Ultraviolet A Induces Generation of Squalene Monohydroperoxide Isomers in Human Sebum and Skin Surface Lipids In Vitro and In Vivo

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At the outermost surface of human skin, skin surface lipids are first-line targets of solar ultraviolet radiation. Therefore, we hypothesized that ultraviolet A and ultraviolet B irradiation induce photo-oxidation of skin surface lipids. To test this, sebum samples were collected from facial skin of 17 healthy volunteers, weighed, and immediately irradiated with either ultraviolet B or ultraviolet A. Squalene, the major sebum lipid, as well as photo-oxidation products were identified in sebum lipid extracts by high-performance liquid chromatography analysis. Upon ultraviolet A exposures squalene was depleted in a concentration-dependent manner, whereas an unidentified sebum lipid photo-oxidation product was detected. Using high-performance thin layer chromatography, high-performance liquid chromatography, atmospheric pressure chemical ionization mass spectrometry, and nuclear magnetic resonance, unidentified sebum lipid photo-oxidation product was identified as a mixture of squalene monohydroperoxide isomers. Squalene monohydroperoxide isomers purified from sebum were identical with squalene monohydroperoxide isomers synthesized by preparative photo-oxidation of squalene. Squalene monohydroperoxide isomers were formed even after small suberythemogenic doses of ultraviolet A (5 J per cm²). Whereas physiologic baseline levels of squalene monohydroperoxide isomers in human skin were only slightly above detection limits, squalene monohydroperoxide isomer levels were strongly increased by suberythemogenic doses of ultraviolet A both in vitro and in vivo. High-performance liquid chromatography results could be complemented by a straightforward thin layer chromatography method for rapid screening of lipid peroxide formation in human sebum/skin surface lipids. In conclusion, specific squalene monohydroperoxide isomers were identified as highly ultraviolet A sensitive skin surface lipid breakdown products that may serve as a marker for photo-oxidative stress in vitro and in vivo. Key words: lipids/peroxidation/photo-oxidation/sebum/squalene monohydroperoxide/ultraviolet.

1999b). It was postulated that the biologic role of the high concentrations of vitamin E present in SSL may serve as protection against photo-oxidation. To investigate the susceptibility of SSL to photo-oxidative stress, human sebum was collected and subjected to defined doses of UVA and UVB. This study was based on preliminary results that had revealed the accumulation of unexpectedly high amounts of an unidentified lipid photo-oxidation product in human sebum (USLPP) upon physiologic and suberythematogenous levels of UVA.

The goal of this study was: (i) to develop a sensitive and reliable method for detecting SSL photo-oxidation products; (ii) to identify the nature and source of USLPP; (iii) to investigate the wavelength dependency of SSL photo-oxidation; and (iv) to quantify and compare the formation of SSL photo-oxidation products in vitro and in vivo.

**MATERIALS AND METHODS**

**Chemicals**

All chemicals and solvents were of the highest analytical or high performance liquid chromatography (HPLC) grade, unless specified otherwise. HPLC grade ethanol, methanol, benzene, toluene, and ethyl acetate were from Roth GmbH (Karlsruhe, Germany). Rose Bengal disodium salt (5′,6′-tetrachloro-4′,5′-tetrabromo fluorescein disodium salt) was from VEB Laborchemie (Apolda, Germany) and squalene was from Sigma-Aldrich Chemie (Steinheim, Germany).

**Volunteers**

SSL of the face were collected from 17 healthy volunteers [nine females, eight male; average age 30.7 ± 6.7 y (mean ± SD); Fitzpatrick skin types I–IV (I/II: one, II/III: 14, III/IV: two)]. Permission was granted by the local ethic committee of the Friedrich-Schiller-University of Jena. Exclusion criteria were any history of dermatologic disorders and any current medical problems or systemic medication. The University of Jena. Exclusion criteria were any history of dermatologic disorders and any current medical problems or systemic medication. The participants were not allowed to apply creams and ointments on the face or to take any oral or topical anti-oxidant supplementation 2 wk prior to and during the time of the study.

**Sebum collection**

Samples of sebum were collected from the forehead of the volunteers using Sebutapes® (Cuderm, Dallas, TX) as previously described (Thiele and Packer, 1999). Briefly, forehead skin was cleaned prior to sample acquisition using a sterile gauze ball (Gazin®, Lohmann & Rauscher International GmbH, Rengsdorf, Germany) soaked in 1 ml 70% ethanol solution. Each tape was weighed before and after sebum collection. The sebum collection time was 1 h for every tape. The average amount of sebum collected per Sebutape® in 1 h was 1.03 ± 0.4 mg (n = 202; mean ± SD). Immediately after collection and weighing, sebum-enriched tapes were irradiated with defined doses of UVB and UVA and, subsequently, stored in Eppendorf tubes at −80°C until further use.

**Irradiation protocol**

UVA radiation was performed using a Dermalight Ultra1 type UVAI 24 kW phototherapy system (Dr Hönle, Munich, Germany; spectrum 340–440 nm, irradiance 80 mW per cm² at a distance of 50 cm). UVB irradiation was carried out with a PL-S 9 W/12 (UV21) UVB light source (Philips, Aachen, Germany), with an emission peak at 363 nm. According to information supplied by the manufacturer, the UVC portion of this light source was less than 0.5%. The UVB irradiance was 0.33 mW per cm² as measured using a Waldmann UV meter (Waldmann, Villingen-Schwenningen, Germany). All in vivo irradiation experiments were carried out on ice.

**Sebum lipid extraction**

Sebum-enriched Sebutapes® were extracted in 1 ml HPLC grade ethanol by vortexing for 1 min in 1.5 ml Eppendorf tubes. Then, tapes were removed and the remainder centrifuged at 2920 × g and 4°C for 10 min 750 μl of each supernatant were transferred into an Eppendorf tube and directly subjected to HPLC analysis.

**In vivo UVA/UVB irradiation**

The foreheads of healthy volunteers (n = 4; all skin types II according to Fitzpatrick scale) were divided into two equal areas. Prior to suberythematogenous exposures to UVA (20 J per cm²), and UVB (60 mJ per cm²), respectively, SSL of the left forehead were collected using a sterile cotton swab (Medip® hygienic wooden applicators, Servoprax GmbH, Wesel, Germany) soaked in 100 μl ethanol.

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After UVA/UVB exposures, the same procedure was performed on the contralateral forehead area. Cotton applicator heads were transferred into 1.5 ml Eppendorf tubes and SSL extracted in 1 ml HPLC grade ethanol by vortexing for 1 min. Thereafter, cotton applicator heads were removed and samples centrifuged at 2920 × g and 4°C for 10 min. Supernatant aliquots of 250 μl were immediately subjected to HPLC analysis. As this cotton swab method involves the use of ethanol, a highly volatile organic solvent, accurate weighing of very small amounts of SSL is not possible. Thus, for standardization purposes, squalene monohydroperoxide (SqmOOH) amounts detected in SSL extracts were related to the colored amounts of squalene. In the case of the experiments represented by Fig 6, this is valid because we have demonstrated that squalene levels are not significantly depleted by 20 J per cm² UVA or 60 mJ per cm² UVB (Fig 1).

**HPLC analysis of squalene and SqmOOH in human sebum**

HPLC was performed using a Gynkotek HPLC system (Dionex-Soferon GmbH, Gernering, Germany). This system included an autosampler (Gina 50), pump (M480G), degasser, and UV/Vis detector (UVD 340, all from Gynkotek/Dionex-Soferon) and a Luna 3μ C18 column (250 × 4.6 mm, Phenomenex®, Hösbach, Germany). The mobile phase consisted of HPLC grade ethanol and methanol (1:1, v/v). The flow rate was 1.2 ml per min. Control standards of squalene were prepared from commercially available pure squalene and was detected by in line UV detection at 210 nm. Peak integration and quantitation was performed by Gynkotek Software 5.6 (Dionex-Soferon GmbH). For identification of unknown lipid peaks, fractions corresponding to these peaks (such as USLPP) were collected and subjected to further analysis. Atmospheric pressure chemical ionization mass spectrometry (APCI MS) spectra of squalene and USLPP.
were obtained on a PE ScieX API 165 single quadrupole mass spectrometer equipped with a Atmospheric Pressure Chemical Ionization (APCI) interface (Applied Biosystems, Langen, Germany). 1H nuclear magnetic resonance (NMR) spectra of USLPP were recorded on a Varian VXR 500 spectrometer equipped with a 2 mm probe, with approximately 100 resonance (NMR) spectra of USLPP were recorded on a Varian VXR 500 interface (Applied Biosystems, Langen, Germany). 

**Synthesis and purification of SqmOOH**

Squalene (200 g, 97%; Sigma-Aldrich) was dissolved in a mixture of methanol (40 ml) and benzene (160 ml), then rose Bengal sodium salt (150 mg) was added. The mixture was transferred to a rotating 1000 ml round flask, which was irradiated with a daylight bulb (250 W). The flask was kept at room temperature, and oxygen was bubbled through the reaction mixture at a flow rate of 15–20 l per h. After 8 h, the reaction was stopped and the solvent removed under reduced pressure. The residue was suspended in toluene (100 ml), and silica gel (40–63 μm; 150 g) was added for adsorption of rose Bengal. The suspension was left overnight, the solids filtered off and the solution evaporated under reduced pressure to provide an oily residue (207 g). A portion (957 g) of this residue was separated on a silica gel column (40–63 μm; 400 × 38 mm i.d.), eluted with benzene/ethyl acetate (93 : 7, v/v) with a flow rate of 7.5 ml per min. Fractions of 100 ml were collected, monitored by peroxide test and thin layer chromatography (TLC) analysis (details see below), and combined on the basis of a similar TLC pattern. The fraction containing the SqmOOH (elution volume 1200–2400 ml; 435 g) was further purified by preparative pressure liquid chromatography on a Lobar reversed phase (RP)-18 column (40–63 μm, 440 × 37 mm i.d.; Merck, Darmstadt, Germany). The preparative chromatography system consisted of a K-1001 HPLC pump, a K-2501 UV/Vis detector (both Knauer, Berlin, Germany), a six-port injector, a LKB SuperFrac fraction collector and a LKB Rec-2 chart recorder (both Pharmacia, Freiburg, Germany). Aliquots were dissolved in 20 ml of a methanol/ethanol mixture (1 : 1, v/v) and separated with the same eluent. Flow rate was 6 ml per min, and detection was at 230 nm. Fractions of 12 ml were collected and analyzed by on a Hypersil ODS HPLC column (5 μm, 250 × 4 mm i.d.) with methanol/ethanol (1 : 1, v/v) at a flow rate of 1 ml per min and detection at 210 nm. The HPLC system consisted of a HP 1050 pump, HP 1040M Series II photodiode array detector, and a HP Chemstation (Agilent, Waldbronn, Germany). Fractions were combined on the basis of the HPLC profiles. Purification of 18 g of crude SqmOOH afforded a purified SqmOOH mixture (500 mg; RP HPLC purity >95%). 1H NMR (400 MHz, CDCl3) δ: 1.29 (CH3-OOH), 1.56 (CH3), 1.95–2.07 (CH2=CH), 2.68 (HOOC-CH=CH=CH), 4.28 (–CH=OOH), 5.00 (CH3-C), 5.05 (–CH=CH=); 5.5–5.6 (CH=CH–CH=CH–OOH), 7.30 (C=OOH), 7.82 (C=OOH), 7.88 (C=OOH). APCI MS (positive ion mode) m/z 443 [M+H]+, 425 ([M+H]–18)+, 409 ([M+H]–34)+.

**High performance thin layer chromatography (HPTLC) analysis of sebum lipids and squalene hydroperoxides**

Analysis was carried out on silica gel 60 F254 coated HPTLC plates (Merck). The sample concentrations were 1 mg per ml for squalene, purified squalene hydroperoxides and sebum samples. For the synthesis squalene hydroperoxide mixture, sample concentrations for sebum samples were 10 mg per ml. Sample volumes of 6 μl (5 μl for squalene hydroperoxides from sebum) were applied on to the HPTLC plate with the aid of a AS 30 sample applicator (Desaga, Heidelberg, Germany). The plates were developed with benzene/ethyl acetate (93 : 7, v/v) over a distance of 9 cm. Visualization of compounds was performed using Godin's reagent (Godin, 1992). 1H NMR spectra were measured in CD3OD with presaturation of the residual solvent peak. For the 1D spectrum, 1054 transients were accumulated, and mild line broadening function (0.25 Hz) applied prior to Fourier transformation. The adiabatic total correlation spectroscopy (Petti et al, 2000) spectrum was recorded using a mixing time of 0.065 s. 1H (500 MHz, CDCl3) δ: see Fig 2. APCI MS (positive ion mode) m/z 443 [M+H]+, 425 ([M+H]–18)+, 409 ([M+H]–34)+.

**Figure 2. Identification of USLPP as squalene monohydroperoxide isomers in skin surface lipids**
Hamburger, 1996). A typical chromatogram obtained with UV detection at 210 nm is shown in Fig 2, together with APCI mass spectra of SqmOOH (peak at 4.50 min) and squalene (peak at 9.50 min), recorded on-line. The mass spectrum of SqmOOH showed a weak quasi molecular ion at m/z 443, and diagnostic fragment ions at m/z 425 and m/z 409 resulting from the elimination of water and hydrogen peroxide from the parent ion. In contrast, the spectrum of the squalene peak showed a single protonated ion at m/z 411. USLPP was purified by repeated injections of sebum samples. The small amount of \( \approx 100 \mu g \) of purified material available precluded extensive NMR experiments, and, in particular, the measurement of \(^{13}C\) spectra. The \(^1H\) signals in the ID spectrum were assigned with the aid of adiabatic H,H total correlation spectroscopy experiment (Peti et al, 2000), comparison with reference spectra of squalene, and known chemical shift rules (Pretsch et al, 2000). The assignments to the three different hemiterpene substructures occurring in the SqmOOH mixture are shown in Fig 3.

In view of structural confirmation of USLPP and for further investigation of its biologic properties, access to larger amounts was required. SqmOOH was synthesized by photo-oxidation of squalene using a white light source and rose Bengal as a photosensitizer. Column chromatography of the crude squalene hydroperoxide reaction mixture on silica gel afforded a SqmOOH fraction, which was purified by RP chromatography to 97% purity. The analytical HPLC chromatogram of the synthetic material is shown in Fig 2(A). The retention time in RP HPLC was identical with that of an authentic sample of USLPP in irradiated sebum (Fig 2(A)). \(^1H\) NMR, APCI MS, and ESI MS data were comparable with those obtained for USLPP, which, hence, was identified as SqmOOH.

UV dose-dependent increase of SqmOOH in human sebum A negative correlation between SqmOOH and squalene was found upon exposures of sebum to increasing doses of UVA (correlation coefficient \( r = -0.97; p < 0.005 \); Fig 4). Very low baseline levels of SqmOOH (0.765 \pm 0.23 nmol SqmOOH per mg sebum, Fig 5) were observed in samples of facial sebum collected by using Sebutapes\(^R\). Owing to a lack of significant squalene depletion (Fig 1A), and only very moderate formation of SqmOOH (Fig 5A) detected upon UBV exposures, no such correlation was obtained for UBV. SqmOOH levels increased slightly after exposure to UBV, however, more dramatically to UVA (Fig 5). Remarkably, formation of SqmOOH was found to be a highly sensitive marker of photo-oxidation particularly when compared with

Statistical analysis Statistical analysis was carried out by repeated measures paired one-way ANOVA (Graph Pad Instat\(^R\), Graph Pad Software, Inc., San Diego, CA) and a Bonferroni post-test.

RESULTS

Wavelength and concentration-dependent depletion of squalene Human sebum squalene levels were not significantly depleted by UBV irradiation (Fig 4A), but were decreased by UVA in a concentration-dependent manner (Fig 1B). A significant reduction of sebum squalene levels to 71% of the initial squalene level was found after a single dose of 40 J per cm\(^2\) UVA (n = 10, p < 0.05).

Identification of USLPP as SqmOOH isomers Parallel to the decrease of squalene the generation of an initially USLPP was observed (Fig 2). To identify USLPP, samples of UV-irradiated sebum were analyzed by APCI MS. In APCI, analyte ions are generated at atmospheric pressure via chemical ionization in the gas phase. This ionization technique is suited for HPLC-MS analysis of lipophilic and poorly functionalized molecules such as squalene and other isoprenoids (Zhou and Hamburger, 1996). A typical chromatogram obtained with UV
vitro, genic doses of UV A (5 J per cm$^2$) increased SqmOOH levels to a slight increase of SqmOOH levels (UVB: 4.9 ± 2.8 pmol SqmOOH per mg sebum; Fig 5A). Using a different method for extraction of SSL by cotton swabs soaked in ethanol, SqmOOH levels were determined in human SSL prior to and after in vivo irradiation. The low baseline levels (31 ± 10 pmol SqmOOH per mmol squalene) confirmed findings from the in vitro experiments using Sebutapes.

**Figure 5.** Dose dependent increase of squalene monohydroperoxides (SqmOOH) in human sebum immediately after irradiation with UVB (A) or UV A (B). Sebum was collected from healthy volunteers using Sebutapes, irradiated and immediately subjected to lipid extraction. Subsequently, SqmOOH levels were measured by HPLC using UV detection as described. Shown are means ± SEM, (A) n = 7, (B) n = 10, ***p < 0.001 versus untreated controls.

**Figure 6.** Squalene monohydroperoxides are generated in skin surface lipids upon UV A exposure of human skin in vivo. Detection of squalene depletion: even very low suberythematogenic doses of UVB (60 mJ/cm$^2$ UVB, and 20 J/cm$^2$ UV A, respectively). After irradiation, ethanol extracts from Sebutapes (n = 10; sebum) or human forehead skin (n= 4; skin surface lipids) were analyzed for SqmOOH content as described. Shown are means ± SD.

**Figure 7.** TLC system for the detection of SqmOOH in sebum.

Developing a TLC system for the detection of SqmOOH in sebum: Preliminary tests with various solvent mixtures showed that best separations were achieved by combination of normal phase TLC on silica gel with benzene/ethyl acetate mixtures as eluents. Benzene/ethyl acetate (93: 7, v/v) was finally chosen, as a suitable fingerprint of the entire spectrum of sebum lipids was obtained by single development. Detection of SqmOOH in UV A-irradiated sebum was straightforward without the need for tedious sample preparation prior to analysis by HPLC or TLC. The presence of SqmOOH in sebum was detected in as little as 60 μg of material. Lane 1 in Fig 7(A) shows a representative chromatogram of untreated sebum. The large spot at the top of the chromatogram (Rf 0.7) represents squalene. Other major spots at Rf 0.6 and 0.4 corresponded to sterol esters and fatty acids, the spot near the starting line (Rf 0.1) to polar lipids. Tentative identifications were based on chromatographic mobility relative to squalene and SqmOOH, and on the characteristic staining of fatty acids and isoprenoids with Godin’s reagent. The UV-irradiated sebum (lane 2) showed a distinctly different pattern in the Rf range between 0.23 and 0.37, where several bands appeared. A purified sample of “USLPP” (lane 3) produced an identical pattern of at least five bands. The chromatogram of synthetic SqmOOH mixture (lane 4) had a fingerprint comparable with that of “USLPP”. Given the much higher concentration applied here, minor spots from polar side products were detected between Rf 0 and 0.17. The staining reagent used in Fig 7(A), Godin’s reagent, is a rather unspecific but all-purpose reagent for staining of isoprenoids and cholesterol and cholesterol-like lipids. Compounds containing a hydroperoxy moiety were selectively stained with a redox dye. Figure 7(B) shows the identical chromatogram stained with N,N-DPDD reagent (Smith and Hill, 1972). Peroxides oxidize the colorless aromatic diamine to a pink semiquinone diimine (Jork et al, 1993). Lanes 2–4 show a pattern of stained bands at Rf 0.23–0.37, confirming that they all bear a hydroperoxy function. No N,N-DPDD-positive bands are observed on lanes 1 (nonirradiated sebum) and 5 (squalene). The colored bands at Rf 0–0.17 in lane 4 confirm that the synthetic SqmOOH contained minor amounts of the more polar dihydroperoxides and trihydroperoxides.

**DISCUSSION**

This study demonstrates that: (i) the identity of the previously reported human sebum photo-oxidation product USLPP is a mixture of defined SqmOOH deriving from sebaceous squalene; (ii) SqmOOH formation in human skin is strongly induced by UVA radiation and correlates well with squalene depletion; (iii) whereas physiologic baseline levels of SqmOOH in human skin are only slightly above detection limits, SqmOOH levels are strongly increased by suberythematogenic doses of UVA both in vitro and in vivo; and (iv) HPLC results could be complemented by a simple TLC method useful for rapid screening of lipid per-oxide formation in human sebum/SSL.

Whereas this study has focused on the major sebum lipid, squalene, the susceptibility of other SSL to oxidative stress has been investigated by other groups. In principle, compounds containing double bonds, such as squalene and cholesterol are susceptible to photo-oxidation. Accordingly, analysis of SSL extracted from human skin after solar UV exposure exhibited increased levels of cholesterol 7-hydroperoxides (Yamazaki et al, 1999). Compared with squalene, however, cholesterol concentrations in human sebum are relatively low (Nicolaides, 1974). Little is
known about the susceptibility of human skin wax esters to photo-oxidation. Typically, they contain long chain saturated fatty acids, which are not very susceptible to photo-oxidation.

Nevertheless, it has been demonstrated that waxes derived from plant outer cell wall surfaces can be oxidized by other environmental oxidants, such as ozone (Cross et al., 1998).

In previously published studies SSL peroxidation products have been measured indirectly using the thiorbarbituric acid assay (Ohsawa et al., 1984; Nazzaro-Poro et al., 1986; Dennis and Shibamoto, 1989), TLC (Nazzaro-Poro et al., 1986) or chemiluminescence combined with high pressure chromatography (Someya et al., 1994; Chiba et al., 1999). In these investigations, the exact identity of SSL/squalene peroxidation products actually formed on human skin as well as the wavelength dependency under physiologic conditions remained unclear (Chiba et al., 1999). In the present, a simple and fast HPLC-based method was developed for simultaneous detection of squalene and SqmOOH using UV detection. Spectroscopic data and chromatographic behavior of SqmOOH were identical to those of USLPP and hence corroborated the identity of the compound isolated from human sebum. Whereas SqmOOH eluted as a single peak from the RP HPLC column, a separation of at least five of six possible SqmOOH isomers was achieved by normal phase chromatography on a silica gel HPTLC plate (Fig 7). Whereas peroxidation of squalene induces a mixture of up to six positional isomers, NMR assignments were only possible for partial structures. The H chemical shifts were in accord with the three partial structures occurring in SqmOOH (Fig 3). The TLC assay developed here provided a good resolution over the entire polarity range of sebum lipids, and clear separation of SqmOOH from other sebum constituents. The combination of two staining reagents, Godin’s reagent and N,N-DPDD, a selective stain for peroxides, provided valuable complementary information on the composition and structure of sebum lipids. SqmOOH bands were readily identified, and traces of hydroperoxides were not masked by other lipids, as it may occur with general purpose stains. TLC methods for skin lipid analysis have been reported previously; however, the focus was on stratum corneum lipids (Bonte et al., 1995; Lavrijsen et al., 1995; Bleck et al., 1999) and the protocols involved time-consuming instrumented development techniques (Jaecklein and Issaq, 1988) and unspecified staining reagents. Previously, Nazzaro-Poro et al. (1986) presented a similar HPTLC detection method using a different analog to N,N-DPDD, DEPD (N,N-diethyl-1,4-phénylen-diámonium sulfit) as staining reagent for investigation of the lipoxygenase activity of *Pityrosporum* in SSL. The HPTLC assay developed here can be employed as a rapid and simple screen for SqmOOH in sebum, and as such, is a useful complement to the HPLC method presented in this study.

Previously, *in vitro* studies suggested that both UVB and UVA radiation of squalene induce squalene hydroperoxides (Ohsawa et al., 1984; Saint-Leger et al., 1986a; Dennis and Shibamoto, 1989; Kohno et al., 1995); however, in these studies unphysiologically high doses of UVB were applied. Similarly, Picardo et al. (1991b) reported depletion of squalene and the generation of unspecified SSL peroxidation products after exposure to high doses of solar-simulated irradiation and attributed these effects mostly to UVB exposure. Our study demonstrates that, under physiologically relevant conditions using suberythematogenic doses, UVA induces SqmOOH at rates at least one order of magnitude higher than UVB (Figs 4, 5, 6). Accordingly, squalene levels in human sebum are strongly depleted by suberythematogenic doses of UVA (Fig 1). Hence, it may be proposed that previously reported UVB-induced formation of squalene/SSL peroxidation products is due to “contaminating” UVA irradiation. In fact, nearly all “UVB lamps” used in photobiology will emit UVB and UVA (Difley, 2002). The inverse correlation between squalene depletion and SqmOOH formation (Fig 4) further confirms that natural sebaceous gland squalene is the substrate for SqmOOH formation observed upon UVA exposure. As UVA-induced oxidative stress in skin is believed to be mediated via reactive oxygen species generated by photosensitizers (Thiele et al., 1998), these data strongly point to an involvement of physiologic photosensitizers. Accordingly, exposure of purified squalene alone to suberythematogenic doses of UVB or UVA does not yield detectable levels of SqmOOH (data not shown). In the presence of the photosensitizer rose Bengal, however, exposure to visible light yields high amounts of SqmOOH. Hence, this method was used in this study to generate SqmOOH standards (Figs 2 and 7). As the latter finding suggests the involvement of singlet oxygen (Klotz et al., 1997), it is likely that UVA-induced SqmOOH formation *in vivo* is also mediated via singlet oxygen (Sies, 1986). Intriguingly, *in vitro* data demonstrate that squalene itself is a good quencher of singlet oxygen (Kohno et al., 1995). With respect to the pathophysiology of acne, bacterial porphyrins were proposed to mediate squalene oxidation in skin (Saint-Leger et al., 1986b). Porphyrins are readily excited by light with a wavelength of

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**Figure 7.** HPTLC analysis confirming generation of specific squalene monohydroperoxide isomers in UVA irradiated human sebum. Visualization of analytes with Godin’s reagent (A); with N,N-DPDD reagent (B). The following samples were separated: Lane 1: sebum taken without UV irradiation; 2: sebum taken after irradiation with 80 J/cm2 UVA; 3: SqmOOH, isolated by collection of HPLC fraction from irradiated sebum; 4: synthetic SqmOOH; 5: squalene. The amount of sebum separated in lanes 1 and 2 is 60 μg. SQ: squalene; SE: sterol esters; FA: fatty acids; PL: polar lipids; X: SqmOOH isomers.
about 400 nm (the Soret band) and, in the presence of oxygen, will cause photodynamic effects mediated by singlet oxygen (Spikes, 1975). The involvement of endogenous photosensitizers, such as porphyrins, and subsequent formation of singlet oxygen would explain the wavelength-dependent formation of SqnOOH with higher sensitivity in the UVA and rather a low sensitivity in the UVB range.

To date, little is known about the in vivo relevance of squalene hydroperoxide formation in human skin upon physiologically relevant doses of UV exposure. It has been suggested that lipid peroxides of SLL and especially of squalene may play an important part in the pathology of acne (Saint-Leger et al., 1986a, b; Chiba et al., 2000), sunburn (Dennis and Shibamoto, 1989), pityriasis versicolor (Nazzaro-Poro et al., 1986), and skin aging (Chiba et al., 1999).

Picardo et al. (1991a) reported an induction of immune tolerance to contact hypersensitivity in a mice ear model after applying a mixture of squalene photo-oxidation products, which were, however, exclusively attributed to UVB irradiation. Saint-Leger et al. (1986b) detected squalene oxidation products in human comedones and suggested that squalene oxides may play an important part in the pathology of comedones. Interestingly, repetitive topical application of squalene hydroperoxides was demonstrated to induce comedone formation in albino rabbit ears and to increase skin roughness as well as wrinkle formation in hairless mouse skin (Chiba et al. 1999, 2000). Furthermore, Chiba et al. (2001) reported a depletion of glutathione and an increased sensitivity to SqnOOH induced oxidative stress in cultivated human fibroblasts. To the best of our knowledge, the role of squalene peroxides in other photoinduced or photoaggravated diseases, such as polymorphic light eruption, lupus erythematosus, or Darier's disease, has not yet been investigated. As topical application of anti-oxidants appears to reduce the development and severity of polymorphic light eruption (Hadhshieh et al., 1997), however, a reduced formation of SqnOOH may be involved in the protective mechanism.

This study has further characterized and quantified specific squalene hydroperoxide isomers occurring upon defined doses of UVA in vitro and in vivo. In particular, the present results demonstrate that in unirradiated skin, baseline levels of SqnOOH are very low, whereas physiologically relevant UVA exposure increases the concentration of this compound between one and two orders of magnitude (Figs 5 and 6). This is remarkable as conventional methods for the detection of lipid peroxidation, in particular those measuring the lipid peroxidation product malondialdehyde, showed very low sensitivity to suberythemalogenic doses of UVA or UVB in human skin in vivo (Thiele et al., 1998, 2001). In conclusion, specific SqnOOH isomers were identified as highly UVA sensitive SSL breakdown products, which may serve as markers for photo-oxidative stress in vitro and in vivo. Future studies will have to focus on the investigation of the epidermal/sebaceous gland penetration of SqnOOH as well as their biologic role in skin.

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