ELSEVIER

International Journal of Pharmaceutics 162 (1998) 129–136

international journal of pharmaceutics

Photooxydative damage of skin lipids in liposomes (hSCLLs)—interference of sterols with the lipid peroxidation chain[1]

Jürgen Lasch [a,*], Ute Schönfelder [a], Mathias Walke [a], Sebastian Zellmer [a], Dieter Beckert [b]

[a] *Institute of Physiological Chemistry of the Martin-Luther-University Halle, Medical Faculty, Hollystrasse 1, D-06097 Halle, Germany*

[b] *Interdisciplinary Group 'Time-Resolved Spectroscopy' at the University Leipzig, D-0403 Leipzig, Germany*

Accepted 7 November 1997

Keywords: Human stratum corneum lipids; Irradiation damage; Photooxidation; Lipid hydroperoxides; 'Photoprotective' sterols; Oxysterols; Lipid radicals

1. Introduction

The lipid peroxidation chain starts with the generation of ROS (reactive oxygen species) and proceeds with the oxidative degradation of glycerophospholipids, sphingolipids, glycolipids, unsaturated fatty acids and sterols. The products of lipid peroxidation are toxic (Pryor, 1980), mutagenic (Mukai and Goldstein, 1976) and carcinogenic (Shamberger et al., 1974).

This is especially relevant to the skin. Human skin (surface and weight of skin of the standard individuum (70 kg/1.7 m) are 1.6 m^2 and 3 kg without subcutis and fat) is the organ which is directly and intensively exposed to ultraviolet radiation (UV). Photoprotection against sunburn and associated irradiation-induced damages of the human skin is mainly attributed to the darkening of the biochrome melanin by its oxidation. After irradiation with UV light, free radicals appear in the skin (Pathak and Stratton, 1968). These authors, studying the electron spin resonance signals of irradiated skin, concluded that darkening represents oxidation in the melanin polymer, which appears to take place through the formation of stable free melanin radicals. Short- and long-term dangers caused by UVA (320–400 nm) and UVB (280–320 nm) are well known but the molecular mechanisms of the primary biological effects are still poorly understood.

* Corresponding author. Tel.: +49 345 5573818; fax: +49 345 5573811; e-mail: juergen.lasch@medizin.uni-halle.de

[1] This paper is dedicated to Benno Parthier, president of the Gesellschaft Deutscher Naturforscher 'Leopoldina', on the occasion of his 65th birthday.

PII S0378-5173(97)00420-1

2. Material and methods

2.1. Materials

Cholesterol (Ch), cholesterol-3-sulfate (ChS), ceramide type III (Cer III) and type IV (Cer IV), stearic acid (SA) and egg yolk lecithin (EYL) were products of Sigma (St. Louis, MO). It must be stressed that the ceramides type III and IV of Sigma are not identical with the ceramides 3 and 4 of the recently updated (Downing-Wertz)-classification of human skin ceramides published by Robson et al. (1994), which ranges from ceramide 1 to 7 according to their position on the TLC plate. Ceramide III corresponds to ceramide 2 and ceramide IV to ceramide 5 of the human skin (Motta et al., 1994) what has caused a lot of semantic confusion. In liposomes prepared with commercial lipids, the Roman numerals of Sigma are used, whereas in 'natural' lipid vesicles like the hSCLLs (human Stratum Corneum Lipid Liposomes) the Arabic numerals are relevant.

Lipid extraction of human stratum corneum was done by a modified Bligh and Dyer method (Bligh and Dyer, 1959).

Thiobarbituric acid (TBA), the stable lipid hydroperoxide, 13(S)-[9Z,11E]-hydroperoxyoctadecadienoic acid was from Sigma, 2′,7′-dichlorodihydro-fluorescein diacetate from Molecular Probes, (Eugene, OR) and n-butanol puriss. p.a. from Fluka (Buchs, Switzerland).

The chemiluminescent substrate luminol (MW 177.16 g/mol) was from Boehringer (Mannheim, Germany) and the luminescence enhancer, 6-hydroxybenzothiazole (MW 151.2 g/mol), from Sigma (Deisenhofen, Germany).

The spin trap DMPO (5,5 dimethyl-1-pyrroline-*N*-oxide), PC (β-linoleoyl-γ-stearoyl-L-α-phosphatidylcholine), charcoal and EDTA were obtained from Sigma. DMPO was diluted 10-fold with argon-saturated ultrapure water (Milli-Q) and purified with neutral decolourizing charcoal. Aliquots were frozen and stored under argon to slow down thermal signal growth (autooxidation).

2.2. Preparation of liposomes (VET)

Liposomes were prepared by the Vesicle Extrusion Technique (MacDonald et al., 1991) with the extrusion apparatus LiposoFast™ from Avestin (Ottawa, Ont.). Pore size of the polycarbonate membrane was 200 nm.

2.3. Oxidation of liposomal lipids

Lipid peroxidation in liposomal membranes was induced by UV irradiation. The UV lamp (HBO 200 W mercury lamp; Osram, München, Germany) has a typical line emission spectrum. Two filters (KG 3, UG 11, Schott, Mainz, Germany) were fitted into the light path so that light < 280 nm and > 400 nm was cut off. Light intensities were measured at sample distance (15 cm) with an Optometer (Giga-Hertz Optik, München, Germany) and yielded a light intensity of 0.211 mW/cm^2 in the UVB region (280–320 nm) and 52.1 mW/cm^2 in the UVA region (320–410 nm). For technical reasons, ESR spectra had to be taken at slightly different irradiation intensities. Exposure intensities at the front of the ESR cavity were: 22 mW/cm^2 (UVA) and 5.2 mW/cm^2 (UVB).

Liposomes (300 μl) were filled into quartz glass fluorescence cuvettes (Hellma, Müllheim, FRG) and placed at a distance of 15 cm in front of the UV light source. A volume of $10 \times 15 \times 2$ mm ($w \times h \times d$) was irradiated. The various samples were irradiated up to 5 h. The total lipid concentration was held constant at 10 mg/ml.

2.4. Measurement of the oxidative degradation of lipids

Six techniques were used: HPTLC (Zellmer and Lasch, 1997), the TBARS-test of Draper and Hadley, 1990, the dichlorodihydrofluorescein test (Cathcart et al., 1984), the chemiluminescence technique (Kricka and Thorpe, 1990) and electron spin resonance spectroscopy. The chemiluminescence reaction we exploited and adapted is illustrated in Fig. 1 .The proportionality of the luminometer counts to concentrations of LOOH was verified by calibration with the stable commercial hydroperoxyoctadecadienoic acid.

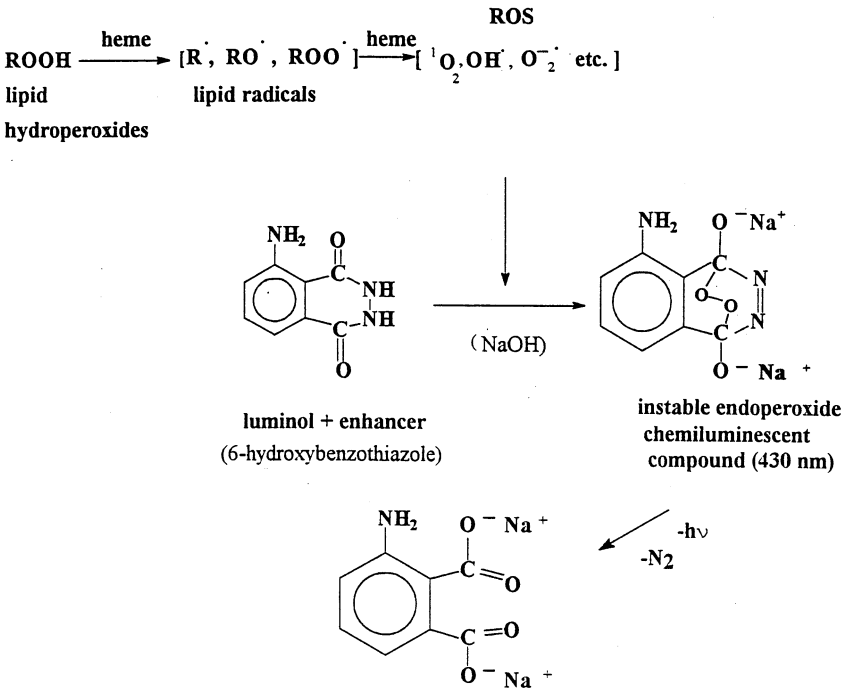

Fig. 1. Chemiluminescent reaction of reactive oxygen species (ROS) generated with the catalyst hemin from lipid hydroperoxides. Adapted from Fortschr. Chem. Forsch. (1974) 46, 61–139.

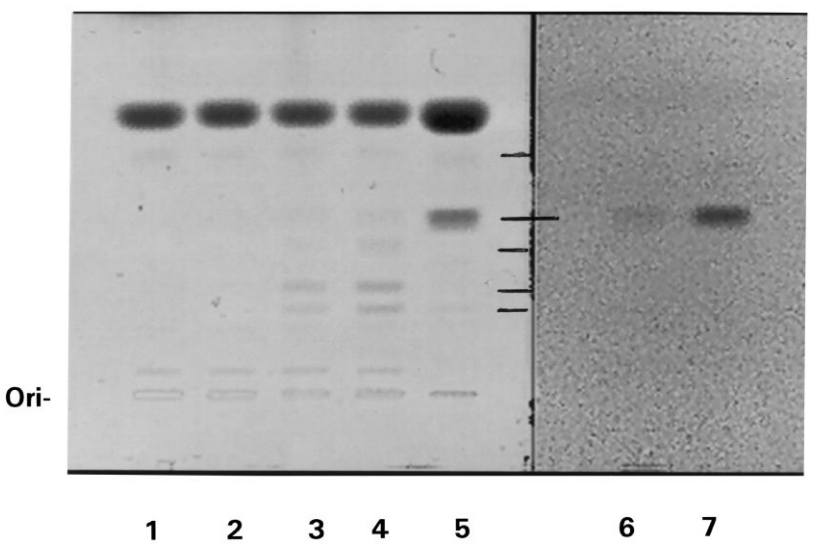

Fig. 2. HPTLC chromatoplate of oxidised cholesterol/DPPC (50/50 mol%) liposomes. Solvent: n-hexane/ethylacetate (1/1), stationary phase: silica 60 F_{254}. Irradiation times: lane 1, 0 h; lane 2, 3 h; lane 3, 6 h; lanes 4, 5, 6 and 7, 8 h. Lanes 5 and 7 are irradiated cholesterol standards. Postchromatographic staining: $CuSO_2/H_3PO_4$/MeOH (10, 8, 5%) and TMPD (tetramethylphenylendiamine). Exposure to UV light results in the formation of 5 spots (lines) with R_f values of 0.16, 0.19, 0.24, 0.33 and 0.38. Lanes 6 and 7 are stained for hydroperoxides with TMPD, i.e. the spots with R_f 0.38 are cholesterol-hydroperoxides.

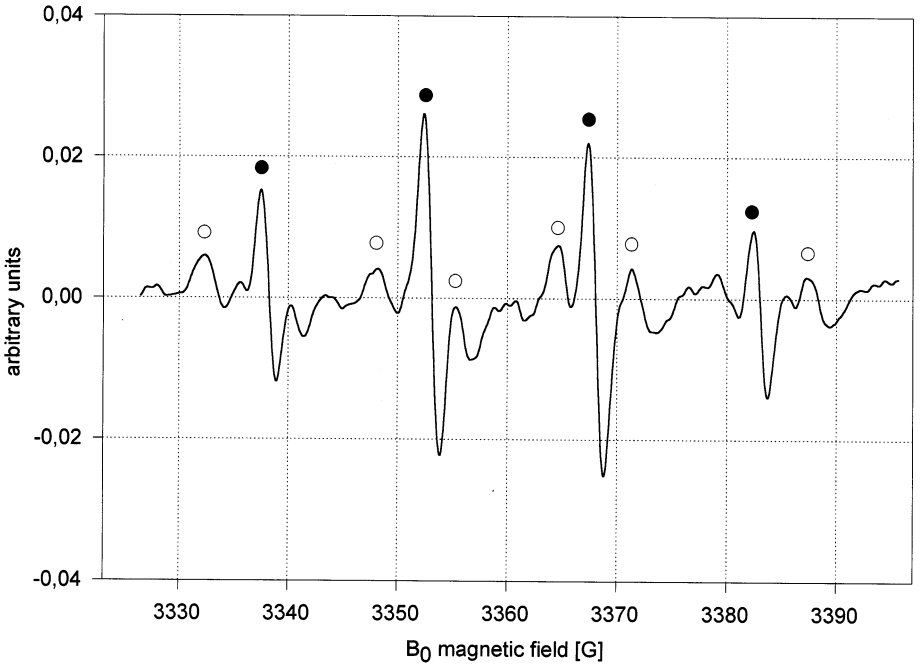

Fig. 3. ESR spectrum of a DMPO spin adduct generated in a dispersion of human skin lipid liposomes by steady-state UV exposure. The spectrum was recorded 30 min after UV irradiation. Ten spectra were averaged and smoothed by gliding averaging using a ten point averaging window: ○, DMPO-L˙ adduct; ●, DMPO-OH˙ adduct.

3. Results

If cholesterol is oxidised as a component of DPPC liposomes, the irradiated lipids show new bands on HPTLC plates which are most likely cholesterol oxidation products and intermediates marked by arrows in Fig. 2. The right hand part of the chromoplate was stained with N,N,N′,N′-tetramethyl-*p*-phenylendiamine, (TMPD) which specifically stains hydroperoxides (Korytowski et al., 1991). Lanes 5 and 7 are the irradiated cholesterol standard (legend to Fig. 2) and lanes 4 and 6 irradiated liposomal lipids (DPPC and cholesterol, 8 h irradiation).The spots stained in lanes 6 and 7 are clearly cholesterolhydroperoxides. We cannot identify the other products and/or intermediates (arrows) which will include epoxides and hydroxy derivatives of cholesterol.

The hyperfine splitting constants of DMPO (spin trap) adducts in the ESR spectrum taken from irradiated skin lipid liposomes (Fig. 3) are: $a_N = 15.5$ G, $a_H = 15.5$ G (typical for DMPO-OH˙) and $a_N = 15.5$ G and $a_H = 22.5$ G (typical for DMPO-L$^\bullet$). Obviously, the radicals L$^\bullet$ and OH$^\bullet$ are formed during the UV irradiation.

Initial lipid concentrations decrease with time of exposure of lipid vesicles to ultraviolet light (Fig. 4). This happens only if oxygen is present (Fig. 5).

In one set of experiments the percentage cholesterol in the liposomal membranes was varied. Generally all samples with 0 wt.% cholesterol were more oxidised than those containing 50 wt.% cholesterol.

The variation of the cholesterol concentration influences the oxidative degradation of the other membrane lipids. In liposomes without cholesterol (●) lipids were decomposed faster than in liposomes consisting of 50 (wt.%) cholesterol (○). The lipid degradation in hSCLLs (▽) was similar to liposomes containing 50 wt.% cholesterol.

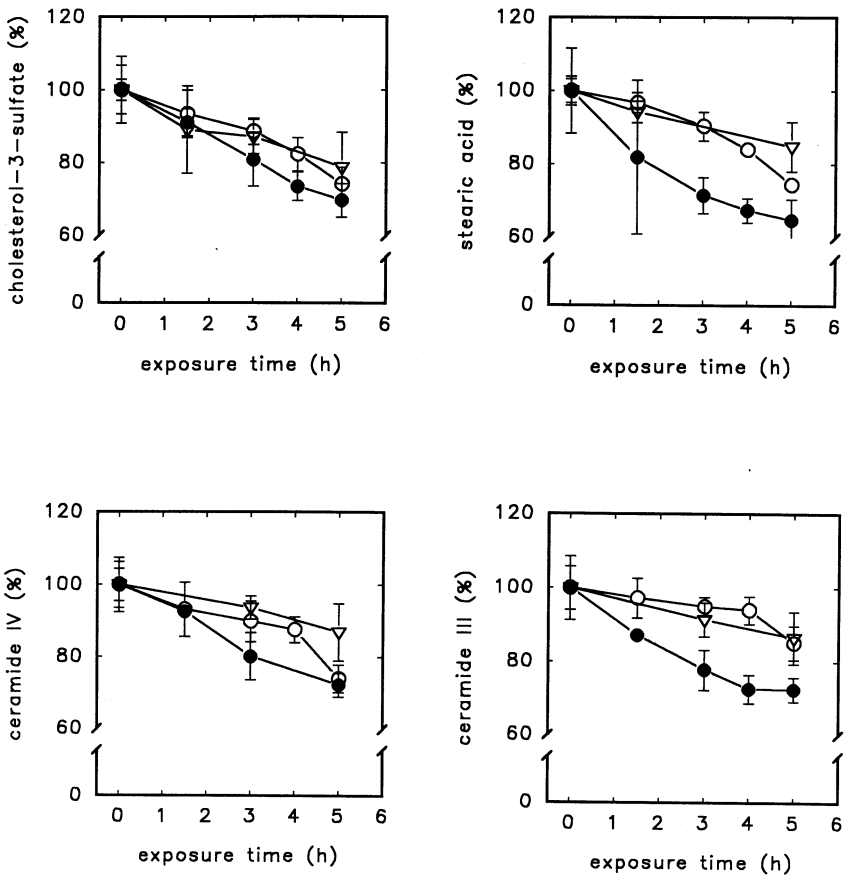

Fig. 4. Decrease of the concentration of various lipids during UV irradiation in liposomal membranes of various cholesterol contents. Symbols: ●, 0 wt.% cholesterol; ○, 50 wt.% cholesterol; hSCLL are represented by hollow triangles.

The protective action of cholesterol with respect to the oxidation of other membrane lipids was corroborated by measuring the formation of thiobarbituric reactive substances after photooxidation. The result is shown in Fig. 6. In the absence of cholesterol, higher amounts of TBARS were formed (●) as in the presence of 50 wt.% cholesterol (○).

Lipidhydroperoxides (LOOH) were measured after 30, 60 and 90 min photo-oxidation of the corresponding lipid vesicles. The results of the chemiluminescent assay are summarised in Table 1.

Lipid hydroperoxide formation during exposure to UV light was also determined by the DCF fluorescent assay. A high cholesterol content in liposomes enhances the generation of hydroperoxides (○) compared to liposomes without cholesterol (●). This difference is due to cholesterol oxidation. In completely saturated control liposomes (DPPC) no lipid hydroperoxides are formed (▽) (Fig. 7).

4. Discussion

There are three lines of evidence which corroborate that we follow photooxidation of lipids: (i) Exclusion of oxygen inhibits all measured lipid losses (Fig. 5), (ii) the luminol-based chemiluminescence assay and the DCF fluorescent assay are specific for hydroperoxides (Zamburlini et al.,

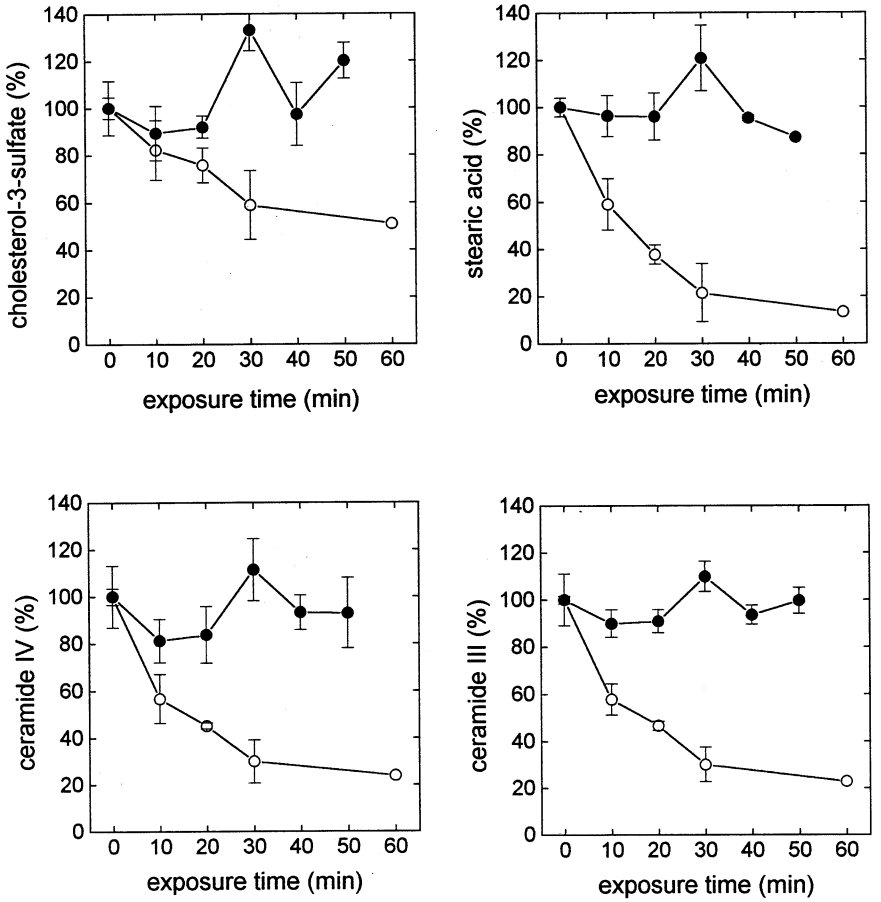

Fig. 5. Control measurements of UV-induced lipid decompositions by exclusion of oxygen. Air and nitrogen atmosphere are compared. Irradiation under nitrogen atmosphere, ●; and air, ○. Liposome sample V.

1995), and (iii) the amount of TBARSs as a measure lipid peroxidation—'end products' like malondialdehyde and 4-hydroxynonenal (Esterbauer and Cheeseman, 1990) depends on the cholesterol content.

In general, we conclude, that sterols with their high concentration in the lipid barrier of the human stratum corneum, are effective scavengers of reactive oxygen species. On the other hand, sterols themselves can be oxidised to cholesterol hydroperoxides (Halliwell and Gutteridge, 1991) or epoxidised (Watabe et al., 1984), i.e. sterols compete with other susceptible lipids for the reactive oxygen species. Little is known, however, about the patho-physiological effects of sterol oxidation products and their metabolic inactivation (Watabe et al., 1984). Stratum corneum lipids are photooxidised to lipid hydroperoxides about 30 times less than egg lecithin which is rich in unsaturated fatty acids (Table 1). Even the addition of 20 wt.% of cholesterol to the hSCLLs does not substantially improved stability towards photooxidation (Table 1), probably because the high intrinsic sterol concentration already provides a substantial protection. The obvious deviation of lipid oxidation in hSCLLs from the rules discernible in model liposomes must be caused by 'packing constraints'. The lipids of the hSCLLs are rigidly packed or even riveted as shown previously by the appearence of cross fractions during freeze fracture electron microscopy (Lasch et al., 1994). The reproducible decrease of stearic acid

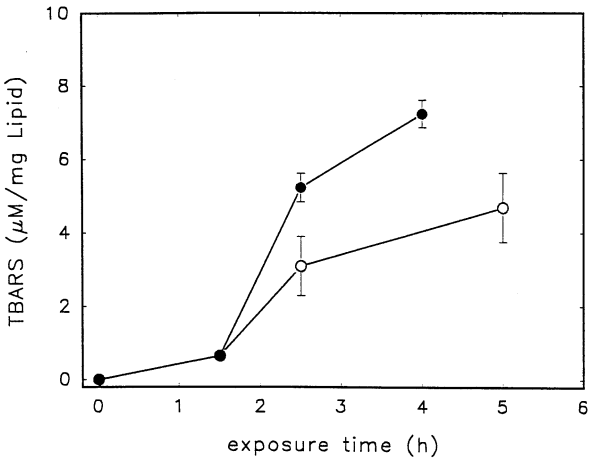

Fig. 6. TBARS formation in human stratum corneum derived liposomes without cholesterol (●) and 50 wt.% cholesterol (○), respectively. TBARS measured according to Draper and Hadley, 1990 by florescence spectroscopy.

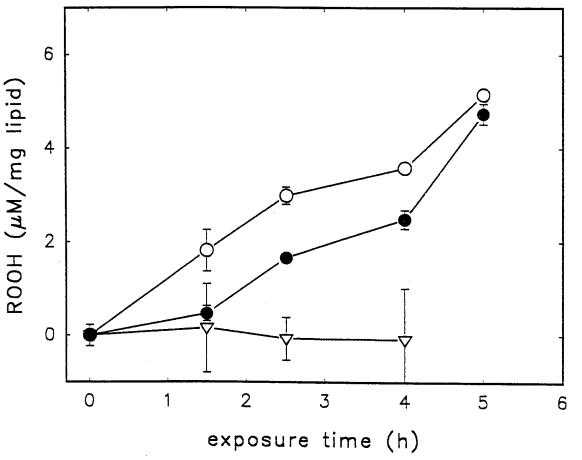

Fig. 7. Lipid hydro peroxide determination in human stratum corneum derived liposomes by the DCF flourescent assay according to Cathcart et al., 1984. More ROOHS were formed in the presence of 50 wt.% cholesterol (○) than in liposomes devoid of cholesterol (●). With DPPC control liposomes no hydroperoxide signal could be detected (▽).

during photooxidation is a surprising result and difficult to explain. Peroxy-linked dimers between linoleate 13-hydroperoxide and palmitate were reported (Frankel, 1987). In our system, unsaturated ceramides instead of linoleic acid are present. UV irradiation can split hydroperoxides (Beasley and Anderson, 1964) generating the alkoxyradical ($LO^{\bullet}$) and the reactive $OH^{\bullet}$ radical which in turn can abstract hydrogen from saturated lipids leaving lipid radicals (alkyl radicals), $L^{\bullet}$ and water.

The ESR spectrum (Fig. 3) shows clearly that indeed lipid radicals are formed even during a rather mild UV irradiation.

Acknowledgements

This study was supported by the DFG grants of the INK (Innovation College) B2, the SFB 197/A8, the individual project Ze 376/1-1 and the Fonds der Chemischen Industrie. U. Schönfelder

Table 1
Increase of lipid hydroperoxides after 60 min photooxidation

Sample	Increase[a] of maximal RLU/s after 60 min photooxidation	% Increase with respect to $t = 0$
HsclLs	30.7	67
hSCLLs + cholesterol (20 wt.%)	33.9	73
Lipid mixture: Cer/Ch/ChS/PA = 31/50/4/15 (wt.%)	30.6	75
EYL liposomes	1018.0	323

Luminescence (RLU/s) was always measured 30 s after addition of the luminescent substrate luminol mixed with the enhancer 6–hydroxybenzothiazole.

[a] The values of the non-irradiated samples measured under identical conditions were subtracted.

holds a grant of the Kultusministerium of Sachsen-Anhalt.

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