and assayed for cellular content of α-tocopherol. Values are the means and SE of at least three separate experiments. All values after 8 h are significantly different from baseline ($P < 0.01$). The concentrations at 0 and 24 h are equivalent to 0.242 and 0.899 ng tocopherol/μg protein, respectively.

formed cells can be enriched with tocopherol by adding the vitamin to the growth media or diet. For example, isolated hepatocytes have a remarkable increase in cellular tocopherol when 25 μM tocopherol succinate is added to the growth media (22). Similarly, the addition of tocopherol to the diet of rats leads to a 30-fold increase in microsomal tocopherol (20). Our studies indicate that the cellular tocopherol content of a cultured neoplastic cell can be increased by supplementing the growth media with tocopherol. Furthermore, *in vitro* supplementation can increase tocopherol concentrations to a level equal to or greater than *in vivo* concentration determined in the same cell type grown in the ascites fluid of mice fed diets containing a standard amount of the vitamin. Taken together these results suggest that tocopherol should be added to the growth media in quantities greater than those in current use.

Cellular Uptake Does Not Depend on PUFA Content

Logically, modification of cellular fatty acid content might affect cellular tocopherol content. The cell might compensate for greater oxidizibility, due to greater polyunsaturation, by increasing its antioxidant content. Our established model for the modification of cellular fatty acids provided us with an opportunity to study the effect of cellular lipid polyunsaturation on tocopherol accumulation. To examine this question, L1210 cells were incubated in media containing one of four supplemental fatty acids in order to modify their fatty acid composition. Table I shows the fatty acid composition of the cells following incubation for 48 h in enriched media. Cells were considerably enriched with the fatty acid that was added to the media. For example, cells incubated in media containing 22:6 had 34% of 22:6 in their total cellular lipids compared to unmodified cells, which were only 1.1% 22:6. Similarly, the enrichments in 20:4, 18:3, and 18:1 were to 34, 33, and 66%, which represents a 288, 6520, and 75% increase, respectively. The degree of modification was similar to that described in our previous reports (5, 15).

We found no significant difference in cellular tocopherol content between fatty acid modified cells of any type and unmodified cells (Fig. 3A). When 18:1 or 22:6 modified cells were incubated in media enriched with various concentrations of tocopherol, there was a concentration-dependent increase in cellular tocopherol of both (Fig. 3B). However, there was no greater enrichment of the 22:6 enriched cells, which are rich in PUFA and have a high number of *bis*-allylic positions. Thus, the PUFA content of cultured L1210 cells appears to have no influence on tocopherol uptake. There may be no cellular sensor to signal that additional tocopherol is needed for the polyunsaturation provided by the experimental manipulation of lipid content.

Relative Tocopherol Deficiency Is Based on bis-Allylic Positions

Recently we have shown that the oxidizability of cellular lipids as measured by free radical-mediated peroxidative events is determined by the number of *bis*-allylic positions contained in the cellular lipids of intact

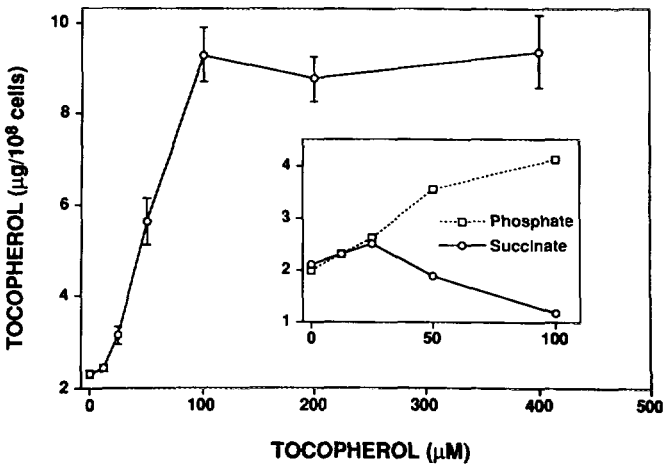

FIG. 2. Concentration dependence of cellular α-tocopherol accumulation. L1210 cells were incubated for 24 h with α-tocopherol acetate. Shown are the mean and SE of at least three separate experiments. The concentrations at 0 and 100 μM are equivalent to 0.222 and 0.899 ng tocopherol/μg protein, respectively. (Inset) Comparison of uptake and accumulation of cellular α-tocopherol after the addition of α-tocopherol succinate or α-tocopherol phosphate to the media. Above 25 μM the succinate ester was toxic as measured by trypan blue dye exclusion so the values are no longer satisfactory estimations of accumulation.

TABLE I

Fatty Acid Composition of L1210 Cells (in mol%)[a]

	Fatty acid added to media				
	Unmodified	18:1ω9	18:3ω3	20:4ω6	22:6ω3
Individual acids[b]					
16:0	15.8 ± 0.5	10.2 ± 0.6	13.1 ± 1.1	15.7 ± 0.5	18.9 ± 1.7
16:1	5.4 ± 0.4	3.6 ± 0.2	3.8 ± 1.0	2.3 ± 0.1	3.1 ± 0.1
18:0	15.5 ± 0.3	8.0 ± <0.1	16.1 ± 1.7	14.4 ± 0.2	16.6 ± 1.4
18:1ω9	37.5 ± 0.2	65.6 ± 0.4	12.3 ± 5.0	8.5 ± 3.6	14.2 ± 1.0
18:2ω6	4.8 ± 0.1	1.2 ± 0.1	3.4 ± 0.6	1.6 ± 0.1	2.0 ± 0.2
18:3ω3	0.5 ± 0.5	0.8 ± 0.5	33.1 ± 2.5	0.7 ± 0.5	0.1 ± 0.1
20:1ω9	1.9 ± 0.1	4.0 ± 0.1	0	0.2 ± <0.1	0.1 ± 0.1
20:4ω6	8.9 ± 0.1	2.1 ± <0.1	8.0 ± 3.3	34.5 ± 2.4	2.9 ± 0.2
22:4ω6	0.8 ± 0.1	0	0.8 ± 0.5	17.2 ± 1.1	2.1 ± 0.8
22:6ω3	1.1 ± <0.1	0.3 ± <0.1	0.7 ± 0.1	0.5 ± <0.1	34.4 ± 3.6
24:0	0	0	0	0	0
Others	7.8	4.2	8.7	4.4	5.6
Classes of fatty acids					
%PUFA[c]	21.5 ± 0.6	6.9 ± 0.3	51.5 ± 0.8	57.1 ± 4.3	45.2 ± 3.7
%MUFA	45.2 ± 0.5	73.9 ± 0.5	17.9 ± 3.7	11.3 ± 3.7	17.7 ± 1.1
%SAT	33.0 ± 0.3	19.2 ± 0.6	30.6 ± 3.1	31.6 ± 0.5	37.1 ± 3.3
%ω3	3.7 ± 0.5	1.9 ± 0.4	37.8 ± 2.8	2.0 ± 0.6	35.6 ± 3.8
%ω6	17.8 ± 0.3	5.0 ± 0.1	13.7 ± 2.2	54.2 ± 3.6	8.4 ± 0.6
Indices					
MBI[c]	0.54 ± 0.02	0.16 ± 0.01	1.18 ± 0.02	1.70 ± 0.12	2.02 ± 0.20
DBI	1.21 ± 0.02	0.97 ± 0.01	1.87 ± 0.05	2.38 ± 0.13	2.65 ± 0.23

[a] L1210 cells were grown for 48 h in media supplemented at 32 μM with the fatty acid designated in the column headings. Cells were washed and extracted with $CHCl_3$:CH_3OH, 2:1 (v/v). After alkaline hydrolysis, fatty acids in the saponifiable fraction were methylated and the methyl esters separated by gas–liquid chromatography. Mole percents of various fatty acids were calculated on the basis of actual amounts of fatty acids determined from integrated peak areas on chromatograms, an internal standard and respective molecular weights of fatty acid methyl esters.

[b] Expressed as mole percent of total fatty acids. Fatty acids are designated as number of carbon atoms:number of double bonds. Values are the mean ± SE of three independent experimental sample determinations.

[c] PUFA, polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; SAT, saturated fatty acids; MBI, methylene bridge index, which is the mean number of *bis*-allylic positions per fatty acid; DBI, double bond index, which is the mean number of double bonds per fatty acid.

cells (5). Our current observation that cells with vastly different levels of PUFA, i.e., widely ranging MBI, have similar tocopherol content suggests that there is a relative "deficiency" of tocopherol in the cells with high PUFA levels. In order to define this relationship in terms of the cellular MBI, we determined the number of tocopherol molecules per *bis*-allylic position (Table II). PUFA enrichment resulted in a modulation of the number of double bonds and, most importantly, a spectral range of more than one log in the total number of cellular *bis*-allylic positions, an MBI ranging from 0.16 in the unmodified cells to 2.02 in modified cells (Table I). The tocopherol:*bis*-allylic position ratio, which estimates the amount of antioxidant available per susceptible position, decreases as the extent of polyunsaturation increases (Table II). Thus, the highest ratio of tocopherol molecules to *bis*-allylic positions is found in the 18:1-enriched cells, which would seem to require the least tocopherol, and the lowest by the 22:6-enriched cells, which are most vulnerable to peroxidation processes and would seem to require the most tocopherol.

Since the amount of α-tocopherol available for each *bis*-allylic position decreases with increased polyunsaturation of the cellular lipids, PUFA enrichment results in an enhancement of vulnerability to oxidation. The relative contribution to heightened oxidation in cell lipids, due to the number of double bonds per se, compared to the contribution due to the decrease in antioxidants available per *bis*-allylic position cannot be quantitated. In the present work, calculated tocopherol to *bis*-allylic position ratios range from about 0.0001 to 0.0011, depending upon the extent of polyunsaturation. Thus, there is one tocopherol molecule available for about 10,000 to 1000 vulnerable double bond configurations measured as *bis*-allylic positions. In various tissues of rats fed a semipurified diet of 20% corn or soybean oil, Evarts and Bieri found ratios of 0.0003–0.001 tocopherol to total PUFA (≈3000–1000 total PUFA to tocopherol) (27). Gruger and Tappel found 0.0005 mole-

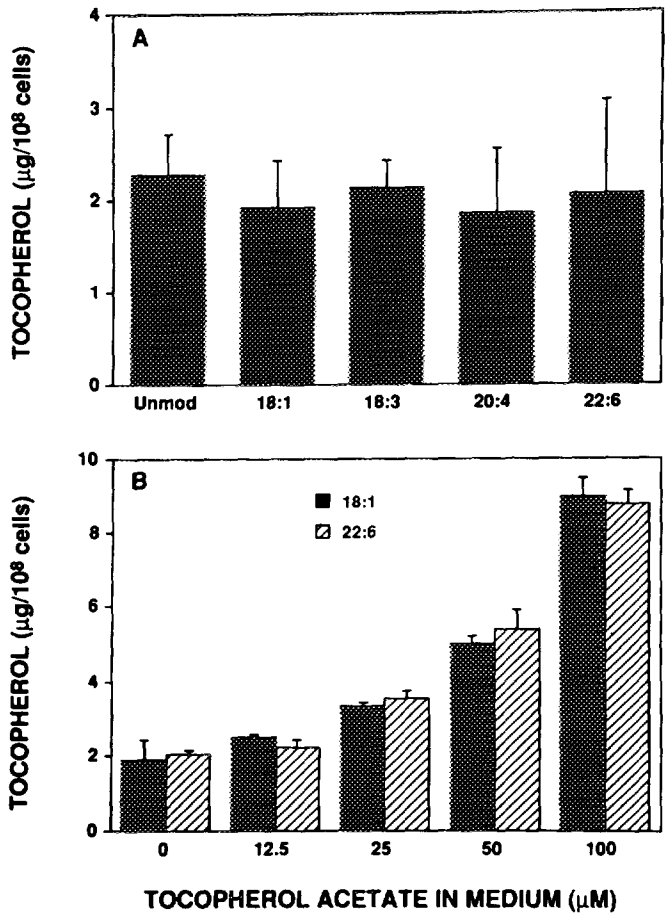

FIG. 3. The effect of fatty acid modification on cellular α-tocopherol content and uptake from tocopherol-supplemented media. (A) Cellular content of α-tocopherol in fatty acid modified cells in standard growth media. Cells were incubated for 48 h in media enriched with fatty acids of various degrees of polyunsaturation prior to determination of cellular content of tocopherol. Shown are the means and SE of at least three separate experiments. None of the differences is significant. The concentrations of tocopherol expressed as ng tocopherol/μg protein were 0.219 (unmodified), 0.186 (18:1), 0.206 (18:3), 0.179 (20:4), and 0.199 (22:6). (B) Effect of supplementation of growth media with α-tocopherol acetate. 18:1 and 22:6 modified cells were incubated in media enriched with α-tocopherol acetate at concentrations from 0–100 μM for 24 h. Values are the mean and SE of at least three separate experiments. The concentrations of tocopherol expressed as ng tocopherol/μg protein in 18:1 enriched cells were 0.186 (0 μM tocopherol), 0.243 (12.5 μM tocopherol), 0.323 (25 μM tocopherol), 0.484 (50 μM tocopherol), and 0.868 (100 μM tocopherol); in 22:6 enriched cells the concentrations of tocopherol expressed as ng tocopherol/μg protein were 0.199 (0 μM tocopherol), 0.215 (12.5 μM tocopherol), 0.343 (25 μM tocopherol), 0.522 (50 μM tocopherol), and 0.848 (100 μM tocopherol). Additional studies at 200 and 400 μM (not shown) also showed no difference in 18:1 and 22:6 modified cells.

cules of α-tocopherol per PUFA molecule (≈2100 polyunsaturated fatty acid molecules per α-tocopherol) in mitochondrial membranes (28). Leedle and Aust studied lung and liver microsomal lipids and found that the ratio of tocopherol to PUFA was 0.008 (lung) and 0.0008 (liver) [PUFA/tocopherol 131 (lung) or 1235 (liver)]; furthermore, the ratio of tocopherol to PUFA in lung was less than necessary to inhibit peroxidation (0.003–0.004) of the same lipids in liposomes as measured by malondialdehyde production (19). Those liposomes with a higher α-tocopherol to polyunsaturated fatty acid ratio resisted lipid peroxidation. Tirmenstein and Reed estimated the presence of 1 molecule of tocopherol to 970 molecules of PUFA (ratio = 0.001) in isolated rat liver nuclei, and a threshold was identified above which was an inhibition of NADPH-induced lipid peroxidation (29), but the extent of polyunsaturation was not experimentally varied in that study. However, these studies did not measure *bis*-allylic positions. Cheeseman *et al.* found a ratio of α-tocopherol to *bis*-allylic methylene positions of 0.00076 in normal intact liver and 0.00318 in Yoshida hepatoma cells (30). The ratio was also higher in the Novikoff hepatoma (31), suggesting that the higher ratio is associated with the malignancy. Our report taken together with these studies seems to confirm the importance of the tocopherol to *bis*-allylic positions ratio as an additional determinant of oxidative vulnerability. Regardless of the exact cause, it can be concluded that L1210 cells that contain fatty acids which are highly polyunsaturated, have a much lower ratio of tocopherol molecules to *bis*-allylic hydrogen positions. This would seem to make them more vulnerable to oxidative attack.

Oxidative Stress Consumes Cellular Tocopherol

Cells that are more susceptible to oxidative stress due to an increased number of *bis*-allylic positions would be expected to demonstrate a decline in tocopherol to a greater extent than less unsaturated cells when presented with a stress. Indeed, when we exposed L1210 cells that were enriched with either 18:1 or 22:6 to the oxidative stress posed by 100 μM Fe^{2+}, we observed a dramatic difference in the rate and extent of tocopherol disappearance (Fig. 4). The cells modified with 18:1 showed no significant difference over time from control. The 22:6 cells with no added Fe^{2+} were not significantly different from the 18:1 cells. However, the 22:6 modified cells that were exposed to 100 μM Fe^{2+} lost their tocopherol at a higher rate than the others. Tocopherol depletion in the 22:6 modified cells was complete by 15 min. This is consistent with the oxidation-associated tocopherol depletion being dependent upon the number of *bis*-allylic positions contained in the cellular lipids.

Oxidation chemistry within the cell has previously been reported to result in a decrease in cellular tocopherol content especially when cells contain appreciable PUFA. For example, mitochondrial tocopherol levels decrease when iron is present in the medium (32). Tocopherol is oxidized more rapidly by linoleic micelles than laurate micelles in the presence of iron (33). When incorporated into egg yolk phosphatidyl choline lipo-

TABLE II

Contents of α-Tocopherol and *Bis*-Allylic Positions per Cell and the Derived Ratios

	Unmod	18:1	18:3	20:4	22:6
α-Tocopherol molecules/cell ($\times 10^{-7}$)	3.28 ± 1.0	2.78 ± 1.2	3.08 ± 0.7	2.68 ± 1.6	3.00 ± 2.3
bis-allylic positions/cell ($\times 10^{11}$)	0.68 ± <0.1	0.25 ± <0.1	2.07 ± 0.3	2.93 ± 0.6	2.97 ± 0.6
α-Tocopherol:*bis*-allylic ratio ($\times 10^{3}$)	0.482	1.112	0.149	0.091	0.101
bis-allylic:α-Tocopherol ratio	2075	899	6711	10989	9901

Note. Values are mean ± SE of at least three independent determinations.

somes, which contain PUFA, tocopherol is oxidized more rapidly by xanthine/xanthine oxidase than when incorporated into dimyristoyl liposomes (34). The same is true *in vivo*. Rats eating omega-3 polyunsaturated diets had lower cell tocopherol content in liver and heart than animals eating linoleic acid (35). Similarly, Chautan and co-workers varied the omega-6 to omega-3 ratio in the diet of rats and found that the omega-3 fatty acids in the diet were associated with lower levels of tocopherol in the liver and blood (36). Therefore, there is evidence that tocopherol levels in cells and tissues decrease when conditions for heightened oxidation are present.

In our studies, the cells modified with 22:6 demonstrated a greater than 10-fold decrease in their tocopherol molecule:*bis*-allylic position ratio as compared to cells modified with 18:1. This would seem to indicate that the 22:6 cells would be at least 10 times more vulnerable to an oxidative stress that leads to lipid peroxidation. Indeed, as measured by the depletion of cellular tocopherol upon exposure to Fe^{2+}, the 22:6 modified cells displayed more than 10-fold less tocopherol over time. Therefore, cells with more lipid polyunsaturation, e.g., a higher MBI, need more cellular tocopherol to protect the oxidatively vulnerable PUFA.

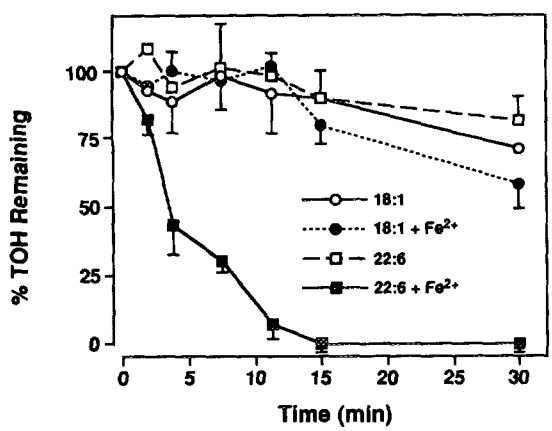

FIG. 4. Effect of oxidative stress on cellular α-tocopherol. L1210 (5×10^7) cells were enriched with 22:6 or 18:1 by adding 32 μM of the fatty acid to the growth media for 48 h. During the final 24 h, 12.5 μM tocopherol acetate was added. The enriched cells were washed and then incubated in 0.9% NaCl with or without 100 μM Fe^{2+} and the cellular tocopherol determined at the time points shown. Shown are the levels as a percentage of time zero and values are the mean and SE of three determinations. The values at time zero were 18:1, 1.84 ± 0.6 μg/10^8 cells; 18:1 + Fe^{2+}, 1.48 + 0.4 μg/10^8 cells; 22:6, 1.70 + 0.1 μg/10^8 cells; and for 22:6 + Fe^{2+}, 2.0 + 0.5 μg/10^8 cells.

In summary, we have optimized a tocopherol assay and used it to study cultured cell tocopherol content. From this study we conclude that most cells grown in tissue culture are relatively deficient in α-tocopherol and that this deficiency should be taken into account when performing experiments that involve oxidative mechanisms. We also conclude that when the L1210 cell is modified to produce a more polyunsaturated membrane, this modification results in a decrease in the ratio of α-tocopherol:*bis*-allylic positions. This lower ratio produces an increased vulnerability to an oxidative stress as measured by tocopherol depletion. These observations may help understand the etiology of diseases with an oxidative mechanism. These data lead us to suggest that manipulation of fatty acid content and/or tocopherol content could increase the efficacy of treatments based on oxidative mechanisms. Finally, further attention should be paid to *in vitro* tocopherol levels, especially when investigations are compared to or used to design *in vivo* experiments.

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