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7-Dehydrocholesterol Enhances Ultraviolet A-Induced Oxidative Stress in Keratinocytes: Roles of NADPH Oxidase, Mitochondria and Lipid Rafts

Antonio Valencia, Anpuchchelvi Rajadurai, A. Bjorn Carle, and Irene E. Kochevar*

Wellman Center for Photomedicine, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, USA

Abstract

Long wavelength solar UVA radiation stimulates formation of reactive oxygen species (ROS) and prostaglandin E₂ (PGE₂), which are involved in skin photosensitivity and tumor promotion. High levels of 7-dehydrocholesterol (7-DHC), the precursor to cholesterol, cause exaggerated photosensitivity to UVA in patients with Smith-Lemli-Opitz syndrome (SLOS). Partially replacing cholesterol with 7-DHC in keratinocytes rapidly (<5 min) increased UVA-induced ROS, intracellular calcium, phospholipase A₂ activity, PGE₂, and NADPH oxidase activity. UVA-induced ROS and PGE₂ production were inhibited in these cells by depleting the Nox1 subunit of NADPH oxidase using siRNA or using a mitochondrial radical quencher, MitoQ. Partial replacement of cholesterol with 7-DHC also disrupted membrane lipid raft domains, although depletion of cholesterol, which also disrupts lipid rafts, did not affect UVA-induced increases in ROS and PGE₂. Phospholipid liposomes containing 7-DHC were more rapidly oxidized by a free radical mechanism than those

*Corresponding author Wellman Center for Photomedicine, Massachusetts General Hospital, Thier-224. 55 Fruit Street, Boston, MA 02114, USA. Tel: (617) 726-8828, Fax: (617) 726-3192, E-mail: kochevar@helix.mgh.harvard.edu

Abbreviations

AY

trans-1, 4 bis(2-chlorobenzyl-aminoethyl) cyclohexane dihydrochloride

CD

methyl- β -cyclodextrin; carboxy-H₂DCFDA, 5-(and-6)-carboxy-2', 7'-dichlorodihydrofluorescein diacetate

Chol

cholesterol

cPLA₂

cytosolic phospholipase A₂

7-DHC

7-dehydrocholesterol

DPI

diphenyleneiodonium

HK

human keratinocytes

SLOS

Smith-Lemli-Opitz syndrome

UVA

ultraviolet A radiation (320–400 nm)

PGE₂

prostaglandin E₂

containing cholesterol. These results indicate that 7-DHC enhances rapid UVA-induced ROS and PGE₂ formation by enhancing free radical-mediated membrane lipid oxidation and suggests that this mechanism might underlie the UVA-photosensitivity in SLOS.

Keywords

oxidative stress; ultraviolet radiation; reactive oxygen species; Smith-Lemli-Opitz syndrome; NADPH oxidase; lipid rafts; keratinocytes

Introduction

Ultraviolet A (UVA, 320-400 nm) radiation from the sun induces formation of reactive oxygen species (ROS) in keratinocytes, the major cell type in the epidermis. UVA-induced ROS initiate diverse responses in keratinocytes including synthesis of pro-inflammatory arachidonic acid metabolites, which are believed to contribute to promotion in the development of UV-induced non-melanoma cancers [1], the most commonly diagnosed neoplasm. The molecular mechanisms by which UVA-induced ROS initiate processes in skin cells include inactivation of tyrosine phosphatases by oxidation of an essential cysteine residue [2] and by calpain-mediated cleavage [3], activation of AP-1, AP-2, STAT1 and other transcription factors [4, 5], activation of growth factor receptors [6] and release of ceramide from membrane sphingomyelin [7]. ROS formation is initiated when chromophores in the cells absorb the energy of UVA photons. In general, the photoexcited chromophores rapidly transfer the energy to oxygen to form very short-lived singlet oxygen or react with other cellular molecules by electron transfer to eventually form superoxide anion [4,8]. Subsequent biochemical steps amplify the ROS, which often are responsible for the cellular responses. Although several types of ROS have been implicated in these responses, the cellular source(s) for the ROS produced after UVA exposure have not been defined and include NADPH oxidase and mitochondria.

Oxidation of membrane phospholipids and cholesterol are well-recognized primary effects of ROS in cells [9]. The initial oxidation products are fatty acid and sterol hydroperoxides, which decompose in the presence low levels of transition metal ions, e.g. Fe²⁺, to form free radicals that initiate further lipid oxidation by a chain reaction. Oxidized lipids and sterols influence signal transduction involving the plasma membrane as well as releasing products that modify signaling [10,11]. Oxidation of cholesterol can have particularly significant effects on UVA-initiated signal transduction processes initiated in the lipid raft domains of the plasma membrane because cholesterol is a major lipid component of lipid rafts. Many GPI-anchored and other membrane proteins involved in cell signaling are present in these tightly packed, highly ordered domains, leading to the concept that lipid rafts play an important role in the process of signal transduction [12-14]. Cholesterol oxidation products differ in molecular shape from cholesterol and are more polar. Consequently, these cholesterol derivatives do not support the formation of lipid rafts in model membranes [15,16]. High doses of UVA induce inflammation in normal skin at least partially by processes involving plasma membrane signaling suggesting that alteration in lipid rafts might influence the response of keratinocytes to UVA.

Recently, patients suffering from a congenital disorder, Smith-Lemli-Opitz syndrome (SLOS), were reported to show a rapid (< 5 min) and highly exaggerated response to solar UVA radiation that lasts up to 48 h [17,18]. The multiple congenital anomalies of SLOS patients are caused by mutations in 7-dehydrocholesterol reductase (Δ^7 -reductase), which converts 7-dehydrocholesterol (7-DHC) to cholesterol (Chol) resulting in low levels of Chol and extremely high levels of 7-DHC in the plasma of SLOS patients [19,20]. Incorporating high levels of Chol into the diet of SLOS patients has been reported to decrease their photosensitivity [21].

Since 7-DHC does not absorb UVA radiation (Fig. 1A), which could initiate photochemical reactions leading to photosensitivity, it appears to cause UVA skin photosensitivity by a unique, but currently unknown, mechanism. The structure of 7-DHC is very similar to that of Chol (Fig. 1B). The additional double bond in the C7-C8 position forms a conjugated system with the C5-C6 double bond present in Chol. Because the allylic hydrogens associated with this conjugated system are likely to be more rapidly abstracted than those in Chol, 7-DHC may be more rapidly oxidized than Chol. Consequently, in addition to a potential effect on signal transduction processes in lipid rafts, 7-DHC may enhance UVA-initiated photoprocesses in keratinocytes by increasing free radical chain oxidation of the membrane lipids.

In this study we tested the hypothesis that partially replacing Chol with 7-DHC in keratinocytes enhances rapid UVA-induced synthesis of prostaglandin E₂ (PGE₂), which might account for the rapid UVA photosensitivity in SLOS patients. We demonstrate that partially replacing Chol with 7-DHC greatly increases the rapid production of ROS after UVA treatment and that the major initial source of these ROS is NADPH oxidase containing the Nox 1 homologue of the catalytic subunit. A quencher of mitochondrial radicals, MitoQ, also partially blocks UVA-induced ROS. Partial exchange of 7-DHC for Chol disrupted membrane lipid raft domains, despite the nearly identical chemical structures of 7-DHC and Chol. However, depletion of Chol, which also disrupts lipid rafts, did not enhance UVA-induced ROS formation. Liposomes containing 7-DHC were more rapidly oxidized than those containing Chol. Taken together, these results indicate that the more rapid oxidation of 7-DHC, rather than its ability to alter membrane signal transduction in lipid rafts, accounts for the enhanced ROS production after UVA. The ROS are essential for a rapid UVA-induced increase in intracellular calcium and subsequent activation of PLA₂ leading to rapid production of PGE₂, a potential mediator of UVA-initiated skin damage.

Materials and methods

Human keratinocyte cultures

Human keratinocytes (HK), immortalized by expression of the catalytic subunit of telomerase, were a gift from Dr. James Rheinwald (NIH Harvard Skin Disease Research Center). Cells were plated in serum-free keratinocyte medium (SFKM) with phenol red (Gibco Invitrogen) supplemented with recombinant EGF (2.5 µg/500 ml from Gibco), bovine pituitary extract (25 mg/500 ml, Gibco), 0.3 mM CaCl₂, 50 mg streptomycin and 50,000 units of penicillin per 500 ml of media (Sigma, St. Louis, MO). HK were incubated at 37°C, 5% CO₂ and medium was replaced every 48 h until the cells reached 50-60% confluence.

Cholesterol was partially replaced by 7-DHC using our previously described procedure [22]. Briefly, HK were treated for 24 h with 1 µg/ml AY9944 [trans-1, 4 bis(2-chlorobenzyl-aminoethyl) cyclohexane dihydrochloride from Sigma] to block cholesterol synthesis. Then Chol was partially removed using 5 mM methyl-β-cyclodextrin (CD) for 1 h. Finally, 5 µM of 7-DHC (Steraloids Inc., Newport, RI) was added for 24 h. These cells are referred to as SLO-HK. For some experiments 10 µM Chol (Steraloids Inc.) was added instead of 7-DHC after the first two steps in order to replace the extracted Chol. These control cells are referred to as Chol-HK. Keratinocytes were also depleted of Chol using AY9944 and CD, without addition of Chol or 7-DHC; these cells are called AY+CD. Cell viability was measured after depleting Chol using 1-10 mM CD, and 5 mM was chosen because it showed good Chol depletion with minimal loss of cell viability. Sterols were prepared in absolute ethanol and added to cells to give a final ethanol concentration of 0.1%. This ethanol concentration was not cytotoxic.

UVA irradiation

The fluorescent UVA broadband lamps (320-420 nm, PUVA 180; Herbert Waldman, Werk für Lichttechnik Schwenningen, Germany) used had an irradiance of 5.1 mW/cm². Cells were irradiated in HBSS after removing the culture plate lids and the temperature remained ~32° C. Immediately after irradiation medium was replaced with fresh SFKM medium or the appropriate treatment medium.

Detection of reactive oxygen species

Cells that were treated to produce SLO-HK, AY + CD, Chol-HK or left untreated (HK) were incubated for 30 min at 37°C with 5 µM of carboxy-H₂DCFDA [5-(and-6)-carboxy-2', 7'-dichlorodihydrofluorescein diacetate, Molecular Probes Inc.] without AY in 12- and 6-multiwell plates. Cells were washed twice with HBSS, covered with HBSS (without AY) and then were irradiated with 1 J/cm² UVA (~180 sec exposure). For ROS measurements at 60 min and longer times after UVA, carboxy-H₂DCFDA was added after the irradiation, keeping the incubation time at 30 min before measurements. Our previous study indicated that the presence of carboxy-H₂DCFDA during the irradiation did not influence the level of fluorescence after irradiation [22]. The fluorescence was detected using a dual scanning microplate spectrofluorometer (Spectra MAX Gemini EM, Molecular Devices) with 480 nm excitation and 530 nm emission.

Preparation of cell fractions

Cellular homogenates from 3×10⁶ cells per sample were obtained in lysis buffer (8 parts of 150 mM K, Na phosphate buffer pH 7.4, 1 mM MgCl₂, 1 mM EGTA, 2 mM NaN₃, 1 mM DTT and 2 parts of glycerol containing 50 mM octylglycoside). Lysates were centrifuged at 1000 rpm for 10 minutes at 4°C. The supernatant was centrifuged at 25,000 rpm in a Beckman Coulter Optima™ L-90K Ultracentrifuge using a NVT 90 rotor for 1 h at 4°C. The pellet was recovered in resuspension buffer (65 mM K, Na phosphate buffer pH 7.4, 1 mM MgCl₂, 1 mM EGTA, 2 mM NaN₃, 1 mM DTT, 20 µg/ml leupeptin, 10 µg/ml pepstatin A, 10 µg/ml aprotinin and 2 mM PMSF) and used as the membrane fraction. The supernatant was centrifuged at 65,000 rpm using the NVT 90 rotor for 1h at 4°C. The supernatant cytosolic fraction was used for determination of NADPH oxidase activity. Protein content was determined by Bradford assay.

NADPH oxidase activity assay

The SOD-inhibitable activity of NADH oxidase was determined by ferricytochrome c reduction in octylglycoside-containing buffer [23]. Membrane and cytosolic fractions (20 and 60 µg of protein per fraction, respectively) were incubated in the reaction mixture (0.1 mM cytochrome c, 65 mM K, Na phosphate buffer pH 6.8, 2 mM EGTA, 1 mM MgCl₂ and 10 µM FAD) plus 100 µM of SDS for 2 min at 24°C. Then, superoxide anion production from NADPH oxidase was induced by addition of 0.2 mM of NADPH. The absorbance of reduced cytochrome c was measured at 550 nm using a spectrophotometer (Agilent 8453 UV-visible Spectroscopy System, Agilent Technologies, Foster City, CA). Results are expressed as absorbance of cytochrome c reduced at 550 nm.

Silencing Nox1 by RNA interference

Specific small interfering RNA (Stealth™-siRNA duplex oligoribonucleotides from Invitrogen™ Life Technologies, Carlsbad, CA) were designed by selecting two different duplex sequences of the human Nox1 gene, which is the catalytic subunit of a non-phagocytic NADPH oxidase expressed by keratinocytes [24,25]. The first duplex primer used for Nox1 was designated as Nox1-A (sense 5'-ACAAUAGCCUUGAUUCUCAUGGUA-3', anti-

sense 5'-UUACCAUGAGAAUCAAGGCUAUUGU-3') starting at 750 bp, and the second duplex primer was designated as Nox1-B (sense 5'-GCAUAUUGUUGGUCAUGCAGCAUU-3', anti-sense 5'-AAUGCUGCAUGACCAACAAUUAUUGC-3') located at 1642 bp. A scrambled StealthTM siRNA duplex for Nox1 (sense 5'-ACACCGAAGUUUCUUGUACGUAAA-3', anti-sense 5'-UUAUACGUACAAGAAACUUCGGUGU-3') was used as a negative control. Silencer^R FAM labeled negative control siRNA (Ambion, Austin, TX) was used to determine the transfection efficiency.

To achieve optimal transfection efficiency, various parameters, including the amounts of transfection reagent (Lipofectamine-2000TM from InvitrogenTM Life Technologies), siRNA, cell density, and the length of exposure of cells to Lipofectamine2000TM-siRNA complexes, were optimized. At 24 h before transfection, HK were transferred onto six-well plates (8 × 10⁵ cells per well) and transfected with 100 nM of each StealthTM-siRNA duplex using Lipofectamine-2000TM transfection reagent for 4 h in serum-reduced media (OptiMem from Gibco Invitrogen) without antibiotics. Then, complete SFKM medium was added to the HK. Gene silencing was monitored after incubation for 72 h, and a maximal decrease in Nox1 expression was observed at 48 h with both Nox1-A and Nox1-B primers. Transfection efficiency was monitored by flow cytometry based on FAM-labeled transfected cells, resulted in 75% transfection efficiency with Nox1-A StealthTM-siRNA and 70% with Nox1-B StealthTM-siRNA. Cell death was measured by flow cytometry using 7-amino-actinomycin D, an early apoptotic marker that intercalates in the DNA of apoptotic cells, resulting in 9% and 13% cell death for Nox1-A and Nox1-B respectively. SLO-HK were produced as described above with AY9944 treatment was begun 8 h after the transfection, followed by CD and 7-DHC treatments.

Analysis of lipid raft aggregation by immunofluorescence

Lipid rafts were analyzed by confocal microscopy using commercial kit from Molecular Probes Inc. (Vybrant® Alexa Fluor® 594 Lipid Raft Labeling kit). Briefly, fluorescein-labeled cholera toxin subunit B (CT-B) is allowed to bind to ganglioside GM1 that selectively partitions into lipid raft domains. Cells were then treated with an anti-CT-B antibody that crosslinks the CT-B that are in proximity in lipid rafts, thus forming larger patches that can be detected by fluorescence microscopy. HK were plated in 8-chamber pre-coated glass slides (Lab-Tek II Chamber Slides, Nalgen Nunc Intl., Naperville, IL) and treated as described above to obtain the SLO-HK, Chol-HK or AY+CD. Cells (~20,000 cells) were incubated with 1 µg/ml fluorescent CT-B in pre-chilled complete KSFM for 10 minutes 4°C. After washing the cells twice with HBSS, crosslinking the fluorescent CT-B-labeled lipid rafts with the anti-CT-B was done by incubating the anti-CT-B in pre-chilled complete KSFM (200-fold dilution) for 15 min at 4°C. Cells were washed twice with HBSS and fixed in paraformaldehyde (4%) for 15 min at 4°C followed by cell permeabilization with 0.1% Triton X-100 in PBS. Cells were washed twice with PBS and blocked for 1 h in blocking solution (PBS supplemented with 4% normal goat serum; Jackson Immuno Research Laboratories Inc., West Grove PA). Primary antibodies against caveolin-1 (Cell Signaling, Beverly MA) dilution 1:1000 and flotillin-2 (Santa Cruz Biotechnology Inc.) dilution 1:500 were incubated in blocking solution overnight at 4°C. Secondary antibody anti-rabbit IgG-FITC was used at 1:1000 dilution in blocking solution for 2 h at room temperature, followed with three washes with PBS. Finally cells were mounted with ProLong (Invitrogen). Co-localization of caveolin-1 or flotillin-2 with fluorescent CT-B labeled lipid rafts was analyzed from individual images obtained using a Bio-Rad Radiance 2100 confocal laser-scanning microscope with krypton-argon and blue diode lasers. Images were acquired through a 60x Nikon Plan Apo objective (numerical aperture 1.4) with oil immersion on an inverted Nikon Eclipse TE300 fluorescent microscope

using 485/525 nm excitation-emission for caveolin-1 and flotillin-2, and 590/617 nm excitation-emission for fluorescent CT-B-labeled lipid rafts. Green fluorescence indicates the presence of caveolin-1 and flotillin-2, and red fluorescence indicates lipid rafts. As a negative control, co-immunostaining of CT-B plus Na⁺/K⁺-ATPase (a non-lipid raft associated protein) was performed using 1:1000 dilution of monoclonal anti-Na⁺/K⁺-ATPase (clone XVIF9-G10 obtained from Affinity Bioreagents, Inc., Golden, CO.) and 1:2000 anti-mouse IgG-biotinyl as the secondary antibody.

Fractionation of detergent resistant membranes (DRM)

A biochemical fractionation of cells by floating sucrose gradient was used as previously described for DRM analysis [14,26]. Briefly, cells grown in 15 cm dishes were treated as described above to obtain SLO-HK, Chol-HK and HK ($4-5 \times 10^6$ cells per plate). After washing with ice-cold DEB (10 mM Tris, 150mM NaCl, pH 7.5), cells were recovered in TEB (DEB plus 1% Triton X-100 and EDTA-free Complete protease inhibitor tablets, Roche Diagnostics GmbH, Mannheim, Germany). The cell lysate was homogenate using a Dounce homogenizer, then nuclei and heavy cellular material were discarded by low speed centrifugation for 5 min. Supernatant was mixed in equal volume with 80% sucrose in DEB and added to the bottom of 12 ml centrifuge tube (Ultra-clear™ centrifuges tubes 14 x 89 mm from Beckman, Fullerton, CA). A linear sucrose gradient 40%-5% was created using a gradient maker SG15 (Hoefer Inc., San Francisco, CA) and poured over the top of cell homogenate. Samples were centrifuged at 39,000 rpm in a Beckman Coulter Optima™ L-90K Ultracentrifuge using a SW41 rotor for 3-6 h at 2°C. Then, 1 ml fractions were recovered and centrifuged at 56,000 rpm using the NVT 90 rotor in the Beckman Coulter Optima™ L-90K Ultracentrifuge for 1 h at 2°C in polyallomer centrifuge tubes (OptiSeal™ 13 x 48 mm, Beckman, CA). The pellet was recovered in 30-50 µl of the lysis buffer described above.

Liposome oxidation

Liposomes were prepared from Chol (or 7-DHC) plus 1-oleoyl-2-palmitoyl-sn-glycero-3-phosphatidylcholine (OPPC) (50:50 molar ratio), or from Chol (or 7-DHC) plus 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphatidylcholine (SAPC) plus OPPC (50:25:25 molar ratio) in ethanol by slow injection into rapidly stirred PBS at 40°C. Total lipid concentration in the liposome suspension was 1.32 mM. OPPC and SAPC were from Avanti Polar Lipids Inc. (Alabaster, AL). Liposomes (final concentration = 0.66 mM lipid) were oxidized using the water-soluble radical initiator AMPA [2,2'-azobis(2-methylpropionamidine) dichloride] (Aldrich) (20 mM) in PBS at 40°C. The partial pressure of dissolved oxygen was measured with a Licox pO₂ Monitor (GMS, Gesellschaft für Medizinische Sondentechnik, Kiel-Mielkendorf, Germany), using a Revoxode Oxygen Catheter-Micro-Probe (Licox C1.R) and corrected for temperature.

Prostaglandin E₂ release

Prostaglandin E₂ (PGE₂) release was assayed using a commercial EIA kit (Cayman Chemical; Ann Arbor, MI). The PGE₂ release was detected by the absorption of 5-thio-2-nitrobenzoic acid at 412 nm, which is the product of non-enzymatic reaction between PGE₂-acetylcholinesterase and acetylthiolcholine plus 5-5'-dithio-bis-(2-nitrobenzoic acid). For these experiments 6-well plates were used containing ≈400,000 cells at 80% confluence. Results are express as pg of PGE₂ released per cell.

Phospholipase A₂ activity

Activity of the cytosolic, calcium dependent phospholipase A₂ (cPLA₂ Type IV) was measured using a commercial kit (Cayman Chemical). Keratinocytes seeded in 6-well plates (≈400,000)

were UVA-irradiated in HBSS and samples were collected in lysis buffer without protease inhibitors. Results are expressed as micromoles of arachidonoyl thio-PC hydrolyzed per minute per ml.

Intracellular calcium levels

Intracellular calcium ($[Ca^{2+}]_i$) was measured using the fluorescent probe Calcium OrangeTM-AM from Molecular Probes Inc. Cells in 6-well plates ($\approx 520,000$ per sample) were incubated with $10 \mu M$ of Calcium OrangeTM-AM for 30 min at $37^\circ C$. Then the fluorescence was detected in a dual scanning microplate spectrofluorometer (Spectra MAX Gemini EM, Molecular Devices) using 530 nm excitation and 575 nm emission wavelengths. To obtain the total free $[Ca^{2+}]_i$ in the samples, the following formula was applied: $[Ca^{2+}]_i = K_d (F - F_{min}) / (F_{max} - F)$

when K_d is the dissociation constant for Calcium OrangeTM (185 nM), F is fluorescence of experimental samples, F_{min} is the fluorescence in absence of calcium and F_{max} is the fluorescence of calcium-saturated probe. Calibration was done using ionomycin ($1 \mu M$ in DMSO; Molecular Probes Inc.) and BAPTA-AM ($5 \mu M$ 1 h preincubation; Molecular Probes Inc.).

Immunoblots

Cells were homogenized in lysis buffer (250 mM Tris-HCl pH 6.8, 150 mM NaCl, 4% [w/v] SDS, 0.5 mM EGTA, 5 mM DTT, 20 $\mu g/ml$ leupeptin, 10 $\mu g/ml$ pepstatin A, 10 $\mu g/ml$ aprotinin and 2 mM PMSF) and centrifuged at 1,500 rpm for 5 min at $4^\circ C$. Supernatants were subjected to SDS-PAGE and the resolved proteins were transferred to PVDF membranes at 100 mV for 1 h at $4^\circ C$. Membranes were blocked with 5% non-fat milk in TBS (PBS plus 0.1% (v/v) Tween 20) for 1-2 h. Primary antibodies were incubated in 2.5 % non-fat milk in TBS overnight at $4^\circ C$ followed by 1 h incubation with HPRT-linked secondary antibody. Bands were visualized using enhanced chemiluminescence system according to the manufacturer (Cell Signaling, Beverly MA). Anti-caveolin-1 was from Cell Signaling and anti-flotillin-2 was from Santa Cruz Biotechnology Inc.

Measurements of 7-dehydrocholesterol and cholesterol

7-DHC levels were measured as previously described [22]. Briefly, cells were homogenized, pelleted and extracted with ethanol. After sonication, cold water was added followed by hexane and the mixture was vortexed. After centrifugation, the clear hexane layer was collected and used for UV measurement using a spectrophotometer (Agilent 8453 UV-visible Spectroscopy System, Agilent Technologies, Foster City, CA). Absorption peaks at 271, 282 and 294 nm were used for analysis of 7-DHC. Quantification of cellular 7-DHC was based on the absorbance value at 282 nm using a calibration curve with 0-40 μM 7-DHC in hexane. The absorption coefficient for 7-DHC at 282 nm was $910 M^{-1}cm^{-1}$. After subtracting the spectrum of Ch-HK from that lipids extracted from SLO-HK the absorbance at 282 nm was divided by the absorption coefficient to obtain the 7-DHC levels. Results are expressed as moles per cell.

Chol was measured using a commercial kit (Amplex Red Cholesterol Assay Kit, Molecular Probes) based on cholesterol oxidase activity. Results are expressed as total Chol concentration in moles per cell obtained using a standard curve. Since this method does not discriminate between Chol and 7-DHC, the levels of 7-DHC obtained by spectrophotometric analysis were subtracted from the Chol level measured to obtain the actual Chol in cells.

Statistical analysis

Data are expressed as mean $\pm$ standard deviation (SD), and statistical significance of the results was determined by one-way analysis of variance (ANOVA) followed by t test, with statistical significance set at $p < 0.01$.

Results

Generation of reactive oxygen species by UVA radiation

Human keratinocytes (HK) were prepared in which $\sim 50\%$ of Chol was replaced with 7-DHC (called SLO-HK) to mimic HK in SLOS patients [19]. As a control for replacement treatment, HK were prepared in which Chol was $\sim 50\%$ depleted and then resupplied with Chol (called Chol-HK) and cells were depleted of Chol with no replacement (called AY+CD) (Table 1). Cells were irradiated with UVA (1 J/cm^2), a low, non-cytotoxic dose that skin keratinocytes are exposed to in a few minutes on a sunny day. ROS measurements were made using carboxy- H_2DCFDA , which is oxidized to a fluorescent fluorescein derivative in by ROS in cells. SLO-HK showed a rapid increase (5-fold after 2 min) in ROS compared to HK with a maximum increase of 7-fold after 5 min and a persistent ~ 2 -fold increase at 2 h (Fig. 2A). Irradiated HK showed a 2-fold increase in ROS after 5 min that was maintained for 2 h. UVA irradiation also produced a greater increase in ROS in SLO-HK than in AY+CD cells (Fig. 2B). Since both SLO-HK and AY + CD are depleted of Chol, but SLO-HK contain 7-DHC (Table 1), these results clearly indicate that the presence of 7-DHC rather than the absence of Chol is responsible for the increase in UVA-induced ROS in SLO-HK.

Treatment with general antioxidants, 5 mM ascorbic acid (AA) and 1 mM N-acetylcysteine (NAC), 1 h prior to irradiation completely prevented the UVA-induced increase in ROS in SLO-HK as well as in the HK and Chol-HK (Fig. 2B). In addition, pretreatment with $1 \mu\text{M}$ DPI, an inhibitor of the NADPH oxidase family of enzymes, decreased the UVA-induced ROS production by 50% in SLO-HK and totally in HK and Chol-HK. These results indicate that NADPH oxidase is a likely source of the UVA-induced ROS. Mito-Q ($0.1 \mu\text{M}$), a specific mitochondrial free radical quencher [27,28], also significantly decreased the UVA-induced ROS production in SLO-HK. Treatment with DPI and MitoQ together was toxic to the cells precluding investigation of the whether ROS from one source stimulated ROS from the second source.

NADPH oxidase activation is a key step in UVA-induced ROS formation in SLO-HK

The activity of NADPH oxidase was measured in subcellular fractions from HK, SLO-HK and Chol-HK as the SOD-inhibitable reduction of cytochrome c, since the primary product of NADPH oxidase is superoxide anion. As shown in Fig. 3, a rapid activation (1.4-fold increase) occurs 5 min after UVA irradiation in SLO-HK, reaches a maximum of 2-fold at 10-30 min and returns to the baseline after 40 min. 7-Dehydrocholesterol is required for this activity increase since no change was found for Chol-HK and HK (Fig. 3, inset).

Nox1, a homologue of the gp91 phox (Nox2) subunit in phagocyte NADPH oxidase, has been described in normal human keratinocytes [24,25]. Using two different sequences of Nox1 siRNA in SLO-HK, the Nox1 protein was significantly decreased 48 h after transfection (Fig. 4A). The Nox1-A siRNA primer more efficiently silenced Nox1 expression compared to Nox1-B. However, both siRNAs induced a significant decrease of Nox1 protein: 93% using Nox1-A siRNA and 77% using Nox1-B siRNA (Fig. 4A).

After Nox1 siRNA transfection and treatment to obtain SLO-HK, cells were exposed to and the ROS level measured. Nox1 siRNA treatment significantly decreased ($\sim 60\%$) the UVA-

induced ROS in SLO-HK (Fig. 4B). Consistent with the decrease in Nox1 protein (Fig. 4A), the Nox1-A sequence showed a more efficient inhibition of ROS than the Nox1-B sequence (Fig. 4B). This response was specific for Nox1 since scrambled siRNA and transfection agent alone induced the same ROS levels as UVA irradiated, non-transfected SLO-HK (Fig 4B).

The effect of silencing Nox1 on UVA-induced PGE₂ release was measured under the same conditions used for ROS measurements. The release of PGE₂ decreased ~60% after silencing Nox1 by using Nox1-A or Nox1-B siRNA in SLO-HK (Fig. 4C). The PGE₂ level from Lipofectamine-treated UVA-mediated SLO-HK was not significantly different than that for UVA alone indicating a specific response mediated by Nox1.

Lipid raft content of membranes is altered in SLO-HK

Partially replacing Chol with 7-DHC might disrupt lipid rafts in the plasma membrane and, consequently, enhance UVA-induced ROS formation and downstream signaling. To evaluate this hypothesis, we first assessed the influence of 7-DHC on lipid rafts. The presence of lipid rafts in HK membranes was established by the co-localization of ganglioside GM1, which selectively partitions into lipid rafts [29], with caveolin-1 and flotillin-2, proteins associated with lipid rafts [30]. Ganglioside GM1 in lipid rafts specifically binds cholera toxin subunit B (CT-B), which is fluorescently labeled, and antibody against CT-B is used to aggregate the lipid rafts for detection by fluorescence microscopy. HK showed strong fluorescence for the crosslinked CT-B bound to GM1 and for caveolin-1 and flotillin-2, detected by immunofluorescence (Fig. 5A, rows 1 and 6). In all cases a punctuate pattern appears in the plasma membrane and the fluorescence associated with CT-B clearly overlaps with that of the two proteins indicating the presence of lipid rafts. SLO-HK showed less fluorescence associated with caveolin-1 or flotillin-2 in the plasma membrane whereas there is increased fluorescence in the intracellular space (Fig. 5A, rows 2 and 7). Some co-localization of CT-B with these two proteins can be observed, as noted by the diamond arrows in the amplified merged image, indicating the existence, to a lower degree, of lipid rafts in SLO-HK. Examples of the caveolin-1-only spots are shown in the same images as arrow heads and in the merged image for CT-B with flotillin-2 (row 7) spots corresponding to flotillin-2 alone are present (triangle arrow heads). When the cells were reconstituted with Chol after partial extraction (Chol-HK), fluorescence corresponding to lipid rafts (CT-B bound to GM1) co-localized again with caveolin-1 (Fig. 5A, row 3) or flotillin-2 (data not shown). Occasional caveolin-1 spots were also present as shown in the amplified merged image. Since methyl- β -cyclodextrin disrupts lipid rafts by removing Chol, this treatment was used as a negative control for lipid rafts (CD in Fig. 5A). The CD cells almost completely lacked green fluorescence in the plasma membrane corresponding to caveolin-1, and the intracellular caveolin-1 staining increased (Fig. 5A, row 4). The co-localization of caveolin-1 and CT-B was much less conspicuous than in HK and this image was very similar to the image of SLO-HK. The presence of lipid rafts was reflected in the cell morphology; CD treatment caused shrinkage and rounding of the cells, as previously reported [31]. This shape change, which was reversed when Chol was replaced, may be related to the known disruption of the actin cytoskeleton by CD [32]. Another negative control was carried out to confirm the specificity of the association of these proteins with lipid rafts. Fluorescence of crosslinked CT-B bound to GM1 was compared to that for immunofluorescence staining of Na⁺/K⁺-ATPase, which does not associate with lipid rafts and is recovered in the heavy fractions in the separation of detergent-resistant membranes [14,33,34]. As shown in Fig. 5A, row 5, in HK cells the green fluorescence corresponding to Na⁺/K⁺-ATPase and the red fluorescence from the CT-B shows little, if any, overlap confirming the specificity of this method.

Disruption of lipid rafts by partially replacing Chol with 7-DHC was a surprising result because the chemical structures and shapes of the two sterols are nearly identical (Fig. 1B) and previous studies in model membranes and brain homogenates indicated that 7-DHC supported lipid raft formation [15,16,35,36]. Consequently, we further evaluated the effect of replacing Chol with 7-DHC on the profile of proteins associated detergent resistant membranes (DRM) isolated by Triton X-100 treatment of keratinocytes. The proteins in DRM were separated using floating sucrose density gradients. In this procedure, proteins associated with DRM, which include lipid rafts, are found in a more buoyant fraction than detergent-solubilized proteins [14]. Proteins in cell lysates were separated on a 5-35-40% sucrose-DEB discontinuous gradient and DRM are expected at the 5-35% interface [14,26]. After the centrifugation, 1 ml fractions were immunoblotted using antibodies against flotillin-2 and caveolin-1, proteins associated with lipid rafts when Triton X-100 is used [14,37,38]. In HK, caveolin-1 and flotillin-2 associated with the lipid raft fractions, which appeared as a hazy band at the interface between the 5-35% interface (Fig. 5B). However, the same fractions from SLO-HK did not show the same hazy band and had very low levels of caveolin-1 and no flotillin-2. In additional experiments, we used a linear sucrose gradient (40-5%) to separate the DRM fraction from a 1% Brij 98 in 40% sucrose-DEB cell homogenate. Caveolin-1 appeared in the more buoyant fractions (4, 5 and 6) from this gradient separation of the DRM from HK, whereas it appeared in less buoyant fractions (7 and 8) for DRM from SLO-HK (Fig. 5C) consistent with a decrease or absence of lipid rafts in SLO-HK. These results, taken together, indicate that replacing Chol with 7-DHC decreases the ability of keratinocyte membranes to form lipid rafts and DRM, and suggest that enhanced UVA-induced ROS formation may be associated with alterations in signaling pathways initiated in these membrane domains.

Lipid oxidation is enhanced by 7-DHC

To determine whether radical-initiated lipid oxidation was enhanced by the presence of 7-DHC, liposomes were prepared containing Chol (or 7-DHC) plus phospholipid containing mono-unsaturated fatty acids (OPPC) (50:50) or, alternatively, Chol (or 7-DHC) plus phospholipid containing polyunsaturated fatty acid (SAPC) plus OPPC (50:25:25). Lipid oxidation was assessed by measuring the oxygen consumption after addition of a free radical initiator, AMPA. The rate of consumption of oxygen for OPPC-liposomes containing 7-DHC was 10% higher than for Chol-containing liposomes (data not shown). Greater oxygen consumption occurred in the SAPC-OPPC-liposomes compared to OPPC-liposomes in the presence of either 7-DHC or Chol due to the presence of polyunsaturated arachidonic acid in SAPC. SAPC-OPPC-liposomes containing 7-DHC were oxidized ~40% more rapidly than those containing Chol (Fig. 6) suggesting that 7-DHC substantially enhances the rate of free radical initiated oxidation of membrane lipids compared to [38].

Rapid UVA-induced synthesis of prostaglandin E₂ in SLO-HK is regulated by ROS and calcium-dependent phospholipase A₂ activation

Prostaglandin E₂ (PGE₂) is an inflammatory mediator associated with UV- and irritant induced rapid inflammation in skin [39]. UVA (1 J/cm²) exposure produced a rapid, 15-fold increase in PGE₂ after 5 min and 9.5-fold increase after 15 minutes in SLO-HK whereas no changes were induced in HK and Chol-HK (Fig. 7A). A small increase in PGE₂ was observed after UVA treatment of HK treated with AY9944 plus CD (AYCD). The early and rapid increase in PGE₂ release in UVA-treated SLO-HK was partially (60-70%) prevented by AA and NAC (Fig. 7B). Pretreatment with DPI caused a smaller, but significant, inhibition of UVA-induced PGE₂ release (~35%) (Fig. 7B), less than the 65% decrease produced when Nox1 was decreased by siRNA treatment (Fig. 4C). MitoQ also decreased the UVA-induced PGE₂ production by about 35% suggest roles for both ROS produced by NADPH oxidase and mitochondrial radicals in the UVA-induced PGE₂ release.

Since PGE₂ is formed by cyclooxygenase 1 and 2 (COX1 and COX2) from arachidonic acid, cells were pretreated with the COX inhibitors acetylsalicylic acid (ASA, 2 mM) [40] and indomethacin (Indo, 50 μM) [41]. The results showed a decrease of 80% in the PGE₂ release, as expected, from blocking the endogenous activity of COX (Fig. 7C). Cytosolic, calcium-dependent PLA₂ (cPLA₂) which cleaves arachidonic acid from membrane lipids, appears to be the most common phospholipase for PGE₂ production in skin cells during inflammation [42,43]. Treatment with a PLA₂ inhibitor (aristolochic acid, ARC) 3 hours prior UVA irradiation reduced (75%) PGE₂ release from SLO-HK (Fig. 7C). The permeable calcium chelator BAPTA-AM (25 μM) caused a 70% decrease of PGE₂, consistent with the Ca²⁺-dependence of cPLA₂ (Fig. 7C). The cPLA₂ activity in SLO-HK, Chol-HK and HK was measured 5 and 10 min after UVA. A > 2-fold increase in cPLA₂ activity was found in UVA-irradiated SLO-HK compared with unirradiated SLO-HK and UVA-treated Chol-HK (Fig. 7D). Although MitoQ did not influence the UVA-induced cPLA₂ activity, DPI reduced this increase by 80% (Fig. 7D). As expected, the Ca²⁺ chelator BAPTA (BTA in Fig. 7A) completely inhibited cPLA₂ activation. Taken together these results, indicate that UVA-induced PGE₂ formation is enhanced in SLO-HK, mediated by ROS, and dependent on cPLA₂ activation.

UVA induces an increase in free intracellular calcium

Intracellular calcium [Ca²⁺]_i was measured in cells after UVA irradiation using the Calcium Orange-AM fluorescent probe. UVA irradiation caused a rapid (<2 min) increase in [Ca²⁺]_i in SLO-HK that was maximal after 5 min (Fig. 8A) and remained elevated for 30 min. In HK a low but significant increase in [Ca²⁺]_i was observed at 20 to 30 min after UVA. The increase in [Ca²⁺]_i appeared to be caused by 7-DHC in SLO-HK rather than the lower Chol levels produced by treatment with CD, because the [Ca²⁺]_i was not increased in AY+CD cells (Fig. 8B) or Chol-HK cells (data not shown). AA and NAC decreased the UVA-induced [Ca²⁺]_i increase by 30% and ROS from NADPH oxidase and mitochondria appear to be involved in the mechanism since DPI and MitoQ decreased the UVA-induced [Ca²⁺]_i increase by 50-60% (Fig. 8B).

Discussion

In this study we sought to understand how partial substitution of 7-DHC for Chol in HK might influence production of UVA-induced ROS and PGE₂ in keratinocytes in order to postulate a mechanism for the rapid (< 5 min) inflammation appearing in the skin of SLOS patients exposed to very low UVA doses. Our results show that PGE₂ is produced rapidly after UVA treatment in SLO-HK (5 min; Fig. 6A), similar to the initial time frame for skin responses of SLOS patients to UVA [17]. UVA did not increase in PGE₂ in HK or Chol-HK, consistent with the lack of erythema in normal skin exposed to low UVA doses.

UVA treatment caused rapid activation of cPLA₂, the rate-limiting enzyme in PGE₂ synthesis, by SLO-HK but no activity increase after identical treatment of HK or Chol-HK (Fig. 7D). The time course for changes in [Ca²⁺]_i, which is required for cPLA₂ activation, showed a significant rise within 2 min after UVA in SLO-HK, but not in HK (Fig. 8A), and the maximal [Ca²⁺]_i level at 3-10 min correlated with the time course for PGE₂ release from SLO-HK (Fig. 7A). Although UVA-induced increases in [Ca²⁺]_i in fibroblasts and in Ca²⁺-dependent calpain activity in A431 cells have been observed [44,45], the rapid time course shown in Fig. 8A has not been reported previously. Taken together, these results suggest UVA-induced increase in [Ca²⁺]_i, followed by activation of cPLA₂ and synthesis of PGE₂ by COX1/2 as a mechanism for the rapid inflammatory response induced by UVA in SLOS patients' skin.

UVA-induced production of ROS precedes, and is necessary for, the increase in $[Ca^{2+}]_i$ and PGE_2 since AA and NAC partially prevented the $[Ca^{2+}]_i$ and PGE_2 increases (Figs. 7 and 8). Exposure to a low UVA dose produced ROS levels that were clearly greater in SLO-HK than in HK. An increased level of ROS was detected up to at least 2 h suggesting that continued production of ROS was activated by the UVA irradiation. Interestingly, NADPH oxidase activity returns to baseline by 40 min after UVA (Fig. 3) even though the ROS level remains elevated much longer (Fig. 2A). The probe used, carboxy- H_2DCFDA , is not specific for a single type of ROS but reacts with several ROS in cells including H_2O_2 , peroxynitrite, and peroxides [46,47], and consequently can detect different ROS from mitochondria, lipid oxidation and other sources after NADPH oxidase is no longer active.

Our results indicate that Nox1 is a major source of the initial UVA-induced ROS in SLO-HK. Low UVA doses increased NADPH oxidase activity in SLO-HK (Fig. 3). Keratinocytes express Nox1 and gp91 $phox$ (Nox2), homologous catalytic subunits of NADPH oxidase, as well as other subunits (p22 $phox$, p47 $phox$, p67 $phox$) [24,25,48]. A study using murine keratinocytes deficient in Nox2 indicated that ROS from this subunit played only a minor role in UVA-induced apoptosis [48]. Because DPI is not an entirely specific inhibitor for the catalytic subunit of NADPH oxidases [49], we used siRNA to deplete SLO-HK of Nox1. In SLO-HK in which Nox1 was reduced ~90% of its normal level, Nox1 RNAi reduced UVA-induced ROS and PGE_2 levels by ~60% (Fig. 4), a greater inhibition than using DPI (Figs. 2B and 7B). Since DPI does not discriminate between the Nox homologues, this result suggests that NADPH containing Nox1 dominates ROS formation in HK.

The mechanism for activation of NADPH oxidase by UVA is not known. In neutrophils, physiological activation requires phosphorylation of cytoplasmic subunits (p47 $phox$, p67 $phox$) and activation of the small GTPase, Rac2. These subunits translocate to the plasma membrane where they assemble with the membrane-bound subunits (p22 $phox$, Nox2) to produce an active enzyme complex [50]. Although subunits homologous to those in leukocytes have been found in many cell types, the mechanisms for activation of the NADPH oxidase in these cells are still unclear [51]. Activation and translocation of Rac1 to the membrane is essential for activation of a Nox1-containing NADPH oxidase [52] although pre-assembly of Nox1 with NoxO1 (the homologue of p47 $phox$) appeared to form a active complex in HEK293 cells [53]. Activation of growth factor receptors stimulates NADPH oxidase activity in many cells types [54-56]. However, UVA-induced activation of NADPH oxidase is unlikely to involve activation of EGFR because UVA inhibits signaling via EGFR [54]. An alternative mechanism involves ceramide because it is released in keratinocytes by UVA [7,57] and has been reported to activate NADPH oxidase in endothelial cells [58]. The reported time course for UVA-stimulated release of ceramide in normal keratinocytes [7,57] appears to be too slow to cause the rapid increase in NADPH oxidase activity that we measured in SLO-HK (Fig. 3), although more rapid kinetics for ceramide release might be found in SLO-HK. Our studies are now focusing on the mechanisms for UVA-induced ROS formation.

Mitochondria are also a significant source of ROS that stimulate the rapid increase in PGE_2 after UVA since MitoQ, a radical-scavenging ubiquinone that is specifically delivered to the mitochondrial matrix [27,28] inhibited both UVA-induced ROS and PGE_2 (Figs. 2A,7B). Mitochondria contain many chromophores absorbing UVA including cofactors (NAD, FMN) and prosthetic groups (cytochromes, iron-sulfur clusters) of electron transport chain proteins that may generate ROS. Moderate UVA doses have been shown to increase electron transfer to oxygen to form superoxide anion at Complex II/Complex III interface of the electron transport chain [59] and our previous study also implicated mitochondria as a source of the ROS leading to UVA-induced apoptosis in SLO-HK [22]. However, mitochondrial membranes have only low levels of Chol that could be replaced by 7-DHC, suggesting that the effect of 7-DHC is mediated in other membranes. The relationship between ROS produced in

mitochondria to those produce by NADPH oxidase remains to be determined. Mitochondria might also be a source for the UVA-induced increase in $[Ca^{2+}]_i$ (Fig. 8A) since oxidative damage to the proteins of the mitochondrial permeability transition pore allows leakage of Ca^{2+} [60]. However, the mechanism must also involve Nox 1 because the UVA-induced increase in $[Ca^{2+}]_i$ is inhibited ~60% by DPI (Fig. 8B).

7-DHC differs from Chol in at least two ways that might influence the level of UVA-induced ROS and subsequent formation of PGE_2 , namely, 7-DHC participates more readily in free radical lipid oxidation reactions than Chol, and 7-DHC disrupts lipid rafts in the keratinocyte plasma membrane. Our results indicate that the former, but not the latter, appears to account for the higher UVA-induced ROS formation in SLO-HK. Both mechanisms require the initial formation of a low level of singlet oxygen, or other ROS, directly from the chromophore that is subsequently amplified. The presence of an additional conjugated double bond in 7-DHC is the major chemical difference between these two sterols (Fig. 1B). The greater degree of conjugated unsaturation in 7-DHC is predicted to make the allylic hydrogens in 7-DHC more rapidly abstracted by radicals than those in Chol. Addition of oxygen to the carbon-centered radical then produces a peroxy radical, which may abstract a hydrogen to become a sterol hydroperoxide. Subsequent decomposition of this hydroperoxide and free radical chain oxidation reactions of unsaturated fatty acids in phospholipids of the 7-DHC-containing membrane increases formation of secondary ROS that are detected by carboxy- H_2DCFDA . The ROS are then responsible for the observed rapid increase in $[Ca^{2+}]_i$ and synthesis of PGE_2 . Further reactions of the primary radical also leads to formation of a cholesterol triene that has recently been suggested as a chromophore for the photosensitivity in SLOS [61]. Continued chain reaction oxidation of membrane unsaturated lipids also provides a mechanism for production of ROS (>120 min, Fig. 2A) when NADPH oxidase is activated for only <40 min (Fig. 3) although extended production of ROS from mitochondria may also contribute.

7-DHC also disrupted lipid raft domains in SLO-HK suggesting that this phenomenon might contribute to enhanced UVA-induced ROS formation and subsequent PGE_2 synthesis because many proteins involved in cell signaling, including NADPH oxidase (in neutrophils) are present in lipid rafts [62]. Lipid rafts were not present or were markedly reduced in SLO-HK, similar to the effect found when Chol is partially removed from HK using CD, which is a well-established method for disrupting lipid rafts. SLO-HK and AYCD cells behaved identically when the localization of two lipid raft-associated proteins, caveolin-1 and flotillin-2, were compared to the localization of a raft marker, crosslinked CT-B bound to ganglioside GM1 (Fig. 5A) indicating that lipid rafts were not present in SLO-HK. This result was surprising because the shapes of the 7-DHC and Chol molecules are very similar, and because studies using model membranes and rat brain homogenate demonstrated that both 7-DHC and Chol support formation of lipid rafts [15,16,35,36]. Consequently, we used independent methods to assess formation of DRM, which contain lipid rafts, in HK and SLO-HK. As shown in Fig. 5B and 5C, a buoyant lipid raft-containing fraction was present in HK but not SLO-HK. Although the size of lipid rafts in the plasma membrane and their methods for detection are still discussed [63-65], these results provide clear evidence that 7-DHC perturbs the plasma membrane organization in a manner consistent with disruption of lipid rafts. The contrast between the results reported in model membranes and our result in cells suggests that components of lipid rafts found in biological membranes but not in most model membranes, e.g., proteins and sphingomyelin, strongly influence the relative stability of these microdomains and, furthermore, that Chol and 7-DHC do not interact with these components in an identical manner. In neutrophils, intact lipid raft domains are required for activation of NADPH oxidase [62], although our results clearly show that NADPH oxidase is active in SLO-HK where lipid rafts are not present. Partial depletion of Chol (AY+CD cells) did not enhance UVA-induced ROS formation (Fig. 2B) indicating that the presence of 7-DHC, rather than absence of lipid rafts, accounts for the enhanced ROS formation in SLO-HK. This result contrasts with a recent

study in which disruption of lipid rafts appeared to enhance UVA-induced ROS formation [66]. The reason for this discrepancy is not clear.

In summary, our results indicate that NADPH oxidase containing Nox1 as the catalytic subunit and mitochondria are the major cellular sources of the ROS detected shortly after UVA exposure and that they are responsible for the rapid increase in PGE₂ in SLO-HK by increasing [Ca]_i and consequently, cPLA₂ activity. In addition, this study indicates that partial replacement of Chol with 7-DHC enhances UVA-induced, ROS-mediated PGE₂ synthesis because 7-DHC reacts more rapidly and enhances membrane lipid peroxidation better than Chol. We also found that, surprisingly, 7-DHC does not support formation of lipid raft domains in the plasma membrane despite its close structural similarity to Chol. The rapid UVA-induced increase in PGE₂ in SLO-HK mimics the immediate phase in SLOS photosensitivity and may represent the actual mechanism occurring in the skin of SLOS patients exposed to solar radiation [17].

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References

- [1]. Bachelor MA, Bowden GT. UVA-mediated activation of signaling pathways involved in skin tumor promotion and progression. *Semin Cancer Biol* 2004;14(2):131–138. [PubMed: 15018897]
- [2]. Gross S, Knebel A, Tenev T, Neining A, Gaestel M, Herrlich P, Bohmer FD. Inactivation of protein-tyrosine phosphatases as mechanism of UV-induced signal transduction. *J Biol Chem* 1999;274(37):26378–26386. [PubMed: 10473595]
- [3]. Gulati P, Markova B, Gottlicher M, Bohmer FD, Herrlich PA. UVA inactivates protein tyrosine phosphatases by calpain-mediated degradation. *EMBO Rep* 2004;5(8):812–817. [PubMed: 15247926]
- [4]. Grether-Beck S, Olaizola-Horn S, Schmitt H, Grewe M, Jahnke A, Johnson JP, Briviba K, Sies H, Krutmann J. Activation of transcription factor AP-2 mediates UVA radiation- and singlet oxygen-induced expression of the human intercellular adhesion molecule 1 gene. *Proc Natl Acad Sci U S A* 1996;93(25):14586–14591. [PubMed: 8962096]
- [5]. Maziere C, Dantin F, Dubois F, Santus R, Maziere J. Biphasic effect of UVA radiation on STAT1 activity and tyrosine phosphorylation in cultured human keratinocytes. *Free Radic Biol Med* 2000;28(9):1430–1437. [PubMed: 10924861]
- [6]. Knebel A, Rahmsdorf HJ, Ullrich A, Herrlich P. Dephosphorylation of receptor tyrosine kinases as target of regulation by radiation, oxidants or alkylating agents. *Embo J* 1996;15(19):5314–5325. [PubMed: 8895576]
- [7]. Grether-Beck S, Bonizzi G, Schmitt-Brenden H, Felsner I, Timmer A, Sies H, Johnson JP, Piette J, Krutmann J. Non-enzymatic triggering of the ceramide signalling cascade by solar UVA radiation. *Embo J* 2000;19(21):5793–5800. [PubMed: 11060030]
- [8]. Huang CC, Fang JY, Wu WB, Chiang HS, Wei YJ, Hung CF. Protective effects of (-)-epicatechin-3-gallate on UVA-induced damage in HaCaT keratinocytes. *Arch Dermatol Res* 2005;296(10):473–481. [PubMed: 15726391]
- [9]. Girotti AW, Kriska T. Role of lipid hydroperoxides in photo-oxidative stress signaling. *Antioxid Redox Signal* 2004;6(2):301–310. [PubMed: 15025931]
- [10]. Obinata H, Hattori T, Nakane S, Tatei K, Izumi T. Identification of 9-hydroxyoctadecadienoic acid and other oxidized free fatty acids as ligands of the G protein-coupled receptor G2A. *J Biol Chem* 2005;280(49):40676–40683. [PubMed: 16236715]

- [11]. Zmijewski JW, Landar A, Watanabe N, Dickinson DA, Noguchi N, Darley- Usmar VM. Cell signalling by oxidized lipids and the role of reactive oxygen species in the endothelium. *Biochem Soc Trans* 2005;33(6):1385–1389. [PubMed: 16246125]
- [12]. Insel PA, Head BP, Ostrom RS, Patel HH, Swaney JS, Tang CM, Roth DM. Caveolae and lipid rafts: G protein-coupled receptor signaling microdomains in cardiac myocytes. *Ann N Y Acad Sci* 2005;1047:166–172. [PubMed: 16093494]
- [13]. Peres C, Yart A, Perret B, Salles JP, Raynal P. Modulation of phosphoinositide 3- kinase activation by cholesterol level suggests a novel positive role for lipid rafts in lysophosphatidic acid signalling. *FEBS Lett* 2003;534(13):164–168. [PubMed: 12527380]
- [14]. Pike LJ, Han X, Gross RW. Epidermal growth factor receptors are localized to lipid rafts that contain a balance of inner and outer leaflet lipids: a shotgun lipidomics study. *J Biol Chem* 2005;280(29):26796–26804. [PubMed: 15917253]
- [15]. Xu X, Bittman R, Duportail G, Heissler D, Vilcheze C, London E. Effect of the structure of natural sterols and sphingolipids on the formation of ordered sphingolipid/sterol domains (rafts). Comparison of cholesterol to plant, fungal, and disease-associated sterols and comparison of sphingomyelin, cerebroside, and ceramide. *J Biol Chem* 2001;276(36):33540–33546. [PubMed: 11432870]
- [16]. Megha; Bakht O, London E. Cholesterol precursors stabilize ordinary and ceramide- rich ordered lipid domains (lipid rafts) to different degrees: Implications for the bloch hypothesis and sterol biosynthesis disorders. *J Biol Chem*. 2006
- [17]. Anstey AV, Ryan A, Rhodes LE, Charman CR, Arlett CF, Tyrrell RM, Taylor CR, Pearse AD. Characterization of photosensitivity in the Smith-Lemli-Opitz syndrome: a new congenital photosensitivity syndrome. *Br J Dermatol* 1999;141(3):406–414. [PubMed: 10583043]
- [18]. Charman CR, Ryan A, Tyrrell RM, Pearse AD, Arlett CF, Kurwa HA, Shortland G, Anstey A. Photosensitivity associated with the Smith-Lemli-Opitz syndrome. *Br J Dermatol* 1998;138(5):885–888. [PubMed: 9666840]
- [19]. Ruan B; Wilson WK, Pang J; Gerst N; Pinkerton FD, Tsai J, Kelley RI, Whitby FG, Milewicz DM, Garbern J, Schroepfer GJ Jr. Sterols in blood of normal and Smith- Lemli-Opitz subjects. *J. Lipid Res* 2001;42:799–812. [PubMed: 11352988]
- [20]. Tint GS, Irons M, Elias ER, Batta AK, Frieden R, Chen TS, Salen G. Defective cholesterol biosynthesis associated with the Smith-Lemli-Opitz syndrome. *N Engl J Med* 1994;330(2):107–113. [PubMed: 8259166]
- [21]. Azurdia RM, Anstey AV, Rhodes LE. Cholesterol supplementation objectively reduces photosensitivity in the Smith-Lemli-Opitz syndrome. *Br J Dermatol* 2001;144(1):143–145. [PubMed: 11167696]
- [22]. Valencia A, Kochevar IE. Ultraviolet A induces apoptosis via reactive oxygen species in a model for Smith-Lemli-Opitz syndrome. *Free Radic Biol Med* 2006;40(4):641–650. [PubMed: 16458195]
- [23]. Shpungin S, Dotan I, Abo A, Pick E. Activation of the superoxide forming NADPH oxidase in a cell-free system by sodium dodecyl sulfate. Absolute lipid dependence of the solubilized enzyme. *J Biol Chem* 1989;264(16):9195–9203. [PubMed: 2542302]
- [24]. Chamulitrat W, Schmidt R, Tomakidi P, Stremmel W, Chunglok W, Kawahara T, Rokutan K. Association of gp91phox homolog Nox1 with anchorage-independent growth and MAP kinase-activation of transformed human keratinocytes. *Oncogene* 2003;22(38):6045–6053. [PubMed: 12955083]
- [25]. Chamulitrat W, Stremmel W, Kawahara T, Rokutan K, Fujii H, Wingler K, Schmidt HH, Schmidt R. A constitutive NADPH oxidase-like system containing gp91phox homologs in human keratinocytes. *J Invest Dermatol* 2004;122(4):1000–1009. [PubMed: 15102091]
- [26]. Badizadegan K, Wheeler HE, Fujinaga Y, Lencer WI. Trafficking of cholera toxin-ganglioside GM1 complex into Golgi and induction of toxicity depend on actin cytoskeleton. *Am J Physiol Cell Physiol* 2004;287(5):C1453–1462. [PubMed: 15294854]
- [27]. Kelso GF, Porteous CM, Coulter CV, Hughes G, Porteous WK, Ledgerwood EC, Smith RA, Murphy MP. Selective targeting of a redox-active ubiquinone to mitochondria within cells: antioxidant and antiapoptotic properties. *J Biol Chem* 2001;276(7):4588–4596. [PubMed: 11092892]

- [28]. Adlam VJ, Harrison JC, Porteous CM, James AM, Smith RA, Murphy MP, Sammut IA. Targeting an antioxidant to mitochondria decreases cardiac ischemia- reperfusion injury. *FASEB J* 2005;19(9): 1088–1095. [PubMed: 15985532]
- [29]. Mitsuda T, Furukawa K, Fukumoto S, Miyazaki H, Urano T, Furukawa K. Overexpression of ganglioside GM1 results in the dispersion of platelet-derived growth factor receptor from glycolipid-enriched microdomains and in the suppression of cell growth signals. *J Biol Chem* 2002;277(13):11239–11246. [PubMed: 11782461]
- [30]. Macdonald JL, Pike LJ. A simplified method for the preparation of detergent-free lipid rafts. *J Lipid Res* 2005;46(5):1061–1067. [PubMed: 15722565]
- [31]. Jans R, Atanasova G, Jadot M, Poumay Y. Cholesterol depletion upregulates involucrin expression in epidermal keratinocytes through activation of p38. *J Invest Dermatol* 2004;123(3):564–573. [PubMed: 15304097]
- [32]. Kwik J, Boyle S, Fooksman D, Margolis L, Sheetz MP, Edidin M. Membrane cholesterol, lateral mobility, and the phosphatidylinositol 4,5-bisphosphate-dependent organization of cell actin. *Proc Natl Acad Sci U S A* 2003;100(24):13964–13969. [PubMed: 14612561]
- [33]. Besshoh S, Bawa D, Teves L, Wallace MC, Gurd JW. Increased phosphorylation and redistribution of NMDA receptors between synaptic lipid rafts and post-synaptic densities following transient global ischemia in the rat brain. *J Neurochem* 2005;93(1):186–194. [PubMed: 15773918]
- [34]. Eckert GP, Igbavboa U, Muller WE, Wood WG. Lipid rafts of purified mouse brain synaptosomes prepared with or without detergent reveal different lipid and protein domains. *Brain Res* 2003;962(12):144–150. [PubMed: 12543465]
- [35]. Keller RK, Arnold TP, Fliesler SJ. Formation of 7-dehydrocholesterol-containing membrane rafts in vitro and in vivo, with relevance to the Smith-Lemli-Opitz syndrome. *J Lipid Res* 2004;45(2): 347–355. [PubMed: 14594996]
- [36]. Berring EE, Borrenpohl K, Fliesler SJ, Serfis AB. A comparison of the behavior of cholesterol and selected derivatives in mixed sterol-phospholipid Langmuir monolayers: a fluorescence microscopy study. *Chem Phys Lipids* 2005;136(1):1–12. [PubMed: 15904906]
- [37]. Yu C, Alterman M, Dobrowsky RT. Ceramide displaces cholesterol from lipid rafts and decreases the association of the cholesterol binding protein caveolin-1. *J Lipid Res* 2005;46(8):1678–1691. [PubMed: 15863835]
- [38]. Girotti, AW. in *Sterols and Oxysterols*. In: Fliesler, SJ., editor. *Chemistry, Biology, and Pathobiology*. Research Signpost; Trivandrum: 2002. p. 121–139.
- [39]. Argenziano G, Zalaudek I, Corona R, Sera F, Cicale L, Petrillo G, Ruocco E, Hofmann-Wellenhof R, Soyer HP. Vascular structures in skin tumors: a dermoscopy study. *Arch Dermatol* 2004;140(12):1485–1489. [PubMed: 15611426]
- [40]. Shiff SJ, Shivaprasad P, Santini DL. Cyclooxygenase inhibitors: drugs for cancer prevention. *Curr Opin Pharmacol* 2003;3(4):352–361. [PubMed: 12901943]
- [41]. Kalgutkar AS, Marnett AB, Crews BC, Rummel RP, Marnett LJ. Ester and amide derivatives of the nonsteroidal antiinflammatory drug, indomethacin, as selective cyclooxygenase-2 inhibitors. *J Med Chem* 2000;43(15):2860–2870. [PubMed: 10956194]
- [42]. Burke JR, Davern LB, Stanley PL, Gregor KR, Banville J, Remillard R, Russell JW, Brassil PJ, Witmer MR, Johnson G, Tredup JA, Tramposch KM. BMS-229724 is a tight-binding inhibitor of cytosolic phospholipase A2 that acts at the lipid/water interface and possesses anti-inflammatory activity in skin inflammation models. *J Pharmacol Exp Ther* 2001;298(1):376–385. [PubMed: 11408565]
- [43]. Tanaka S, Sato T, Akimoto N, Yano M, Ito A. Prevention of UVB-induced photoinflammation and photoaging by a polymethoxy flavonoid, nobiletin, in human keratinocytes in vivo and in vitro. *Biochem Pharmacol* 2004;68(3):433–439. [PubMed: 15242810]
- [44]. Maziere C, Morliere P, Louandre C, Conte MA, Gomilla C, Santus R, Antonicelli F, Hornebeck W, Maziere JC. Low UVA doses activate the transcription factor NFAT in human fibroblasts by a calcium-calcineurin pathway. *Free Radic Biol Med* 2005;39(12):1629–1637. [PubMed: 16298688]
- [45]. He D, Sassa S, Lim HW. Effect of UVA and blue light on porphyrin biosynthesis in epidermal cells. *Photochem Photobiol* 1993;57(5):825–829. [PubMed: 8337255]

- [46]. Possel H, Noack H, Augustin W, Keilhoff G, Wolf G. 2,7-Dihydrodichlorofluorescein diacetate as a fluorescent marker for peroxynitrite formation. *FEBS Lett* 1997;416(2):175–178. [PubMed: 9369208]
- [47]. Afri M, Frimer AA, Cohen Y. Active oxygen chemistry within the liposomal bilayer. Part IV: Locating 2',7'-dichlorofluorescein (DCF), 2',7'-dichlorodihydrofluorescein (DCFH) and 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) in the lipid bilayer. *Chem Phys Lipids* 2004;131(1):123–133. [PubMed: 15210370]
- [48]. He YY, Huang JL, Block ML, Hong JS, Chignell CF. Role of Phagocyte Oxidase in UVA-Induced Oxidative Stress and Apoptosis in Keratinocytes. *J Invest Dermatol* 2005;125(3):560–566. [PubMed: 16117799]
- [49]. Baker CJ, Deahl K, Domek J, Orlandi EW. Oxygen metabolism in plant/bacteria interactions: effect of DPI on the pseudo-NAD(P)H oxidase activity of peroxidase. *Biochem Biophys Res Commun* 1998;252(2):461–464. [PubMed: 9826552]
- [50]. Babior BM, Lambeth JD, Nauseef W. The neutrophil NADPH oxidase. *Arch Biochem Biophys* 2002;397(2):342–344. [PubMed: 11795892]
- [51]. Hordijk PL. Regulation of NADPH oxidases: the role of Rac proteins. *Circ Res* 2006;98(4):453–462. [PubMed: 16514078]
- [52]. Park HS, Lee SH, Park D, Lee JS, Ryu SH, Lee WJ, Rhee SG, Bae YS. Sequential activation of phosphatidylinositol 3-kinase, beta Pix, Rac1, and Nox1 in growth factor-induced production of H₂O₂. *Mol Cell Biol* 2004;24(10):4384–4394. [PubMed: 15121857]
- [53]. Cheng G, Lambeth JD. NOXO1, regulation of lipid binding, localization, and activation of Nox1 by the Phox homology (PX) domain. *J Biol Chem* 2004;279(6):4737–4742. [PubMed: 14617635]
- [54]. He YY, Huang JL, Gentry JB, Chignell CF. Epidermal growth factor receptor down-regulation induced by UVA in human keratinocytes does not require the receptor kinase activity. *J Biol Chem* 2003;278(43):42457–42465. [PubMed: 12930839]
- [55]. Wang H, Kochevar IE. Involvement of UVB-induced reactive oxygen species in TGF- β biosynthesis and activation in keratinocytes. *Free Radic Biol Med* 2005;38(7):890–897. [PubMed: 15749385]
- [56]. Reich H, Tritschler D, Herzenberg AM, Kassiri Z, Zhou X, Gao W, Scholey JW. Albumin activates ERK via EGF receptor in human renal epithelial cells. *J Am Soc Nephrol* 2005;16(5):1266–1278. [PubMed: 15829704]
- [57]. Maziere C, Conte MA, Leborgne L, Levade T, Hornebeck W, Santus R, Maziere JC. UVA radiation stimulates ceramide production: relationship to oxidative stress and potential role in ERK, JNK, and p38 activation. *Biochem Biophys Res Commun* 2001;281(2):289–294. [PubMed: 11181043]
- [58]. Zhang DX, Zou AP, Li PL. Ceramide-induced activation of NADPH oxidase and endothelial dysfunction in small coronary arteries. *Am J Physiol Heart Circ Physiol* 2003;284(2):H605–612. [PubMed: 12424096]
- [59]. Gniadecki R, Thorn T, Vicanova J, Petersen A, Wulf HC. Role of mitochondria in ultraviolet-induced oxidative stress. *J Cell Biochem* 2000;80(2):216–222. [PubMed: 11074592]
- [60]. Cadenas E, Davies KJ. Mitochondrial free radical generation, oxidative stress, and aging. *Free Radic Biol Med* 2000;29(3):4–222.
- [61]. Chignell CF, Kukielszak BM, Sik RH, Bilski PJ, He YY. Ultraviolet A sensitivity in Smith-Lemli-Opitz syndrome: Possible involvement of cholesta-5,7,9(11)-trien-3 β -ol. *Free Radic Biol Med* 2006;41(2):339–346. [PubMed: 16814115]
- [62]. Shao D, Segal AW, Dekker LV. Lipid rafts determine efficiency of NADPH oxidase activation in neutrophils. *FEBS Lett* 2003;550(13):101–106. [PubMed: 12935894]
- [63]. Yuan C, Furlong J, Burgos P, Johnston LJ. The size of lipid rafts: an atomic force microscopy study of ganglioside GM1 domains in sphingomyelin/DOPC/cholesterol membranes. *Biophys J* 2002;82(5):2526–2535. [PubMed: 11964241]
- [64]. Silvius JR. Fluorescence energy transfer reveals microdomain formation at physiological temperatures in lipid mixtures modeling the outer leaflet of the plasma membrane. *Biophys J* 2003;85(2):1034–1045. [PubMed: 12885650]

- [65]. Nicolau DV Jr, Burrage K, Parton RG, Hancock JF. Identifying optimal lipid raft characteristics required to promote nanoscale protein-protein interactions on the plasma membrane. *Mol Cell Biol* 2006;26(1):313–323. [PubMed: 16354701]
- [66]. Gniadecki R, Christoffersen N, Wulf HC. Cholesterol-rich plasma membrane domains (lipid rafts) in keratinocytes: importance in the baseline and UVA-induced generation of reactive oxygen species. *J Invest Dermatol* 2002;118(4):582–588. [PubMed: 11918702]

Fig 1.
7-Dehydrocholesterol and cholesterol. A, Absorption spectrum of 7-dehydrocholesterol in hexane. B, Chemical structures of 7-dehydrocholesterol and cholesterol.

Fig 2.

Rapid ROS generation by UVA irradiation in SLO-HK is prevented by antioxidants, by a NADPH oxidase inhibitor and by a mitochondrial radical quencher. (A) Time course for ROS production in cells irradiated with 1 J/cm² of UVA using carboxy-H₂DCFDA. (B) ROS inhibition in SLO-HK 5 min after UVA irradiation with 1 mM N-acetylcysteine (NAC), 5 mM ascorbic acid (AA), 1 μ M DPI and 0.1 μ M MitoQ 1 h prior to UVA-irradiation. Samples designated as AY+CD were treated with AY9944 plus CD but were not incubated with sterol. * $p < 0.01$ compared to HK treated in the same manner. Data represent the average of 4-6 independent experiments in which triplicate samples were used.

Fig 3.

Irradiation of SLO-HK with 1 J/cm² of UVA induces the activation of NADPH oxidase. Time course for NADPH oxidase activation measured as cytochrome c oxidation in cell homogenates. SLO-HK were irradiated with 1 J/cm² of UVA, then cell homogenates were collected at indicated times. * p<0.01 compared with non-irradiated SLO-HK (time = 0) representing the average of 4 independent experiments in duplicates. Inset, NADPH oxidase activity was measured 10 min after UVA irradiation of HK, SLO-HK and Chol-HK. * p<0.01 compared with HK. Data represent the average of 4 independent experiments with duplicate samples.

Fig 4.

RNA interference was used to knockdown the expression of Nox1 in SLO-HK. (A) Two different sequences of Stealth™-siRNA directed against the human Nox1 were used, designated as Nox1-A siRNA (sequence starting at 750bp) and Nox1-B siRNA (sequence starting at 1642bp). The maximum decrease of Nox1 expression was found 48 h after the transfection in SLO-HK. Immunoblot is representative of 3 independent experiments. Lower panel indicates the quantification of the bands corresponding to Nox1 levels in the same western blot. (B) UVA-induced ROS formation in SLO-HK is partially prevented by decreasing Nox1 expression by RNAi. ROS were measured using carboxy-H₂DCFDA 5 min after UVA irradiation in Nox1-knocked down SLO-HK using RNAi directed against Nox1 (Nox1-A and Nox1-B). Non-transfected SLO-HK and Lipofectamine-treated SLO-HK were used as

controls. Measurements were made 48 h after the transfection. The dotted line shows the ROS levels of non-irradiated and non-transfected SLO-HK. n=3 for experiment with triplicate samples; * p<0.01 compared to UVA irradiated SLO-HK. (C) PGE₂ release was measured 5 minutes after the UVA irradiation (1 J/cm²) in SLO-HK after knocking down Nox1 expression using two sequences of StealthTM-siRNA (Nox1-A and Nox1-B). * p<0.01 compared to unirradiated SLO-HK and ** p<0.01 compared to UVA irradiated SLO-HK. n=3 for experiments with triplicate samples.

Fig 5.

The plasma membrane of SLO-HK shows altered lipid raft composition compared to HK and Chol-HK. (A) HK, SLO-HK, Chol-HK and CD-treated cells without irradiation were immunostained for either caveolin-1, flotillin-2, or Na⁺/K⁺-ATPase or were treated with fluorescent cholera toxin subunit B (CT-B) and crosslinked with antibody as described in the Materials and Methods. Green fluorescence indicates the presence caveolin-1, flotillin-2 and Na⁺/K⁺-ATPase as indicated, and red fluorescence indicates lipid rafts by the crosslinked fluorescent CT-B bound to ganglioside GM1. Co-localization of CT-B with caveolin-1 or flotillin-2 indicates the presence of lipid rafts. In the amplified merged images, diamond-headed arrows point to the yellow overlap spots; arrows are not shown for HK cells because of nearly complete overlap between caveolin-1 or flotillin-2 with CT-B. Absence of co-localization is shown as green spots (white arrow heads) and was apparent for CT-B with Na⁺/K⁺-ATPase in HK (row 6) and for CT-B with caveolin-1 and flotillin-2 in SLO-HK and CD cells. Lipid rafts (CT-B) without co-localization of flotillin-2 is clear as red spots in SLO-HK and shown as triangle-arrowheads (row 7). Images were obtained with a 60x oil immersion objective by confocal microscopy; bar = 10 μ m. Data are representative of 4 independent experiments. (B) Immunoblot of lipid raft-containing fractions from HK and comparable fractions from SLO-HK obtained from $4-5 \times 10^6$ HK or SLO-HK homogenized in 1% Triton X-100 lysis buffer then centrifuged in non-linear sucrose-DEB gradient (5-30-40%). Fractions shown are from the 5-30% interface. (C) Fractions of detergent resistant membranes obtained from a linear sucrose-DEB gradient (5-40%) separation of $4-5 \times 10^6$ HK and SLO-HK lysed with 1% Brij lysis buffer. Caveolin-1 is found in more buoyant fractions from HK compared to SLO-HK. Immunoblots are representative of 3 independent experiments. (B) Immunoblot of lipid raft-containing fractions from HK and comparable fractions from SLO-HK obtained from $4-5 \times 10^6$ HK or SLO-HK homogenized in 1% Triton X-100 lysis buffer then centrifuged in non-linear sucrose-DEB gradient (5-30-40%). Fractions shown are from the 5-30% interface. (C) Fractions of detergent resistant membranes obtained from a linear sucrose-DEB gradient (5-40%) separation of $4-5 \times 10^6$ HK and SLO-HK lysed with 1% Brij lysis buffer. Caveolin-1 is found in more buoyant fractions from HK compared to SLO-HK. Immunoblots are representative of 3 independent experiments.

Fig 6.

Liposomes containing 7-dehydrocholesterol were oxidized more rapidly than cholesterol-containing liposomes during free radical oxidation. Liposomes containing 0.66 mM SAPC, 0.33 mM OPPC and either 0.33 mM Chol or 7-DHC were incubated at 40°C with the free radical generator, AMPA (20 mM). Partial pressure of dissolved O₂ was measured with an oxygen electrode. Control liposomes (designated AMPA) contained only 0.66 mM SAPC and 0.33 mM OPPC. Results are representative of 3 independent experiments.

Fig 7.

UVA irradiation (1 J/cm²) induces the rapid release of PGE₂ and PLA₂ activation in SLO-HK, but not Chol-HK, in ROS-dependent process. (A) Time course for PGE₂ release after UVA irradiation of SLO-HK, Chol-HK and AY9944 plus CD-treated cells; * p<0.01 compared with non-irradiated HK (time=0). Average from 5 independent experiments done in triplicate. (B) PGE₂ release 5 min after UVA irradiation of SLO-HK without antioxidants (-) and with 1 h antioxidant pretreatments: 5 mM ascorbic acid (AA), 1 mM N-acetylcysteine (NAC), 1 μM DPI and 0.1 μM MitoQ. (C) Five min after UVA irradiation, PGE₂ was measured in SLO-HK preincubated 3 h with the COX inhibitors acetylsalicylic acid (ASA, 2 mM), indomethacin (Indo, 50 μM), the PLA₂ inhibitor aristolochic acid (ARC, 50 μM) and 1 h pretreatment with the calcium chelator BAPTA-AM (BAPTA, 25 μM). In B and C, * p<0.01 compared to HK and ** p<0.01 compared to SLO-HK. Data represent the average of 5 independent experiments. D, Activity of cPLA₂ was measured as indicated in the Material and Methods 5 min after UVA treatment (1 J/cm²). SLO-HK were incubated for 1 h with 1 mM NAC, 5 mM AA, 0.1 μM

MitoQ, 1 μ M DPI and 25 μ M BAPTA-AM (BTA) prior to the UVA irradiation. Cells in which Chol was replaced (Chol-HK) were used as a control. * $p < 0.01$ compared to HK-dark and ** $p < 0.01$ compared with UVA-irradiated SLO-HK. Data represent the average of 5 experiments with triplicate samples.

Fig 8.

Intracellular calcium levels increase after UVA irradiation in SLO-HK. (A) Time trace of intracellular calcium levels was measured using the fluorescent probe Calcium Orange-AM after UVA irradiation (1 J/cm^2) in HK and SLO-HK. (B) Decrease in the intracellular calcium using 5 mM ascorbic acid (AA), 1 mM N-acetylcysteine (NAC), 1 μM DPI and 0.1 μM MitoQ (MQ) 1 h prior UVA irradiation. * $p < 0.01$ compared with control (HK) representing the average of 5 (A) independent experiments with triplicate samples. ** $p < 0.01$ compared with UVA-irradiated SLO-HK representing 3 independent experiments with duplicates.

Table 1

Levels of 7-dehydrocholesterol and cholesterol in experimental groups. Preparation of the cells is described in the Experimental Procedures. Results shown are representative of 6 independent experiments for 7-DHC and 4 independent experiments for Chol measurements.

	7-DHC (moles/cell) $\times 10^{-14}$	Chol (moles/cell) $\times 10^{-14}$
HK	0	7.89 $\pm$ 1.76
AY+CD	0.46 $\pm$ 0.10	1.56 $\pm$ 0.05
SLO-HK	2.75 $\pm$ 0.35	3.12 $\pm$ 0.92
Chol-HK	0.51 $\pm$ 0.09	6.75 $\pm$ 2.13