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Essential Fatty Acid Deficiency in Cultured Human Keratinocytes Attenuates Toxicity Due to Lipid Peroxidation[1,2]

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Human keratinocytes are commonly grown in culture with a serum-free medium. Under these conditions, keratinocytes become essential fatty acid deficient (EFAD), as determined by gas chromatographic analysis of cell phospholipid fatty acid composition. Exposure of EFAD keratinocytes for 2 hr to concentrations of *t*-butyl hydroperoxide (tBHP) up to 2 mM did not result in toxicity assessed by lactate dehydrogenase (LDH) release and only a small indication of lipid peroxidation assessed by the release of thiobarbituric acid-reactive substances (TBARS). Addition of 10 μM linoleic acid (LA) to serum-free medium alleviated the EFAD condition by increasing the phospholipid content of LA and its elongation and desaturation products, arachidonic acid and docosatetraenoic acid. Exposure of LA-supplemented keratinocytes to tBHP resulted in significant LDH (at 1 and 2 mM tBHP) and TBARS (tBHP concentration dependent) release. TBARS release was also significantly elevated in unexposed LA-supplemented keratinocytes (basal release). Cosupplementation with the antioxidant, α-tocopherol succinate (TS) prevented tBHP (1 mM)-induced LDH release in LA-supplemented cultures. TS supplementation also attenuated the effect of tBHP on TBARS release, but when compared to TS-supplemented EFAD cultures, LA supplementation still led to increased tBHP-induced TBARS release. Keratinocyte cultures are potentially useful as an alternative to animals in toxicology research and testing. It is important, however, that the cell model provide a response to toxic insult similar to that experienced *in vivo*. Our results suggest that fatty acid and antioxidant nutrition of cultured keratinocytes are important parameters in mediating the toxic effects of lipid peroxidation.

The growth and maintenance of human keratinocyte cultures commonly involves the use of a serum-free modified MCDB-153 medium that is supplemented with insulin, epidermal growth factor, hydrocortisone, and bovine pituitary extract (Boyce and Ham, 1983). This medium supports optimal cell growth and minimizes terminal differentiation of the cells. We sought to use keratinocyte cultures to investigate chemically induced oxidative stress and resulting toxicity. In preliminary experiments, we found that keratinocytes grown under serum-free conditions were relatively resistant to the lipid peroxidizing and toxic effects of the model prooxidant *t*-butyl hydroperoxide (tBHP), and experienced negligible toxicity at concentrations as high as 2 mM. This was in contrast to reports of tBHP-induced cytotoxicity in other cultured mammalian cells at concentrations from 0.25 to 1 mM (Forman *et al.*, 1987; Masaki *et al.*, 1989; Ochi and Miyaura, 1989). An examination of the growth medium ingredients reveals the absence of the essential fatty acids linoleic and linolenic acid, except the trace amount that might be present in bovine pituitary extract. This absence should lead, after sufficient cell divisions, to a deficiency of these fatty acids and the polyunsaturated fatty acids derived from them. Linoleic acid is of particular importance because it is the starting material for biosynthesis of arachidonic acid, which is metabolized by cells to bioactive eicosanoids (Ziboh and Miller, 1990). Esterified arachidonic acid in cell membranes would also be a major substrate for nonenzymatic lipid peroxidation resulting from xenobiotic toxicity or oxidative stress (Halliwell and Gutteridge, 1989). In the presence of oxygen, the reaction of free radicals with polyunsaturated fatty acids (PUFA) can yield reactive peroxyl free radicals. Peroxyl free radicals can either further propagate the peroxidation of lipids or, in the case of fatty acids with three or more double bonds, undergo reactions leading to fragmentation. Lipid peroxidation compromises the integrity of cell membranes and is thought to be involved in the mechanism of toxic action for some chemicals (Horton and Fairhurst, 1987).

tBHP might be expected to exhibit weak toxic activity in essential fatty acid deficient keratinocytes, due to the rela-

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[2] Mention of product or company name does not constitute endorsement by the National Institute for Occupational Safety and Health.

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tive absence of PUFA to serve as a substrate in lipid peroxidation. Studies with porcine endothelial cells cultured in serum-supplemented medium have shown that supplemental polyunsaturated fatty acids enhance, whereas saturated and monoenoic fatty acids inhibit, lipid peroxidation and toxicity following hydrogen peroxide exposure (Hart *et al.*, 1991). Human keratinocytes cultured in serum-supplemented culture have been shown to be capable of converting exogenously supplied linoleic acid into arachidonic acid (Isseroff *et al.*, 1987). Therefore, linoleic acid supplementation of essential fatty acid-deficient keratinocytes should reverse their deficient state and could make them more susceptible to tBHP-induced lipid peroxidation and toxicity. The purpose of this investigation was to determine the ability of tBHP to induce lipid peroxidation and toxicity in human keratinocytes grown in serum-free medium and the influence of supplementation of the medium with linoleic acid. The fatty acid content of human keratinocytes was determined after growth in serum-free medium and after supplementation with linoleic acid.

MATERIALS AND METHODS

Reagents. Fatty acid-free bovine serum albumin (crystallized and lyophilized), tBHP, α-tocopherol succinate, 2-thiobarbituric acid (TBA), tetraethoxypropane, ethylenediamine tetraacetic acid (EDTA), trichloroacetic acid, butylated hydroxytoluene (BHT), Triton X-100, sodium dodecyl sulfate, and diheptadecanoyl phosphatidylcholine were purchased from Sigma Chemical Company (St. Louis, MO). Linoleic acid and fatty acid methyl ester standards were from Nu-Chek-Prep (Elysian, MN). All organic solvents were HPLC grade or equivalent and purchased from Fisher Scientific (Cincinnati, OH).

Cell cultures and incubations. Cryopreserved primary human epidermal keratinocytes, basal medium, and growth supplements were obtained from Clonetics Corp. (San Diego, CA). Keratinocyte growth medium, also obtained from Clonetics Corp, was a modified MCDB-153 supplemented with bovine pituitary extract (0.4%, v/v), insulin (5 μg/ml), epidermal growth factor (0.1 ng/ml), hydrocortisone (0.5 μg/ml), and gentamicin/amphotericin-B. Biosafety guidelines recommended by Centers for Disease Control (HHS Publication No. 84-8395, 1984) were followed. One week after cultures were initiated, keratinocytes were subcultured into 35 $\times$ 10-mm dishes for lipid peroxidation and toxicity assays and 100 $\times$ 25-mm dishes for phospholipid fatty acid analysis at 5 to 10 $\times$ 10^3 cells/cm^2. After 3 days, the medium was replaced with growth medium with or without linoleic acid (10 μM) and α-tocopherol succinate (2 μM) supplementation. Linoleic acid was complexed to fatty acid-free bovine serum albumin (2:1 molar ratio) to facilitate its uptake. Keratinocytes were then grown for an additional 4 days, with one change of medium after 2 days, prior to use in lipid peroxidation and toxicity experiments. Experiments were also conducted to determine if short-term cultures retained sensitivity to a prooxidant. Cryopreserved first-passage keratinocytes were thawed and seeded into wells of a 24-well plate at approximately 1 $\times$ 10^4 cells/cm^2. Linoleic acid-supplemented cultures did not receive linoleic acid on the day they were thawed (Day 0) and were supplemented with 1 μM on Day 1, 2 μM on Day 2, and 10 μM on Day 3. This supplementation regimen was used to maintain the ratio of linoleic acid to 10^5 cells between approximately 0.2 to 0.5, and avoided any linoleic acid-induced growth reduction or toxicity. On Day 4, the cells were exposed to 2 mM tBHP for 2 hr and the release of thiobarbituric acid-reactive substances (TBARS) and lactate dehydrogenase (LDH) measured.

In experiments involving analysis of phospholipid fatty acid content, linoleic acid supplementation of cultures was the same as that for lipid peroxidation and toxicity experiments. In addition, another group was included in which linoleic acid (10 μM) supplementation was started 3 days after cultures were initiated and lasted for 10 days.

tBHP (1 mM) exposure buffer was prepared in a low calcium (150 μM) Hanks' balanced salt solution (HBSS). For lipid peroxidation and toxicity experiments, keratinocytes were washed twice with HBSS and tBHP exposure buffer (1 ml for 35-mm dishes or 0.25 ml for 24-well plates) was added. Incubations were at 37°C in an atmosphere of 95% air/5% CO_2 for 2 hr. Incubations were terminated by transfer of the incubation buffer to a microcentrifuge tube on ice and addition of 1 ml 0.3 N NaOH/0.1% SDS to the culture dish to lyse the cells for total protein analysis using the BioRad Protein Reagent (Bio-Rad Laboratories, Richmond, CA). The incubation buffer was centrifuged at 1000*g* for 10 min at 4°C to pellet cellular material. Aliquots of the supernatant were utilized for assays of lipid peroxidation and toxicity.

Lipid peroxidation and toxicity assays. The release of TBARS into the incubation buffer was measured by the method of Yagi (1976) as modified by Casini *et al.* (1982). Preliminary work indicated that under our experimental conditions virtually all the TBARS were released into the incubation buffer. An equal volume of cold trichloroacetic acid (12.5%) was added to an aliquot (0.75 ml for 35-mm dishes and 0.15 ml for 24-well plates) of the incubation medium, and centrifuged at 1000*g* for 10 min at 4°C. An aliquot was taken and added to a tube containing an equal volume of TBA reagent (0.6% TBA, 0.01% BHT, 1 mM EDTA), capped, and heated at 100°C for 20 min. The mixture was allowed to cool and TBARS extracted with 1-butanol. Fluorescence was determined in a Perkin–Elmer LS-50 Spectrofluorometer with excitation wavelength of 520 nm and emission wavelength of 553 nm. Tetraethoxypropane was dissolved in 0.01 N HCl to produce malondialdehyde, and this was used to generate a TBARS standard curve. The data are expressed as nmoles TBARS per milligram protein.

LDH activity was measured spectrophotometrically employing a kit from SIGMA Chemical Co. (Procedure No. 228-UV) that is based on the method of Amador *et al.* (1963). The oxidation of lactate was measured through the coreduction of NAD by following the absorbance change at 340 nm with a Gilford Response spectrophotometer. Total LDH activity was measured in three to four dishes of control and linoleic acid-supplemented groups following a 10-min incubation in 0.2% Triton X-100 in HBSS at 37°C. LDH activity is expressed as percentage of total LDH activity.

Phospholipid fatty acid analysis. Lipids were extracted from keratinocyte cultures in 100-mm dishes as described previously (Wey *et al.*, 1986) with the exception that the proportions of chloroform/methanol/acetic acid/water described by Bligh and Dyer (1959) were used. Prior to addition of the internal standard diheptadecanoyl-phosphatidylcholine, aliquots of the lipid extract were taken for determination of lipid phosphorus by the method of Chen (1956). The lipid extract was spotted on aluminum-backed 250-μm silica gel G plates (Whatman, Hillsboro, OR) under a gentle stream of nitrogen. The samples were then exposed to HCl fume for 5 min to hydrolyze 1-*O*-alkenyl bonds of ether(alkenyl)-linked phospholipids and yield the corresponding lysophospholipids. This step eliminated the formation of dimethyl acetals during transesterification. Plates were developed using a mobile phase of petroleum ether/diethyl ether/acetic acid (70/30/1, v/v/v). Under these conditions, the phospholipids and lysophospholipids remain at the origin and neutral lipids and free fatty acids migrate up the plate. After scraping the origin into a glass tube, methanolic HCl (0.6 N) was added and the sample heated at 75°C for 1 hr to transesterify fatty acids to methyl esters (FAME). FAME were extracted into hexane, and the hexane extract was washed once with deionized water and passed over anhydrous sodium sulfate. FAME were separated and quantitated by capillary gas chromatography on a fused silica column (0.32 mm by 30 m, 0.2 μm SP-2330, Supelco Inc., Bellefonte, PA). Injections were made in the

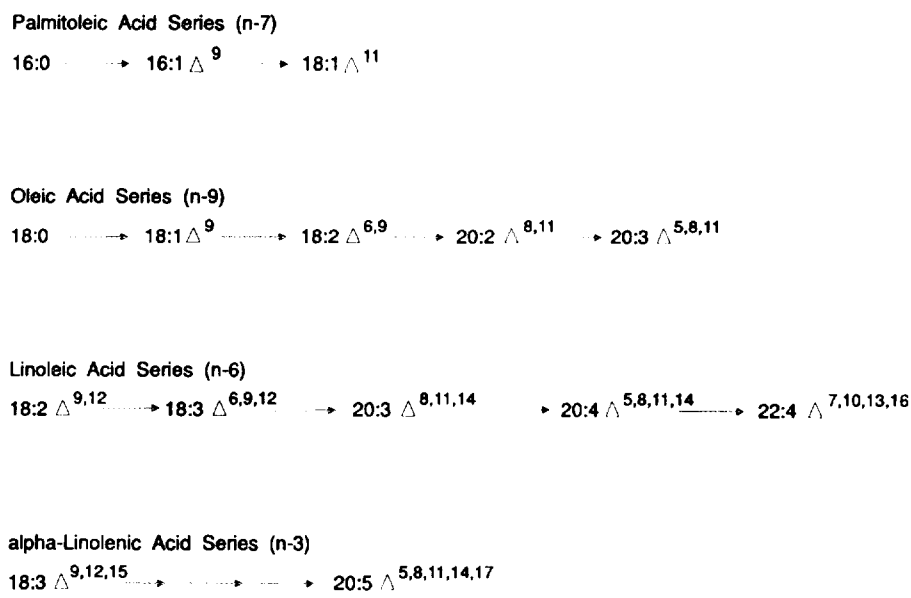

FIG. 1. Unsaturated fatty acid series of mammalian cells. Fatty acids are represented (i.e., $18{:}2\Delta^{9,12}$) as the number of carbons; :, the number of double bonds; Δ, the position of the double bonds from the carboxyl end. Each series is distinguished by the position of the first double bond from the methyl end of the acyl chain, as indicated by the number after *n* in parentheses. Linoleic and α-linolenic acid are essential nutrients and therefore required in the diet of mammals.

split mode (100:1) and oven temperature programmed from 170 to 185°C at 1°C/min and then to 240°C at 3°C/min. Eluting FAME were detected by flame ionization and peak areas electronically integrated. Authentic standards (Nu-Chek-Prep) were used to identify FAME peaks by coelution and to determine their relative detector response factors for use in quantitation. FAME mass was determined as the ratio of FAME peak area to internal standard (methyl heptadecanoate) peak area times the amount of internal standard added. The molar amount of FAME was calculated and normalized by the molar amount of phospholipid as determined by phosphorus analysis. These calculations were only performed for authentic standard-identified FAME. In the case of the essential fatty-deficient keratinocytes, a number of unidentified peaks were observed. Quantitative changes in these peaks are expressed as percentages of total peak area.

Statistical analysis. Statistical significance was determined using one- or two-way analysis of variance and the Scheffe range test. Commercial software (Statgraphics, version 5.0, STSC, Inc., Rockville, MD) was used to perform the analyses.

RESULTS

Phospholipid Fatty Acid Composition

The modified MCDB-153 growth medium commonly used for serum-free culture of keratinocytes does not contain the essential fatty acids, linoleic acid and linolenic acid. As the keratinocytes multiply they become deficient in these fatty acids and their products. To determine the extent of essential fatty deficiency and the ability of linoleic acid supplementation to reverse this condition, the fatty acid composition of keratinocyte phospholipids was analyzed under three conditions: (1) in keratinocytes grown under normal serum-free conditions for 13 to 14 days; (2) in keratinocytes grown in serum-free medium for 10 days and then supplemented with 10 μM linoleic acid bound to fatty acid-free serum albumin for an additional 4 days; (3) in keratinocytes grown in serum-free medium for 3 days prior to supplementation with 10 μM linoleic acid bound to fatty acid-free serum albumin for an additional 10 days. PUFA can be classified into four major series based on the position of the double bond nearest to the terminal methyl carbon of the hydrocarbon chain as shown in Fig. 1. In an essential fatty acid deficiency, biosynthesis of PUFA of the *n*-9 series increases to compensate for the absence of *n*-6 and *n*-3 series PUFA and is used as a marker of the condition (Ziboh and Miller, 1990). Under serum-free conditions, there was nearly a complete absence of *n*-6 and *n*-3 PUFA in keratinocytes (Fig. 2 and Table 1). There were a number of peaks that could not be identified with authentic standards and whose relative size decreased following linoleic acid supplementation. These unidentified peaks, particularly 10, 11, 12, and 13 in Fig. 2, may be oleic acid series (*n*-9) PUFA and result from the essential fatty acid deficiency. Although this interpretation is speculative, it is supported by the progressive reduction in relative size of peaks 10–13 as linoleic acid supplementation is initiated earlier and is present for a longer time (Figs. 2B and 2C). Thus, cells not supplemented with linoleic acid appear to become essential fatty acid deficient (EFAD cells). The fatty acid profiles indicate that linoleic acid is quickly elongated and desaturated to 20:3*n*-6, 20:4*n*-6 (arachidonic acid) and 22:4*n*-6. Keratinocytes grown in serum-free medium containing fatty acid-free albumin (5 μM) possessed a phospho-

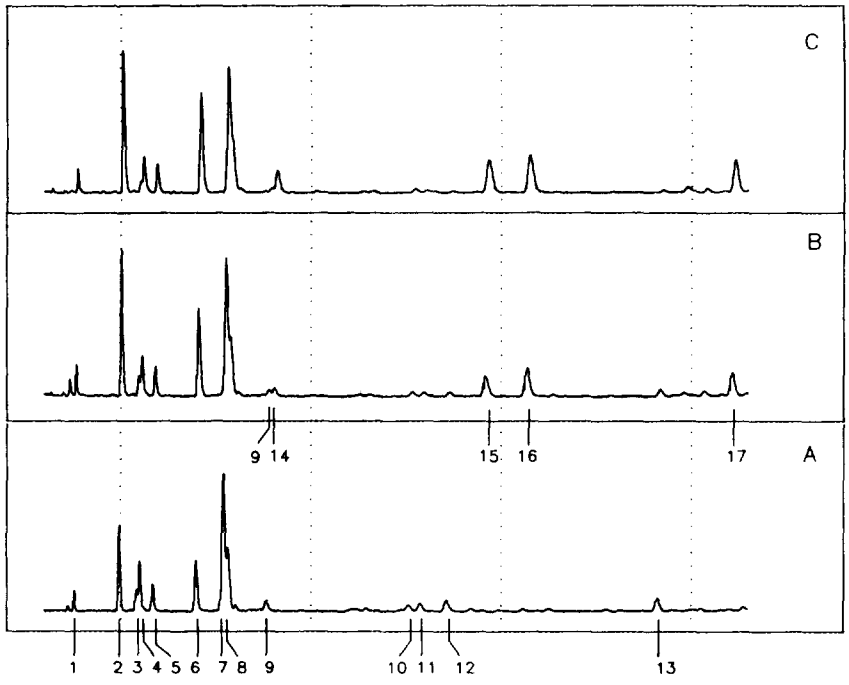

FIG. 2. Representative chromatograms of fatty acid methyl esters derived from cell phospholipids for each of the linoleic acid supplementation groups. (A) Keratinocytes maintained in unsupplemented medium for 14 days. (B) Keratinocytes maintained in unsupplemented medium for 10 days and linoleic acid (10 μM)-supplemented medium for 4 days. (C) Keratinocytes maintained in unsupplemented medium for 3 days and linoleic acid (10 μM)-supplemented medium for 10 days. The following peaks were identified using authentic standards: 1, 14:0; 2, 16:0, 4, 16:1(*n*-7); 5, 17:0; 6, 18:0, 7, 18:1(*n*-9); 8, 18:1(*n*-7); 14, 18:2(*n*-6); 15, 20:3(*n*-6); 16, 20:4(*n*-6); 17, 22:4(*n*-6). Peaks 3 and 9–13 were unidentified. For the purpose of comparison, the ordinate of the chromatogram in each panel has been scaled such that the peak area of the internal standard, 17:0, is identical.

lipid fatty acid profile essentially identical to unsupplemented controls (Table 1).

Toxicity and Lipid Peroxidation

Initially, tBHP-induced TBARS and LDH release were assessed in third-passage keratinocyte cultures after growth for 4 days in linoleic acid-supplemented medium versus serum-free medium. Supplementation with linoleic acid resulted in a significant elevation in the basal (unstimulated) release of TBARS (Table 2). The interexperimental variation for basal and tBHP-stimulated TBARS release possibly reflects the initial PUFA status of the cells and the degree of *n*-6 PUFA replenishment. As a result, unstimulated levels of TBARS release, obtained from corresponding controls in the same experiment, were subtracted from the total TBARS of tBHP-stimulated cultures to yield a net TBARS release due to tBHP exposure (Fig. 3). In EFAD cells, there was a small concentration-dependent increase in TBARS, but no LDH release at any of the tBHP concentrations. In linoleic acid-supplemented cells, the tBHP concentration-dependent increase in TBARS was significantly enhanced when compared to EFAD cells and also resulted in significantly increased LDH release. The tBHP-stimulated release of TBARS and LDH in keratinocytes grown in serum-free medium containing fatty acid-free albumin (5 μM) was essentially identical to unsupplemented controls (data not shown). In the long-term cultures, there was no statistically significant difference in total protein recovered from linoleic acid-supplemented cultures compared to unsupplemented cultures. To determine if the observed effects could be due to an extreme depletion of essential PUFA as a result of long-term culture (13–14 days), an assessment of the toxicity of tBHP on short-term cultures (4 days) was performed. Cryopreserved first-passage keratinocytes were thawed and allowed to grow without supplementation for 1 day and then supplemented with increasing concentrations of linoleic acid for 3 days. Exposure of these cultures to tBHP (2 mM) for 2 hr yielded results similar to those described for "long term" cultures. TBARS release was significantly greater ($p < 0.05$) for linoleic acid-supplemented cultures (nmole TBARS/mg protein, means ± SD: 1.6 ± 0.2, N = 12, tBHP-linoleic acid; 0.9 ± 0.05, N = 12, tBHP-control), and was associated with a significantly increased LDH release ($p < 0.05$) in linoleic acid-supplemented cells (% of total LDH, means ± SD: 31 ± 9%, N = 12, tBHP-linoleic acid; 2 ± 2%, N = 12, tBHP-control). In these short-term cultures, the total protein of linoleic acid-supplemented cultures was significantly greater ($p < 0.05$) than unsupplemented cultures (mg protein per well, means ± SD: 0.20 ±

TABLE 1
Effect of Linoleic Acid Supplementation on Phospholipid Fatty Acid Composition of Cultured Human Keratinocytes[a]

Fatty acid methyl ester[c]	Unsupplemented (n = 10)	FAF-BSA[d] (n = 4)	Linoleic acid supplemented[b] 4 Days (n = 5)	Linoleic acid supplemented[b] 10 Days (n = 4)
14:0	47 ± 8	40 ± 4	49 ± 9	30 ± 8[e]
16:0	223 ± 30	232 ± 7	302 ± 18[e]	192 ± 36[f]
16:1(n − 7)	174 ± 28	175 ± 10	121 ± 8[e]	72 ± 11[e,f]
18:0	237 ± 66	202 ± 9	297 ± 55	254 ± 52
18:1(n − 9)	772 ± 245	682 ± 24	610 ± 101	392 ± 60[e]
18:1(n − 7)	171 ± 154	256 ± 172	84 ± 116	ND[g]
18:2(n − 6)	ND	ND	60 ± 17[e]	79 ± 19[e]
20:3(n − 6)	7 ± 9	8 ± 9	142 ± 15[e]	160 ± 37[e]
20:4(n − 6)	22 ± 18	27 ± 5	181 ± 48[e]	184 ± 42[e]
22:4(n − 6)	ND	ND	135 ± 40[e]	158 ± 33[e]
Total	1653 ± 223	1623 ± 177	1981 ± 247	1521 ± 286

[a] Data are nanomoles fatty acid methyl ester per micromoles lipid phosphorus and are listed as means ± SD. Statistical significance was determined using analysis of variance and comparison of means using the Scheffe range test.
[b] Cells were grown in medium supplemented with 10 μM linoleic acid for the time indicated. Total lipids were extracted and phospholipids separated as described under Materials and Methods.
[c] Only fatty acids for which authentic standards were available are listed.
[d] Fatty acid-free bovine serum albumin (5 μM) added to medium.
[e] $p < 0.05$, compared to the unsupplemented group.
[f] $p < 0.05$, compared to the 4 days group.
[g] ND denotes not detected.

0.04, N = 11, linoleic acid supplemented; 0.13 ± 0.02, N = 12, unsupplemented). This evidence for an apparent growth stimulation by linoleic acid supplementation was unexpected and will require further study. Due to the low numbers of cells in these cultures, a determination of phospholipid fatty acid content was not possible.

Cosupplementation with 2 μM α-tocopherol and linoleic acid for 4 days resulted in an inhibition of TBARS release by 1 mM tBHP (Table 3). However, α-tocopherol supplementation did not completely reverse the effect of linoleic acid supplementation. α-Tocopherol supplementation completely eliminated the increased LDH release caused by 1 mM tBHP in linoleic acid supplemented cells (% of total LDH, means ± SD: 15 ± 5%, tBHP-linoleic acid; 6 ± 1%, tBHP-linoleic acid–α-tocopherol; 6 ± 1%, linoleic acid).

TABLE 2
Effect of Linoleic Acid Supplementation on Basal Release of Thiobarbituric Acid-Reactive Substances

Linoleic acid supplementation[b]	TBARS[a] Experiment 1 (N = 3)	Experiment 2 (N = 4)	Experiment 3 (N = 5)
−	0.011 ± 0.003	0.046 ± 0.012	0.072 ± 0.021
+	0.083 ± 0.022	0.069 ± 0.009	0.103 ± 0.034

[a] Data are nanomoles thiobarbituric acid-reactive substances (TBARS) per milligram protein and are listed as means ± SD. The effect of linoleic acid supplementation was tested for statistical significance by two-way ANOVA, $p < 0.001$.
[b] Cells were grown in medium supplemented with 10 μM linoleic acid for 4 days.

DISCUSSION

The fatty acid composition of cultured cells is influenced by the fatty acid composition of culture medium and serum supplements (Spector and Yorek, 1985). Significant changes in fatty acid composition on establishment of primary cell cultures have been described for human endothelial cells (Lagarde *et al.*, 1984), human tracheal epithelial cells (Alpert and Walenga, 1991), mouse hepatocytes (Ruch *et al.*, 1989), and murine keratinocytes (Isseroff *et al.*, 1985). In all these cases, PUFA of the n-6 series are reduced. This may be due to a lower relative and absolute amount of n-6 PUFA available in the culture medium than was available in the native tissue. There may also be a greater utilization of n-6 PUFA in cultured cells through, for example, the enzymatic oxidation of arachidonic acid in eicosanoid biosynthesis and the nonenzymatic degradation of PUFA due to oxidative stress. Our results indicate that keratinocytes grown in the serum-free medium of the present study become essential fatty acid deficient. Furthermore, this defi-

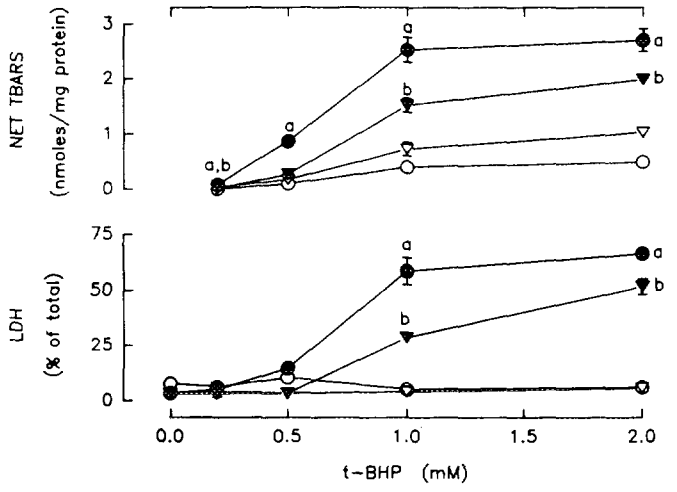

FIG. 3. Release of lactate dehydrogenase (LDH) and thiobarbituric acid-reactive substances (TBARS) by human keratinocytes exposed to 1 mM *tert*-butyl hydroperoxide (tBHP). Cells were grown for 10 days in serum-free medium and then 4 days in serum-free medium with (closed symbols) or without (open symbols) linoleic acid (10 μM) supplementation. Results are shown for two experiments (experiment 1, ○; experiment 2, ▽). For each experiment, statistical comparisons were made between linoleic acid supplemented versus unsupplemented cultures at each tBHP concentration using ANOVA and the Scheffe range test ($p < 0.05$, a for experiment 1 and b for experiment 2). The vertical bars are the SD, and their absence indicates a value smaller than the symbol.

ciency leads to a relative resistance of the cells to tBHP-induced lipid peroxidation and toxicity as measured by the release of TBARS and LDH, respectively.

The resistance of keratinocytes cultured in serum-free medium to the toxic effects of tBHP appears to be due to the sparse amount of cellular PUFA as reflected by the phospholipid fatty acid composition (Fig. 2). Increasing the cellular content of PUFA through linoleic acid supplementation enhanced the sensitivity of keratinocytes to both the lipid peroxidizing and toxic effects of tBHP (Fig. 3). We found that even relatively short-term cultures of keratinocytes were more sensitive to tBHP when supplemented with linoleic acid. These short-term cultures would have gone through four to five population doublings. In the absence of any exogenous source of essential PUFA and assuming no metabolic utilization, the cell content of essential PUFA would be 3 to 6% of the starting value. This is an optimistic estimate since zero metabolic utilization is not a realistic assumption. Our results are consistent with a mechanistic role for lipid peroxidation in the killing of keratinocytes as has been described for rat hepatocytes (Masaki *et al.*, 1989). The findings of our study are also supported by results in porcine endothelial cells supplemented with pure saturated, monounsaturated, or polyunsaturated fatty acids (Hart *et al.*, 1991). In their study, endothelial cells supplemented with polyunsaturated fatty acids experienced enhanced lipid peroxidation (TBARS formation) and toxicity (LDH release) following hydrogen peroxide exposure. Supplementation of the endothelial cells with saturated and monounsaturated fatty acids resulted in less lipid peroxidation and toxicity than unsupplemented controls.

The status of cellular mechanisms that protect against oxidant damage are also important parameters influencing toxicity. Ruch *et al.* (1989), in a study of the effects of culture duration on hydrogen peroxide-induced mouse hepatocyte toxicity, found that decreased susceptibility of hepatocytes to hydrogen peroxide-induced toxicity was associated with decreased percentages of PUFA in cell lipids. However, cellular levels of reduced glutathione and α-tocopherol were increased and may have contributed to the effect. In another study, EFAD rabbit tracheal epithelial cells (Dennery *et al.*, 1990) were supplemented with Excyte III (Miles-Pentex, Kankakee, IL), a commercial mixture of cholesterol esters and phospholipid-rich lipoproteins, and their sensitivity to hyperoxic injury was assessed. This supplementation reversed the EFAD, but resulted in both less toxicity and lipid peroxidation. These results are in apparent contradiction to the results of our study. However, lipoproteins are the major route of α-tocopherol transport *in vivo* (Traber and Kayden, 1984), and the apparent protective effect *in vitro* may have been due to the presence of α-tocopherol in the lipoprotein fraction of Excyte III. In the present study, keratinocytes supplemented with linoleic acid and α-tocopherol released less than half the TBARS of EFAD keratinocytes when exposed to 1 mM tBHP. A comparison of α-tocopherol-supplemented EFAD cells to those supplemented with both α-tocopherol and linoleic acid (Ta-

TABLE 3
tert-Butylhydroperoxide-Induced Lipid Peroxidation in α-Tocopherol-Supplemented Human Keratinocytes[a]

	TBARS[b]	
Supplementation[c]	Control	tBHP-exposed
None	0.072 ± 0.021	0.566 ± 0.060[d,e]
α-Tocopherol (2 μM)	0.071 ± 0.010	0.117 ± 0.025[d,e]
Linoleic acid (10 μM)	0.103 ± 0.034	1.685 ± 0.327[d,e]
α-Tocopherol + linoleic acid	0.048 ± 0.018[f]	0.230 ± 0.083[d,e]

[a] Cultures were exposed to 1 mM *tert*-butyl hydroperoxide (tBHP) or buffered salt solution for 2 hr. Statistical significance was determined by ANOVA and the Scheffe range test.

[b] Values are the net release of thiobarbituric acid reactive substances (TBARS) expressed as nanomoles TBARS per milligram protein (means ± standard deviation, $N = 5$).

[c] Cells were grown in medium supplemented with α-tocopherol or linoleic acid for 4 days.

[d] $p < 0.05$, as compared to all other tBHP-exposed groups.

[e] $p < 0.05$, as compared to the corresponding unstimulated group.

[f] $p < 0.05$, as compared to the unstimulated linoleic acid-supplemented group.

ble 3) shows that with α-tocopherol supplementation the enhancement of TBARS release by linoleic acid supplementation was preserved, albeit at a lower level. The modified MCDB-153 growth medium used in our studies does not contain α-tocopherol. Therefore, EFAD keratinocytes should also be deficient in α-tocopherol and differences in cell α-tocopherol levels would be an unlikely explanation for the attenuation of peroxide-induced lipid peroxidation and toxicity in these cells. Reduced and oxidized glutathione were not measured in our study, and their potential role in the attenuation of lipid peroxidation remains to be determined.

Thus, sensitivity to the prooxidant effects of tBHP can easily be manipulated by nutritional supplementation. It remains to be determined whether supplementation conditions that yield fatty acid compositions identical to those found *in vivo* can be established. The results of studies on human epidermis (Chapkin *et al.*, 1986) indicate that exogenously supplied linoleic acid is not converted to arachidonic acid *in vivo*. However, this metabolic capability was observed in cultured keratinocytes by Isseroff *et al.* (1987) and in the present study. In keratinocytes supplemented with 10 μM linoleic acid for 10 days, the percentages of linoleic and arachidonic acid in phospholipids were 4.96 and 9.73, respectively. Published values for the composition of fatty acids in human epidermal phospholipids (Lampe *et al.*, 1983) or total lipids (Chapkin *et al.*, 1986) indicate that the percentage of linoleic acid is expected to exceed that for arachidonic acid. The reversal of the ratio of linoleic acid to arachidonic acid may be due to the induction of linoleic acid conversion to arachidonic acid by the isolation and culturing of cells. The PUFA composition of a medium supplement to restore the original fatty acid composition of cultured keratinocytes may be complex and will require further research.

Recently, cultured rodent and human keratinocytes have been used for studies on the mechanism of action of dermal toxicants (Hsieh and Acosta, 1991; Smith *et al.*, 1990; Picardo, *et al.*, 1992). In two of these studies keratinocytes were cultured with a source of essential PUFA and evidence for oxidative stress or lipid peroxidation was found. In a study of dithranol toxicity in rat keratinocytes, Hsieh and Acosta (1991) found evidence for involvement of reactive oxygen species, but did not make any measures of lipid peroxidation. The rat keratinocytes were cultured for 3 days in a serum-free medium containing bovine serum albumin. Albumin possesses bound free fatty acids and could have served as a source of linoleic acid. Picardo *et al.* (1992) cultured human keratinocytes on mouse fibroblast feeder layers with serum-free medium and found evidence for lipid peroxidation by the contact allergen, paraphenylenediamine. Lipid peroxidation was inferred from a reduction in the arachidonic acid to palmitic acid ratio of cell phospholipids. Although the fibroblast feeder layer was able to provide some essential PUFA, the ratio for untreated cells (0.2) was still relatively low compared to our findings for linoleic acid supplementation of 4 (0.6) and 10 days (0.96). It is possible that further replenishment of the essential PUFA content in the cell cultures used in the studies just described may have altered the concentration dependencies of the observed toxicities. Our study demonstrates that linoleic acid supplementation of keratinocytes grown in serum-free medium produces viable cell cultures with extreme differences in membrane PUFA content, and we suggest that this model may prove useful for future investigations of the contribution of lipid peroxidation to the toxicity of chemicals. Finally, human cell cultures (Frazier *et al.*, 1989), including human keratinocytes (Babich *et al.*, 1989; Segal *et al.*, 1990), have been promoted as potentially useful alternatives to animals in toxicological research and testing. Therefore, it is important that the cell model gives a response to toxic insult similar to that experienced *in vivo*. Our results suggest that fatty acid and antioxidant nutrition of cultured keratinocytes are important parameters in assessing susceptibility to lipid peroxidation and toxicity, particularly in cells grown in serum-free medium.

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