Antioxidant Balance and Free Radical Generation in Vitamin E-Deficient Mice after Dermal Exposure to Cumene Hydroperoxide

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Organic peroxides are widely used in the chemical industry as initiators of oxidation for the production of polymers and fiber-reinforced plastics, in the manufacture of polyester resin coatings, and pharmaceuticals. Free radical production is considered to be one of the key factors contributing to skin tumor promotion by organic peroxides. In vitro experiments have demonstrated metal-catalyzed formation of alkoxyl, alkyl, and aryl radicals in keratinocytes incubated with cumene hydroperoxide. The present study investigated in vivo free radical generation in lipid extracts of mouse skin exposed to cumene hydroperoxide. The electron spin resonance (ESR) spin-trapping technique was used to detect the formation of α-phenyl-*N*-*tert*-butylnitrone (PBN) radical adducts, following intradermal injection of 180 mg/kg PBN. It was found that 30 min after topical exposure, cumene hydroperoxide (12 mmol/kg) induced free radical generation in the skin of female Balb/c mice kept for 10 weeks on vitamin E-deficient diets. In contrast, hardly discernible radical adducts were detected when cumene hydroperoxide was applied to the skin of mice fed a vitamin E-sufficient diet. Importantly, total antioxidant reserve and levels of GSH, ascorbate, and vitamin E decreased 34%, 46.5%. 27%, and 98%, respectively, after mice were kept for 10 weeks on vitamin E-deficient diet. PBN adducts detected by ESR in vitamin E-deficient mice provide direct evidence for in vivo free radical generation in the skin after exposure to cumene hydroperoxide.

Introduction

Many peroxy compounds are a source of free radicals and are highly regarded as polymerization initiators. They are extensively used in chemical and pharmaceutical industries (*1*) as catalysts, intermediates, and raw materials for a number of products, e.g., reinforced plastics, rubber curing, finishing agents for acetate yarns, dental cements and restoratives, and treatment of acne. In the food industry, organic peroxides are used for bleaching flour, fats, oils, waxes, and milk, and preparation of certain cheeses (*2*−*4*).

Cutaneous exposure to organic hydroperoxides is known to cause delayed reaction in skin resulting in severe erythema, edema, and vesiculation (*5*). A number of hydroperoxides and dialkylperoxides used in industries are effective tumor promoters in mouse skin (*9*−*11*). Organic peroxide-induced lipid peroxidation was implicated as one of the essential mechanisms of toxicity in keratinocytes (*6*−*8*). Free radicals are considered to be one of the key factors contributing to skin tumor promotion by organic peroxides (*11*−*14*). Exposure of mouse keratinocytes and skin flaps in vitro to cumene hydroperoxide was shown to form metal-catalyzed alkoxyl, alkyl, and aryl radicals (*12, 13*). The fact that organic peroxides have a reputation for being potent skin tumor promoters and inducers of epidermal hyperplasia (*15*), their ability to trigger free radicals, may be critical for their carcinogenic properties.

The question can be raised as to whether oxidative stress occurs in vivo to yield free radicals in skin exposed to cumene hydroperoxide. It is well-known that susceptibility to lipid peroxidation is affected by levels of vitamin E in different tissues (*16*−*18*), including skin (*19*−*21*). The present study was designed to detect free radicals in the skin of mice treated with cumene hydroperoxide while given vitamin E-sufficient or vitamin E-deficient diets. We employed electron spin resonance (ESR) with a spin-trapping technique using α-phenyl-N-*tert*-butylnitrone (PBN) to detect the formation of radical adducts in lipid extracts from the skin of mice given topical application of cumene hydroperoxide.

Materials and Methods

Chemicals. Fatty acid-free human serum albumin (hSA), luminol, sodium dodecyl sulfate (SDS), 2,2′-dipyridyl (2,2′-DP), L-ascorbic acid, α-phenyl-*N*-*tert*-butylnitrone (PBN), cumene hydroperoxide (Cum-OOH), and glutathione were purchased from Sigma Chemicals Co. (St. Louis, MO). Cumene hydroperoxide-dimethyl-$^{13}C_2$ (^{13}C-Cum-OOH) was purchased from Cam-

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Table 1. Composition of Diets

typical analysis		chemical composition nutrients		minerals		vitamins	
protein, %	19.3			calcium, %	0.6	thiamin hydrochloride, ppm	20.0
fat, %	10.0	protein, %	19.3	phosphorus	0.4	riboflavin, ppm	20.0
fiber, %	4.3	fat, %	10.0	potassium, %	0.4	nicotinic acid, ppm	90.0
carbohydrate, %	60.6	fiber (crude), %	4.3	magnesium, %	0.065	pyridoxine hydrochloride, ppm	20.0
		carbohydrate, %	60.6	sodium, %	0.2	D-calcium pantothenate, ppm	60.0
ingregients		gross energy, kcal/g	4.1	chlorine, %	0.2	folid acid, ppm	4.0
casein-vitamin, free, %	21.00			fluorine, ppm	5.0	biotin, ppm	0.4
sucrose, %	5.00			iron, ppm	60.0	L-inositol, ppm	200.0
nonnutritive fiber (Solka-flok), %	3.00			zinc, ppm	20.0	vitamin B_{12}, μg/kg	20.0
corn oil, %	5.00			manganese, ppm	65.0	menadione dimethylpyrimidinol	
lard, %	5.00			copper, ppm	15.0	bisulfite, ppm	20.0
dextrin, %	43.65			cobalt, ppm	3.2	vitamin A acetate, IU/g	22.0
DL-methionine, %	0.15			iodine, ppm	0.6	vitamin D, IU/g	2.2
RP vitamin mixture, %	2.00			chromium, ppm	3.0	DL-α-tocopheryl	
chlorine chloride, %	0.20			molybdenum, ppm	0.8	acetate, IU/kg (sufficient diet)	50.0
RP mineral mixture #10, %	5.00			selenium, ppm	0.2	acetate, IU/kg (deficient diet)	<10.0
total, %	100.00						

bridge Isotope Laboratories, Inc. (Andover, MA). Metaphosphoric acid was purchased from Fisher Scientific Co. (Pittsburgh, PA). Methanol, ethanol, chloroform, hexane, and water (HPLC grade) were purchased from Aldrich Chemical Co. (Milwaukee, WI). ThioGlo-1 was obtained from Covalent Inc. (Wobum, MA). 2,2′-Azobis(2-aminodinopropane)-dihydrochloride (AAPH) was purchased from Wako Chemicals USA, Inc. (Richmond, VA).

Animals. Sixty-two female Balb/c AnN Hsd mice (3−4 weeks old) weighing 16−18 g were obtained from specific pathogen-free stocks at Harlan (Indianapolis, IN). Each mouse was housed in an individual ventilated cage under controlled environmental conditions in an AAALAC accredited facility. The animals were weighed biweekly, and the food consumption was recorded monthly.

Vitamin E-Deficient and Vitamin E-Sufficient Diets. Vitamin E-deficient and vitamin E-sufficient diets in pellets were purchased from Test Diet, Purina Mill (Richmond, IN). The sufficient Diet 5755 is a purified, synthetic diet that provides all the essential nutrients to support maintenance, growth, gestation, and lactation in laboratory mice and rats (Table 1). Vitamin E-deficient diet was based on the sufficient Diet 5755 from which vitamin E was removed. Mice were randomly divided into two groups with an equal number of mice in each. The first group was placed on the vitamin E-deficient diet, and the second was given the vitamin E-sufficient diet. Animals were daily supplied with fresh diets stored at 4 °C. The mice were kept on the diets for 10 weeks. Vitamin E deficiency in mice was validated by carrying out measurements of α-tocopherol in plasma and skin homogenates.

Blood and Skin Collections, and Preparations of Homogenate from Skin. The interscapular area of mousebacks was shaved the day before procedures. The area (1.5 × 2.0 cm^2) of mouse skin was evenly painted with Cum-OOH (12 mmol/kg in 100 μL of saline) or 100 μL of saline. Immediately after exposure to Cum-OOH, mice were sacrificed by inhalation of excess CO_2, and blood was promptly drawn by cardiac puncture. Blood was centrifuged, and plasma samples were stored at −80 °C. Skin flaps from the interscapular area of the back of mice (1.5 × 2.0 cm^2) were excised, and samples were taken for ESR studies and biochemical analysis. Skin for biochemical studies was immediately frozen at −80 °C until processed. The skin homogenates were prepared from frozen tissues with ice-cold phosphate-buffered saline (PBS, pH 7.4) using a tissue tearer (model 985-370, Biospec Products, Inc., Racine, WI).

HPLC Assay of α-Tocopherol. Extracts of α-tocopherol from skin homogenates were prepared using a procedure described by Lang et al. (*22*). A Waters HPLC system with a 717 auto sampler, a Hewlett-Packard ODS Hypersil column (5 mm, 200 × 4.6 mm), a Waters 600 controller pump, and a 474 fluorescence detector was used to measure α-tocopherol in samples. The wavelengths employed in the assay were 292 nm (excitation) and 324 nm (emission). Both the excitation and emission slits were 5 nm. Eluent was CH_3OH with the flow rate of 1 mL/min. Under these conditions, the retention time for α-tocopherol was 8.2 min. The minimum detection level of α-tocopherol in samples was 0.1 pmol/mg of protein. The data acquired were exported from the Waters 474 detector using Millennium 2000 software (Waters Assosiates, Milford, MA).

Fluorescence Assay of Glutathione (GSH) and Protein Sulfhydryls. Total thiol concentration in homogenates of skin was determined using ThioGlo-1, a maleimide reagent which produces a highly fluorescent product upon its reaction with SH− groups (*23*). A standard curve was established by addition of GSH (0.04−2.0 μM) to 0.1 M phosphate buffer (pH 7.4) containing 10 μM ThioGlo-1. GSH content was estimated by an immediate fluorescence response registered upon addition of ThioGlo-1 to a tissue homogenate. Total protein sulfhydryls were determined from the additional fluorescence response after the addition of SDS (4 mM) to the same homogenate. A Shimadzu spectrofluorophotometer RF-5000 U (Shimadzu, Japan) was

employed in the assay: excitation 388 nm and emission 500 nm. The data acquired were employed for determination using an excitation slit of 1.5 nm and an emission slit of 5 nm. The wavelengths are exported from the spectrofluorometer using RF-5000 U PC Personal Fluorescence software (Shimadzu, Japan). In a separate series of experiments, we tested the specificity of our ThioGlo-1-based protocol for measurements of GSH and protein cysteines in the homogenates from mouse skin. We performed ThioGlo-1 assays in samples before and after pretreatment with GSH-peroxidase/H_2O_2 (0.2 unit/mL/0.13 mmol/mL). We found that after pretreatment with GSH-peroxidase/H_2O_2, the response to ThioGlo-1 from low molecular weight thiols (in the absence of SDS) decreased by more than 95%, while that of protein SH groups (after addition of SDS) did not undergo any substantial changes (97% of that in the presence of GSH-peroxidase). Given that GSH-peroxidase specifically oxidizes GSH (*24*−*26*), we conclude that assaying with ThioGlo-1 quantitatively determines both GSH (in the absence of SDS) and protein SH groups (in the presence of SDS) in the homogenates from mouse skin.

Chemiluminescence Measurements of Total Antioxidant Reserve. A water-soluble azo-initiator, 2,2′-azobis(2-aminodinopropane)-dihydrochloride (AAPH), was used to produce peroxyl radicals (*27*). Oxidation of luminol by AAPH-derived peroxyl radicals was assayed by the chemiluminescence response. A delay in the chemiluminescence response caused by interaction of endogenous antioxidants with AAPH-derived peroxyl radicals was observed upon addition of homogenates. Based on the known rate of peroxyl radical generation by AAPH, the amount of peroxyl radicals scavenged by endogenous antioxidants was evaluated. The incubation medium contained 0.1 M phosphate buffer (pH 7.4) at 37 °C, AAPH (50 mM), and luminol (0.4 mM). The reaction was started by the addition of AAPH. Luminescent analyzer 633 (Coral Biomedical, Inc., San Diego, CA) was employed for determination.

Measurements of Lipid Peroxidation Products. Lipids were extracted from skin of Balb/c mice following the procedure of Folch et al. (*28*) using chloroform/methanol (2:1, v/v). The solvents were evaporated under nitrogen, and the dried lipids were dissolved in the methanol/hexane (5:1, v/v) mixture. The concentration of lipid hydroperoxides with conjugated dienes in the lipid extracts from skin was determined by measurements of absorbance at 233 nm using a molar extinction coefficient of 2.7×10^4 M^{-1} cm^{-1} (*29*). Accumulation of secondary lipid peroxidation products reacting with 2-thiobarbituric acid (TBARS) in skin homogenates was measured spectophotometrically by absorbance at 535 nm, as described by Buege and Aust (*30*). A molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} was used for calculations (*30*). Measurements were made using 2401-PC Shimadzu spectrophotometer interface with a Dell OptiPlex GX 400 personal computer.

Colorimetric Determination of Ascorbate. Ascorbate concentration in skin homogenates was determined using an L-ascorbic acid determination kit (catalog no. 409 677, Boehringer Mannheim, Germany). The sample and sample blank were each prepared by mixing 50 mL of skin homogenate with 1.0 mL of tetrazolium salt MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide]. An ascorbate oxidase was then added only to the sample blank. Both samples were incubated at 37 °C for 6 min, and the first set of absorbencies (*A*1) was measured at 578 nm. Then PMS (5-methylphenazinium methosulfate), 100 μL, pH 3.5, was added to the same tubes, and the absorbencies (*A*2) were measured after incubation for 15 min at 37 °C. The difference between the sample and sample blank was used to determine the concentration of L-ascorbic acid present in the skin homogenate. A spectrophotometer (Beckman DU640, Fullerton, CA) was used to measure the absorbency at 578 nm. A standard curve was prepared using L-ascorbic acid (Sigma, St. Louis, MO) and 1.5% metaphosphoric acid (Fisher Scientific, Pittsburgh, PA) at pH 4.0.

ESR Study of Generation of Free Radicals in Skin. The skin used in these experiments came from the interscapular shaved area of the back of mice. Equal amounts of the skin flap (1.5×2.0 cm) were collected to study exposure outcomes in control and experimental groups. Backs of mice were injected subcutaneously in the dorsal lumbar area with the spin trap α-phenyl-N-*tert*-butylnitrone (1 mmol/kg, 100 μL) dissolved in saline. Ten minutes after injection, the interscapular area of mouse back was painted with saline (100 μL) or Cum-OOH (12 mmol/kg, in 100 μL of saline). The skin flaps were collected 30 min after Cum-OOH application. Samples were minced and homogenized with 15 mL of chloroform/methanol (2:1 mixture) and 1.0 mL of 2,2′-dipyridyl (30 mM) to prevent oxidation during lipid extraction (*31*). The skin homogenates were then centrifuged (2000 rpm, 10 min, 4 °C), and the chloroform layer was collected. The lipid extracts from skin were dried under nitrogen. Finally, 1 mL of lipid extract from mouse skin was used to detect spin-trapped free radicals. ESR spectra were recorded immediately at room temperature using a quartz flat cell and a Bruker EMX with a Super High Q cavity. Instrumental settings were as follows: microwave power, 20 mW; modulation amplitude, 1.0 G; conversion time, 0.6 s; time constant, 1.3 s. Spectra were recorded on an IBM-compatible computer interfaced with the spectrometer. The determination of the hyperfine coupling constants of PBN radical adducts was done in chloroform/methanol. Authentic PBN radical adducts were prepared as follows and were extracted with 2:1 chloroform/methanol to obtain the relevant hyperfine coupling constants in that solvent. Authentic methyl radical adduct was prepared by adding Ti^{3+} (0.5 mM) to ^{13}C-labeled Cum-OOH (50 mM) in water containing 10 mM PBN. After addition of 1 mL of chloroform/methanol and vortex mixing, the solution was centrifuged for 5 min at 14000*g*, and the organic layer was removed for EPR spectroscopy. Authentic alkoxyl radical adducts [PBN/•OCH_3 and PBN/•$OC(CH_3)_2C_6H_6$] were produced by the addition of 3 mM $FeCl_3$ (dissolved in the corresponding alcohol) to a solution of PBN (100 mM in the corresponding alcohol). After a 10 min incubation at 37 °C, 20 mM diethylenetriaminepentaacetic acid (DTPA) was added to stop any further chemical reaction, and 1 mL of chloroform/methanol solution and 200 μL of H_2O were added to provide a sufficient phase transition. After vortexing, the mixtures were centrifuged for 5 min at 14000*g* in a microcentrifuge, and the organic layer was used for EPR spectroscopy. Hyperfine coupling constants were determined using the WinSim program of the NIEHS public EPR software tools package, which is available over the Internet (*http://EPR.niehs.nih.gov/*). The program was allowed to systematically vary the nitrogen and hydrogen hyperfine coupling constants and the relative concentrations of each species to achieve the best fit to the experimental spectra.

Protein Assay. Measurements of protein in homogenates from mouse skin were run using a Bio-Rad protein assay kit, catalog no. 500-0006 (Richmond, CA).

Statistics. Data were expressed as the mean ± SEM for each group. A one-way ANOVA test was employed to compare the responses between treatments. Statistical significance was set at $p < 0.05$.

Results

Body Weight and Food Intake. Body weight gain and food intake did not vary significantly between groups given the vitamin E-sufficient or vitamin E-deficient diets. The average food intake in mice given the vitamin E-sufficient and vitamin E-deficient diets was 1.4 ± 0.3 and 1.5 ± 0.2 g/mouse/day, respectively. After 10 weeks on their respective diets, average body weight in groups of mice given vitamin E-sufficient and vitamin E-deficient diets was 19.2 ± 0.4 and 20.4 ± 0.8 g, respectively.

Total Antioxidant Reserve of Skin Homogenates of Mice Exposed to Cum-OOH. Accumulation of biomarkers of oxidative stress occurs upon exposure to oxidative insult, when antioxidant defenses are compro-

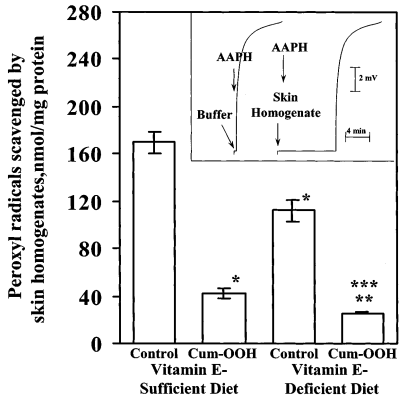

Figure 1. Total antioxidant reserve in the skin of Balb/c mice given vitamin E-sufficient or vitamin E-deficient diets, with or without topical exposure to Cum-OOH. Inset: Characteristic chemiluminescence response generated by AAPH with luminol in the presence and in the absence of skin homogenate. The inset is the original recording showing the measurements of total antioxidant reserve of the skin homogenates. In the presence of AAPH-derived peroxyl radicals, luminol is oxidized and produces a chemiluminescence response. Conditions: Mice were painted with 12 mmol/kg Cum-OOH in 100 μL of saline for 30 min. Values are means ± SEM of 8 mice. *$p < 0.05$ vs vitamin E-sufficient control; **$p < 0.05$ vs vitamin E-deficient control; ***$p < 0.05$ vs mice given vitamin E-sufficient diet and topically treated with Cum-OOH.

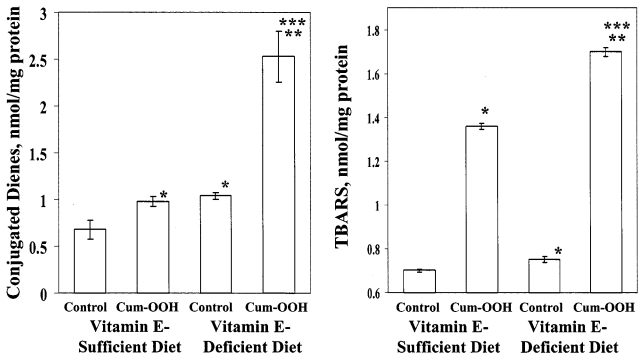

Figure 2. Accumulation of lipid peroxidation products (conjugated dienes and TBARS) in the skin of Balb/c mice given vitamin E-sufficient or vitamin E-deficient diets after exposure to Cum-OOH. Conditions: Mice were painted with 12 mmol/kg Cum-OOH, in 100 μL of saline for 30 min. Values are means ± SEM of 5 mice. *$p < 0.05$ vs vitamin E-sufficient control; **$p < 0.05$ vs vitamin E-deficient control; ***$p < 0.05$ vs mice given vitamin E-sufficient diet and topically treated with cumene hydroperoxide.

Table 2. Vitamin E Content in Animal Tissues

tissue	vitamin E-sufficient diet	vitamin E-deficient diet
skin (pmol/mg of protein)	98.5 ± 11.6	2.37 ± 0.74
plasma (nmol/mL)	7.8 ± 0.9	0.28 ± 0.05

mised. Therefore, we first investigated total antioxidant reserves of skin homogenates from mice exposed to Cum-OOH. We employed a luminol-enhanced chemiluminescence assay. We used a water-soluble azo-initiator, AAPH, to produce peroxyl radicals at a constant rate (*27*). Interaction of peroxyl radicals generated by AAPH with luminol in phosphate buffer (pH 7.4 at 37 °C) resulted in a characteristic chemiluminescence response (Figure 1, inset). Addition of skin homogenates to the oxidation system produced a lag period during which the chemiluminescence response was not observed. The duration of the lag period produced by homogenates from control (saline-exposed) mice was significantly greater than that observed in the presence of homogenates from mice treated with Cum-OOH (Figure 1). The lag period is caused by the competition of all endogenous skin antioxidants with luminol for AAPH-derived peroxyl radicals (*32*). Thus the luminol-enhanced chemiluminescence assay revealed a 34% decrease in the total antioxidant reserve of skin homogenates in untreated mice given vitamin E-deficient diet compared with those in vitamin E-sufficient diet (Figure 1). In addition, a significant 75% decrease in total antioxidant reserves of skin homogenates of mice kept on vitamin E-sufficient diet after topical exposure to 12 mmol/kg Cum-OOH (Figure 1) was observed. The antioxidant reserve of skin samples from vitamin E-deficient mice was reduced by 87% after topical treatment with the same concentration of Cum-OOH ($p < 0.05$).

Lipid Peroxidation in Skin of Mice Exposed to Cum-OOH. A significant increase in conjugated dienes and TBARS in the skin of vitamin E-deficient mice treated with Cum-OOH was seen (Figure 2). The primary (conjugated dienes) and secondary lipid peroxidation products (TBARS) in skin of vitamin E-deficient mice were increased 2.48- and 2.30-fold, respectively, above the levels seen in the control mice. In vitamin E-sufficient mice, the primary (conjugated dienes) and secondary lipid peroxidation products (TBARS) were increased 1.45- and 1.94-fold, respectively, above the levels of the corresponding controls (Figure 2). Using the molar extinction coefficient of 2.7×10^4 M^{-1} cm^{-1} for lipid hydroperoxides at 233 nm, we calculated that 0.30 and 1.54 nmol of lipid peroxides per milligram of protein were accumulated in skin of vitamin E-sufficient and vitamin E-deficient mice, respectively, following topical exposure to Cum-OOH.

Vitamin E Levels in Skin of Mice Given Vitamin E-Deficient and Vitamin E-Sufficient Diets after Topical Exposure to Cum-OOH. Vitamin E deficiency in mice was validated by measuring the level of α-tocopherol in plasma and skin homogenates. Following 10 weeks of vitamin E deprivation, sharp 98% and 96% decreases in the levels of vitamin E in skin and plasma, respectively, were detected (Table 2). A similar decrease of vitamin E in plasma was reported in male Wistar rats after 3 months of vitamin E deprivation; however, vitamin E in rat skin was below the detectable limit after 3 months of dieting (*21*). Topical exposure of mice given the vitamin E-sufficient diet to Cum-OOH (12 mmol/kg, in 100 μL of saline, for 30 min) resulted in a 99.7% decrease in the level of α-tocopherol in skin compared to the saline-treated control, whereas skin α-tocopherol levels were below the detectable level in mice maintained on vitamin E-deficient diet after Cum-OOH treatment (Figure 3).

GSH and Protein Thiols in Skin of Mice Given Vitamin E-Sufficient and Vitamin E-Deficient Diets after Cum-OOH Exposure. As cellular thiols are sensitive indicators of cell/tissue redox status and are directly involved in the reduction of peroxides, we measured levels of GSH and protein thiols after topical exposure to Cum-OOH in the skin of mice given vitamin E-deficient and vitamin E-sufficient diets. The addition of ThioGlo-1 to skin homogenates produced an instantaneous increase in fluorescence due to the formation of the GSH-ThioGlo-1 reaction products. After this, the intensity of the response did not change unless SDS was added to the incubation system to unfold protein SH− groups, at which point a slow increase of observed

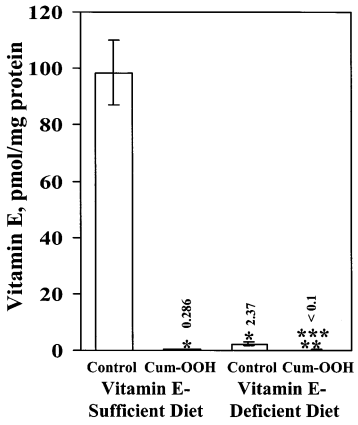

Figure 3. Level of vitamin E in the skin of Balb/c mice given vitamin E-sufficient or vitamin E-deficient diets, with or without topical exposure to Cum-OOH. Conditions: Mice were painted with 12 mmol/kg Cum-OOH, in 100 μL of saline for 30 min. Values are means ± SEM of 8 mice. *p < 0.05 vs vitamin E-sufficient control; **p < 0.05 vs vitamin E-deficient control; ***p < 0.05 vs mice given vitamin E-sufficient diet and topically treated with Cum-OOH.

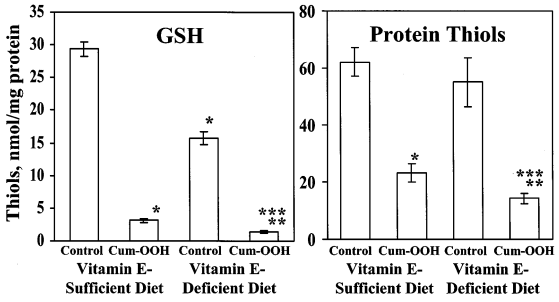

Figure 4. Level of GSH and protein thiols in the skin of Balb/c mice given vitamin E-sufficient or vitamin E-deficient diets, with or without topical exposure to cumene hydroperoxide. Conditions: Mice were painted with 12 mmol/kg Cum-OOH, in 100 μL of saline for 30 min. Values are means ± SEM of 8 mice. *p < 0.05 vs vitamin E-sufficient control; **p < 0.05 vs vitamin E-deficient control; ***p < 0.05 vs mice given vitamin E-sufficient diet and topically treated with Cum-OOH.

fluorescence leveled off after 45−60 min. This latter fluorescence response was due to the interaction of protein SH− groups with ThioGlo-1 (*23*). As shown in Figure 4, GSH levels of skin homogenates were decreased by 46.5% in untreated mice given vitamin E-deficient diet. There were no changes in the level of protein SH− groups in the skin of mice maintained on either diet. In addition, topical exposure of mice given a vitamin E-sufficient diet to Cum-OOH (12 mmol/kg, 30 min) produced a significant (89%) reduction in GSH concentration and a 62.5% decrease of protein sulfhydryls in skin homogenates (p < 0.05). The vitamin E-deficient mice topically exposed to Cum-OOH produced a decrease in GSH (91%) and protein thiol (74%) levels in skin compared to levels seen in vitamin E-sufficient mice treated with Cum-OOH (p < 0.05).

Ascorbate in Skin of Mice Given Vitamin E-Sufficient and Vitamin E-Deficient Diets after Topical Exposure to CuOOH. After 10 weeks of vitamin E deprivation, the level of ascorbate in the skin of mice decreased by 27%. As shown in Figure 5, topical exposure of mice to Cum-OOH (12 mmol/kg, 30 min) given vitamin E-sufficient diet resulted in a 93.8% decrease in the level of ascorbate in skin compared to the saline-treated controls. Skin ascorbate levels were below detectable

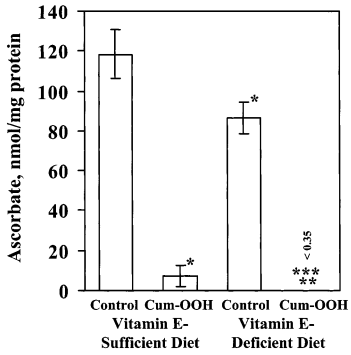

Figure 5. Level of ascorbate in the skin of Balb/c mice given vitamin E-sufficient or vitamin E-deficient diets, with or without topical exposure to cumene hydroperoxide. Conditions: Mice were painted with 12 mmol/kg Cum-OOH for 30 min. Values are means ± SEM of 8 mice. *p < 0.05 vs vitamin E-sufficient control; **p < 0.05 vs vitamin E-deficient control; ***p < 0.05 vs mice given vitamin E-sufficient diet and topically treated with cumene hydroperoxide.

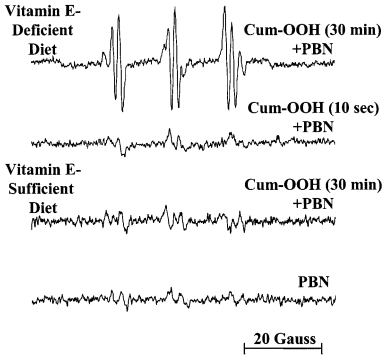

Figure 6. ESR detection of free radicals formed in vivo and spin-trapped with PBN in the skin of BALB/c mice given vitamin E-sufficient or vitamin E-deficient diets, with or without topical exposure to cumene hydroperoxide. Conditions: Prior to exposure, vitamin E-deficient and vitamin E-sufficient mice were injected subcutaneously in the dorsal lumbar area with the spin trap α-phenyl-*N*-*tert*-butylnitrone (1 mmol/kg in 100 μL of saline). 10 min postinjection, the interscapular area of backs of mice was painted with saline (100 μL) or Cum-OOH (12 mmol/kg, in 100 μL of saline). The skin flaps (1.5 × 2.0 cm^2) were collected 30 min after Cum-OOH application. In some control experiments, the skin flaps (1.5 × 2.0 cm^2) were collected 10 s after Cum-OOH application Instrumental conditions: microwave power, 20 mW; modulation amplitude, 1.0 G; time constant, 1.3 s; conversion time, 0.6 s.

levels in mice maintained on the vitamin E-deficient diet after Cum-OOH treatment.

ESR Study of Free Radicals Formed in Vivo in the Skin of Mice Given Vitamin E-Deficient Diet after Treatment with Cum-OOH. We observed a prominent nine-line ESR signal in extracts of the skin of mice given vitamin E-deficient diet 30 min after treatment with Cum-OOH (Figure 6). In contrast, a hardly discernible ESR signal was detectable in the skin from vitamin E-sufficient mice treated with Cum-OOH. To demonstrate that the detected radicals were generated in vivo in response to Cum-OOH treatment, we performed additional control experiments. In these experiments, vitamin E-deficient and vitamin E-sufficient mice were treated with Cum-OOH (12 mmol/kg, in 100 μL of saline) for 10 s. Following the treatment, lipids were extracted and analyzed by ESR identically to the samples prepared from the skin of mice treated with Cum-OOH (12 mmol/kg, in 100 μL of saline) for 30 min. We did not detect free radical generation after 10 s postexposure to

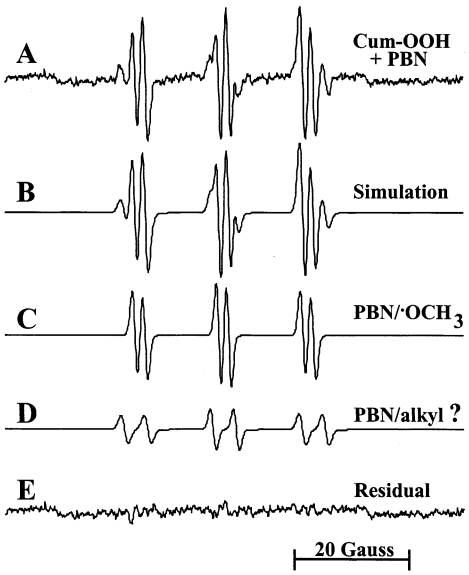

Figure 7. Free radical adducts detected after topical exposure to Cum-OOH in the skin of Balb/c mice given vitamin E-deficient diet. (A) ESR spectra of PBN spin-trapped lipid-derived radical adducts formed in vivo after topical exposure of vitamin E-deficient Balb/c mice to Cum-OOH. (B) Complete computer simulation of spectrum A. (C) PBN/•OCR, a methoxyl radical adduct with hyperfine coupling constants $a^N = 14.65$ G and $a^H = 1.8$ G. (D) Unidentified alkyl PBN radical adduct with hyperfine coupling constants $a^N = 15.5$ G and $a^H = 4.0$ G. (E) Residual, indicating the precision of the simulation spectrum. Conditions: Prior to exposure, vitamin E-deficient and vitamin E-sufficient mice were injected subcutaneously in the dorsal lumbar area with the spin trap α-phenyl-*N*-*tert*-butylnitrone (1 mmol/kg in 100 μL of saline). 10 min postinjection, the backs of mice were painted with saline (100 μL) or Cum-OOH (12 mmol/kg, in 100 μL of saline). The skin samples were collected 30 min after Cum-OOH application. Instrumental conditions: microwave power, 20 mW; modulation amplitude, 1.0 G; time constant, 1.3 s; conversion time, 0.6 s.

Cum-OOH (12 mmol/kg, in 100 μL of saline). In particular, no typical nine-line ESR signal was detected in lipid extracts from mouse skin in vitamin E-deficient or vitamin E-sufficient animals as we have seen after 30 min posttreatment with Cum-OOH (Figure 6).

Analysis of the ESR spectrum showed that it consisted of signals from two radical species (Figure 7). The ESR spectrum was simulated using two species, one with hyperfine coupling constants $a^N = 14.65$ G and $a^H = 1.8$ G, and a secondary species with $a^N = 15.5$ G and $a^H = 4.0$ G. The latter free radical adduct had similar but not identical hyperfine coupling constants to the methyl radical adduct of PBN in methanol (*33*), but none of the hyperfine coupling constants were identical to those of any species in chloroform. Because the cumoxyl radical formed from Cum-OOH either might be trapped or give rise to methyl radical and subsequently methoxyl radical, we prepared authentic methyl, methoxyl, and cumoxyl radical adducts of PBN in Folch solution. Authentic PBN/•CH_3 was prepared from the reaction between ^{13}C-Cum-OOH and Ti^{3+} (*34*). After extraction into Folch solution, the ESR spectrum obtained from the ^{13}C-labeled methyl radical adduct had $a^N = 15.8$ G, $a^H = 3.6$ G, and $a^{13C} = 3.6$ G (Table 3), which are reasonably similar to those detected in mouse skin extracts. However, the authentic methoxyl and cumoxyl radical adducts of PBN (prepared using the iron-catalyzed nucleophilic addition of the corresponding alcohol to PBN) (*35*) did not give hyperfine coupling constants corresponding to those obtained from

Table 3. Hyperfine Coupling Constants of PBN Radical Adducts in Folch Solution ($CHCl_3/CH_3OH$)[a]

radical adduct	a^N	a^H	a^{13C}
•CH_3	15.8	3.6	3.6
•OCH_3	14.4	2.6	–
•$OC(CH_3)_2C_6H_6$	14.3	2.5	–

[a] The PBN radical adducts were prepared by trapping the free radical product of the reaction between cumene hydroperoxide and Ti^{3+} in a small volume of water followed by extraction with chloroform or a mixture with chloroform to form the final solvent system.

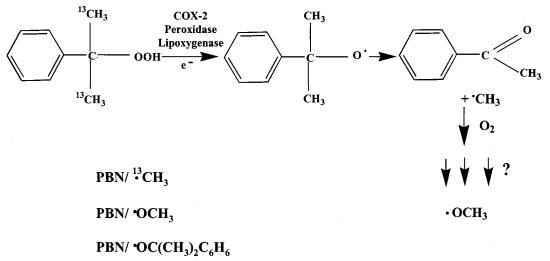

Figure 8. Schema illustrating formation of different free radical species during interaction of Cum-OOH with intracellular peroxidase, COX-2, and lipoxygenase. As shown, a number of different free radical species can be formed and spin-trapped from Cum-OOH. These include formation of methoxyl and methyl radicals that were detected using the ^{13}C-labeled cumene hydroperoxide.

the other adduct detected from the mice (compare the data in Table 3 to the adduct, $a^N = 14.65$ G and $a^H = 1.8$ G). The latter adduct detected in mouse skin extracts remains unassigned.

The reaction between Cum-OOH and a peroxidase, a reduced transition metal, or lipoxygenase is known to result in the formation of cumoxyl radical (Figure 8) (*36*, *37*). The cumoxyl free radical is unstable and readily undergoes a *β*-scission reaction to form a methyl radical and phenyl-methyl ketone (Figure 8). To determine whether the secondary free radical species detected arose from trapping a methyl radical from the Cum-OOH, the experiment was repeated using Cum-OOH labeled at the methyl carbons with ^{13}C. The ESR spectra of skin extracts of mice treated with ^{13}C-Cum-OOH were complex and indicated the presence of at least two species (Figure 9). One component, with $a^N = 15.5$ G and $a^H = 4.0$ G, was somewhat similar to one of the components observed when ^{12}C-Cum-OOH was used (Figure 9). The second component had an additional hyperfine coupling and was effectively computer-simulated with $a^N = 15.9$ G and $a^H = 3.8$ G, and a coupling to a nucleus with $I = 1/2$ and $a = 3.8$ G, i.e., a ^{13}C from the labeled Cum-OOH (Figure 9). The isotopic enrichment of the ^{13}C-labeled Cum-OOH was confirmed by mass spectrometry (data not shown). Because the hyperfine coupling to a ^{13}C nucleus was detected, the latter adduct was assigned to the methyl radical derived from *β*-scission of the cumoxyl radical (Figure 8).

To confirm that the detected hyperfine coupling constants can be assigned to the methyl radical in the present solvent system, authentic PBN/•CH_3 was prepared in various solvents by spin-trapping the free radical product of the reaction between Cum-OOH and Ti^{3+} (*34*). The additional hyperfine coupling from the ^{13}C-labeled Cum-OOH provided an independent assignment of the methyl radical adduct to its set of hyperfine coupling constants. These data are summarized in Table 3. As can be seen from Table 3, the hyperfine coupling constants

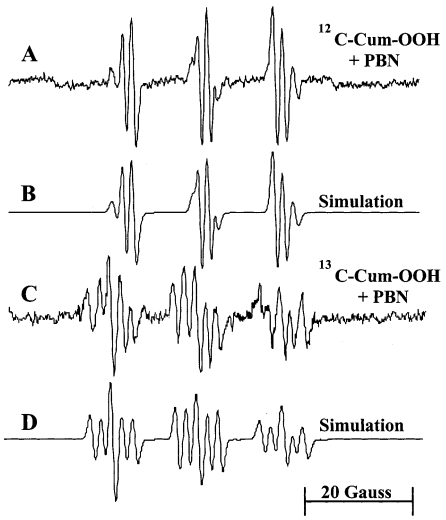

Figure 9. Free radicals detected in the skin of vitamin E-deficient mice after treatment with ^{13}C-labeled cumene hydroperoxide. (A) The ESR spectrum obtained from mice treated with natural isotopic abundance Cum-OOH. (B) Computer simulation of spectrum A calculated with two species: 36% a^N = 15.5 G, a^H = 4.0 G and 64% a^N = 14.65 G, a^H = 1.8 G. (C) Representative ESR spectrum obtained from mice treated with ^{13}C-labeled Cum-OOH. (D) Computer simulation of spectrum C calculated using two species: 65% a^N = 15.9 G, a^H = 3.8 G, a^{13C} = 3.8 G; 35% a^N = 15.5 G, a^H = 4.0 G.

for PBN/•CH_3 are highly dependent upon the solvent system. When the solvent was a mixture of $CHCl_3$ and H_2O, the hyperfine coupling constants from the β-hydrogen and from the ^{13}C-methyl were about the same. These data confirm the simulation and assignment of the spectrum as PBN/•$^{13}CH_3$.

The radical adduct without a hyperfine coupling to a ^{13}C atom had nitrogen and hydrogen hyperfine coupling constants that were very similar to those detected in the ^{13}C-labeled methyl radical adduct. Based upon that similarity, that species can be assigned to the PBN adduct of an alkyl free radical likely formed by peroxidation of lipids in the mouse skin. Those hyperfine coupling constants were similar to those previously detected for one of the species (Figure 7D). The other species detected in ESR experiment is likely that of the methoxyl radical derived from the Cum-OOH (Figures 7C and 8).

Discussion

Cutaneous antioxidants play a pivotal role in the defense mechanisms against reactive oxygen species generated by a variety of environmental and occupational factors, e.g., air pollutants, ultraviolet and X-ray radiation, heat, chemical oxidants and others. Low-molecular-weight antioxidants (vitamins C and E, thioctic acid, glutathione, and coenzyme Q) are widely used for skin care. Their benefits range from treatment of skin maladies such as acne and psoriasis to protection against environmental insults (*38*). The cutaneous antioxidants are regulated by an intimately interlinked network system. In the redox antioxidant network, the low-molecular-weight thiol GSH can synergistically enhance the ascorbate-dependent recycling of vitamin E (*39*−*44*).

Vitamin E acts as an antioxidant "harvesting center" in the membrane that collects reducing equivalents from other antioxidants, particularly vitamin C (*45*−*47*). The

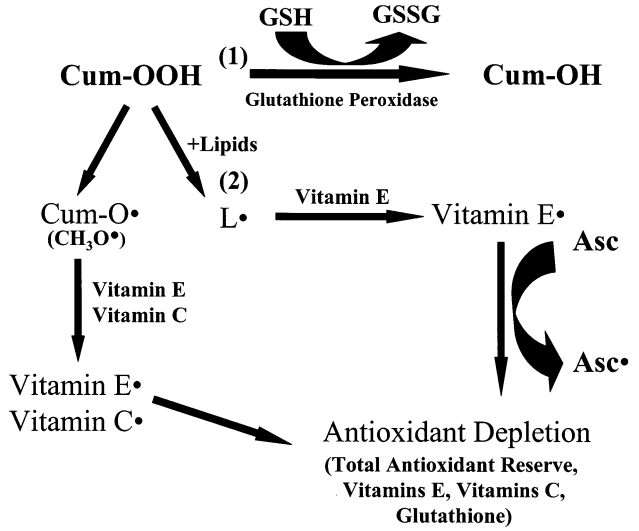

Figure 10. Schema illustrating interaction of Cum-OOH-derived radicals with major antioxidants (vitamin E, vitamin C, and thiols).

dramatic decrease in the level of vitamin C in skin of mice after exposure to Cum-OOH in our study is in accordance with these observations. In vitamin E-deficient mice, low vitamin C levels were further decreased upon exposure to Cum-OOH such that vitamin C was no longer detectable. These results indicate that vitamin C was most likely used for the recycling of vitamin E, resulting in a complete depletion of vitamin C (Figure 10). If reaction 1 (Figure 10) was solely responsible for the metabolism of Cum-OOH, one would expect depletion of GSH without major involvement of vitamin E and vitamin C (Figure 10). No radical formation would also be expected with this pathway. In contrast, our results clearly demonstrate that both vitamin E and vitamin C were strongly involved in the metabolism of Cum-OOH in skin, most likely in their interaction with Cum-OOH-derived and lipid radicals. This was directly confirmed first by ESR detection of radical intermediates in the skin of vitamin E-deficient mice, second by depletion of vitamin E, and third by depletion of ascorbate. The fact that this effect was most prominent in vitamin E-deficient animals suggested that vitamin E plays a pivotal role in interacting with Cum-OOH-derived radicals. This possibly precludes observation of PBN adducts in vitamin E-sufficient animals. In contrast, in animals with low endogenous levels of vitamin E, distinct signals from PBN spin-trapped radical adducts could be observed.

Oxidative stress can be enhanced by a large variety of conditions including nutritional distress, exposure to chemical and physical agents, injury, and hereditary disorders. Vitamin E-deprived animals are generally more susceptible to adverse effects than vitamin E-supplemented ones (*19*−*21*). We observed that topical exposure of skin to Cum-OOH induced oxidative stress in vivo. Decreased antioxidant reserve, depletion of GSH, and diminished ascorbate levels were marked in vitamin E-deficient mice, demonstrating accelerated oxidative stress in skin after Cum-OOH application. This was also evident by accumulation of lipid peroxidation products at a higher rate in skin of vitamin E-deficient mice treated with Cum-OOH. In particular, 0.30 and 1.54 nmol of lipid peroxides per milligram of protein were accumulated in skin of vitamin E-sufficient and vitamin

E-deficient mice, respectively, following topical exposure to Cum-OOH (Figure 2). Additionally, we observed significant oxidation of phospholipids with profound oxidation and externalization of phosphatidylserine when normal human epidermal keratinocytes were exposed to different concentrations of Cum-OOH (*9*).

Free radicals can be generated in vivo as byproducts of normal metabolism via catalytic cycling of numerous enzymes (*45*). It has been suggested that oxygen radicals derived from inflammatory cells are capable of triggering free radical production in skin (*46*, *47*). Organic peroxides are known to cause inflammatory reactions in skin (*5*). One could expect that the radicals detected in skin were formed by activated inflammatory cells. However, one can exclude the role of inflammatory cells in radical adduct formation in mouse skin because no inflammation in vivo was found after 30 min postexposure to Cum-OOH (data not shown). The radicals detected in lipid extracts from skin were not generated during sample preparation or handling as demonstrated by the lack of detectable signals when Cum-OOH was added to mouse skin in vivo for 10 s or ex vivo to skin homogenates. We found the formation of radical adducts in lipid extracts from Cum-OOH-treated skin of mice given a vitamin E-deficient, but not a vitamin E-sufficient diet. The detection of two distinct signals when ^{13}C-labeled Cum-OOH was used indicated formation of at least two distinct free radical species in mouse skin. One of the signals was clearly Cum-OOH-derived since a hyperfine coupling to a ^{13}C-atom was detected (Figure 9). The other signal, which lacked a coupling to a ^{13}C, was present in all experiments. The latter spectrum had very similar nitrogen and β-hydrogen hyperfine coupling constants to those confirmed for the methyl radical adduct from the Cum-OOH in the ex vivo solvent, suggesting it had a similar structure, i.e., a saturated alkyl radical. Ethyl and pentyl free radicals were reported as products of lipid peroxidation (*48*−*50*). In our experiments when the primary lipid antioxidant, vitamin E, was removed from the diet, we detected presumably lipid-derived free radicals in lipid extracts from skin of mice exposed to Cum-OOH. These radicals may be formed from the β-scission of the lipid alkoxyl radicals that either reacted with molecular oxygen to produce peroxyl radicals or abstracted a hydrogen atom from ethane and pentane. The latter ones are well-known indicators of lipid peroxidation (*51*, *52*). Methyl and ethyl radical adducts were previously detected in murine keratinocytes treated with Cum-OOH by spin trapping either with 5,5-dimethyl-1 pyrroline *N*-oxide or with 2-methyl-2-nitrosopropane (*53*). Previous in vivo ESR studies are limited, and the only species so far identified in the skin tissue ex vivo were the signals assigned to a mixture of alkoxyl and carbon-centered radical adducts formed after Cum-OOH application (*13*). Intermediate methyl peroxyl and cumylperoxyl radicals may have been formed during the metabolism of cumene hydroperoxide, but we observed no evidence of those radicals spin-trapped with PBN. The PBN-trapping of peroxyl radicals has been reported to result in the formation of a carbonyl form of PBN with a distinct 3-line spectrum that we did not observe in any of our experiments (*54*, *55*). The slow reaction rate of free radicals with PBN compared to the rate of reaction of peroxyl radicals with lipids might have contributed to the lack of detection of peroxyl radicals in the mouse skin system.

Knowing that the Cum-OOH is lipophilic, we expected that it would all be "consumed" by the skin. However, we also argued that perhaps some amount of administered Cum-OOH might stay on top of the skin as a residue and play a part in chemical reaction in the ex vivo part of the chemical analyses of the study. Anticipating such eventuality, we took skin from the interscapular area of mice (identical to the skin specimens used in the study) and subjected the skin to homogenization as described in the paper after adding Cum-OOH. We found that the values of vitamin E and GSH from this ex vivo procedure are the same as those obtained from similar skin homogenized without the addition of Cum-OOH ex vivo (data not shown). As has been shown by Burkitt and Mason (*31*), the addition of 2,2′-dipyridyl (2,2-DP) to samples is essential to prevent formation of radicals ex vivo. We collected skin samples into a 2,2-DP solution and studied Cum-OOH-induced free radical formation in lipid extracts of skin. We are confident that the values presented in the paper are due to the in vivo effect of Cum-OOH on the skin. The fact that the radical adducts could be detected only in the skin of mice deficient in vitamin E strongly suggests that its formation is associated with lipid peroxidation. Increased levels of primary and secondary lipid peroxidation products and diminished levels of antioxidant reserve and tissue antioxidants in the skin of mice exposed to Cum-OOH were also evident. In this respect, the primary role of vitamin E in preventing free-radical-initiated peroxidative tissue injury is widely accepted. In conclusion, the present study provides the most direct ESR evidence of lipid-derived peroxidation associated with free radical formation after Cum-OOH exposure to skin.

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