Increase of particulate nitric oxide synthase activity and peroxynitrite synthesis in UVB-irradiated keratinocyte membranes

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Here we demonstrate that human keratinocytes possess a Ca^{2+}/calmodulin-dependent particulate NO synthase that can be activated to release NO after exposure to UVB radiation. UVB irradiation (up to 20 mJ/cm²) of human keratinocyte plasma membranes resulted in a dose-dependent increase in NO and L-[^{3}H]citrulline production that was inhibited by approx. 90 % in the presence of *N*-monomethyl-L-arginine (L-NMMA). In time-course experiments with UVB-irradiated plasma membranes the changes in NO production were followed by analogous changes in soluble guanylate cyclase (sGC) activity. In reconstitution experiments, when particulate NO synthase was added to purified sGC isolated from keratinocyte cytosol, a 4-fold increase in cGMP was observed; the cGMP was increased by NO synthesized after UVB irradiation (up to 20 mJ/cm²) of particulate NO synthase. A 5-fold increase in superoxide (O_2^-) and a 7-fold increase in NO formation followed by an 8-fold increase in peroxynitrite ($ONOO^-$) production by UVB (20 mJ/cm²)-irradiated keratinocyte microsomes was observed. UVB radiation (20 mJ/cm²) decreased plasma membrane lipid fluidity as indicated by steady-state fluorescence anisotropy. Membrane fluidity changes were prevented by L-NMMA. Changes in Arrhenius plots of particulate NO synthase in combination with changes in its allosteric properties induced by UVB radiation are consistent with a decreased fluidity of the lipid microenvironment of the enzyme. The present studies provide important new clues to the role of NO and $ONOO^-$ released by UVB-irradiated human keratinocytes in skin erythema and inflammation.

INTRODUCTION

It has been shown that epidermis is structurally and functionally connected with the rest of the body and that circulating neutrophils and lymphocytes can traffic into the epidermis after various types of inflammatory stimulus [1,2]. The wavelengths responsible for erythema are in the UV portion of the solar spectrum, including UVB (290–320 nm) and UVA (320–400 nm). The redness after exposure to UV radiation is best explained by photoexcitation of an epidermal chromophore that absorbs the incident energy with subsequent release of vasoactive agents that migrate to the dermal vasculature to evoke the vasodilatory response [3,4].

NO is produced by NO synthase, which converts L-arginine and oxygen into citrulline and NO. Constitutive NO synthase is a Ca^{2+}/calmodulin-dependent enzyme that is active as a monomer and is responsible for the transient release of minute quantities (picomoles) of NO, whereas inducible NO synthase is not dependent on Ca^{2+}/calmodulin and causes a sustained release of larger amounts (nanomoles) of NO [5,6]. It has been demonstrated that calmodulin-dependent NO synthase activity is also present in the particulate fractions of bovine aortic endothelial cells [7–9]. This localization might render the enzyme more susceptible to activation by physicochemical stimuli, such as a shear stress and/or cholesterol-induced changes in membrane fluidity [10,11].

Peroxynitrite ($ONOO^-$), a product of NO reaction with superoxide (O_2^-), has recently been defined as a potent oxidant and potential mediator of tissue injury [12–15]. Because NO is a vasodilator, sites of NO synthesis should experience increased perfusion and thus the heat and redness that partly define inflammation. Indeed, NO seems to increase vascular leakiness in several tissues [16,17]. $ONOO^-$, in contrast, activates soluble guanylate cyclase (sGC) producing a vasorelaxant effect, thus indicating that the mechanism of O_2^- inactivation of NO is by converting it to a shorter-lived and less potent vasorelaxant species [18].

The present studies demonstrate for first time that human keratinocytes possess a particulate (membrane-bound) NO synthase that is Ca^{2+}/calmodulin-dependent. UVB irradiation of keratinocyte membranes resulted in the activation of particulate NO synthase to release NO and $ONOO^-$, which might be an early key event in keratinocyte dysfunction leading to skin erythema and inflammation.

MATERIALS AND METHODS

Materials

L-[^{3}H]Arginine (54 Ci/mmol) and cGMP radioimmunoassay kit were obtained from the Radiochemical Centre, Amersham (Little Chalfont, Bucks., U.K.); NO (99.99 % pure) was obtained from Messer Griesheim (Gumpoldskirchen, Germany). 2′,5′-ADP-agarose and GTP-agarose were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.); DEAE-Bio-Gel A and Dowex AG 50WX8 (Na^+ form) were obtained from Bio-Rad Chemical Division (Richmond, CA, U.S.A.). (L-NMMA) and superoxide *N*-Monomethyl-L-arginine dismutase (SOD) were obtained from Calbiochem (Lucerne, Switzerland). Other reagents, solvents and salts were of analytical grade and were obtained from Sigma.

Preparation of keratinocyte membranes

Human keratinocytes were cultured in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine and 10 % (v/v) fetal calf serum as previously described [19]. Approx. 10^9 cells were suspended in 50 mM Tris/HCl, pH 7.4, containing 10 mM EDTA, 5 mM glucose, 1.15 % (w/v) KCl, 0.1 mM D,L-

Abbreviations used: DPH, 1,6-diphenyl-1,3,5-hexatriene; HBSS, Hanks balanced salt solution; L-NMMA, *N*-monomethyl-L-arginine; sGC, soluble guanylate cyclase; SOD, superoxide dismutase.

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dithiothreitol, 2 mg/l leupeptin, 2 mg/l pepstatin A, 10 mg/l trypsin inhibitor and 44 mg/l PMSF (buffer 1). The cell suspension was bubbled with helium for 15 min and sonicated, and the homogenate was centrifuged in three subsequent steps at 1000 ***g***, 10000 ***g*** and 105000 ***g*** for 10, 20 and 60 min respectively. The 105000 ***g*** pellet (microsomal fraction) was resuspended in 50 mM Tris/HCl, pH 7.4, containing 0.1 mM EDTA, 0.1 mM dithiothreitol, 2 mg/l leupeptin, 2 mg/l pepstatin A, 10 mg/l trypsin inhibitor, 44 mg/l PMSF and 10 % (v/v) glycerol (buffer 2). For the separation of the plasma membranes from the microsomes, 2 ml aliquots of the microsomal fraction (2.0–2.5 mg of protein) were layered over a 20 ml cushion of 27 % (w/v) sucrose in 10 mM Tris/HCl buffer containing 1 mM EDTA and centrifuged for 3 h at 65000 ***g*** in a SW-27 rotor of a Beckman L5-75 ultracentrifuge. The band (2 ml) at the sucrose interface containing plasma membranes was diluted with 8 ml of buffer 2 (without glycerol), collected by centrifugation at 100000 ***g*** for 60 min, resuspended in homogenization buffer 2 and stored at −70 °C. The designation of the subcellular fractions as microsomes and plasma membranes was based on the determination of the corresponding marker enzyme activities, i.e. NADH-dependent cytochrome *c* reductase (microsomes) and 5′-nucleotidase (plasma membranes) with 25–50 μg aliquots of each fraction as previously described [11]. Solubilization of membranes was performed with 0.1 % (w/v) Lubrol-PX at 4 °C for 12 h under magnetic stirring, in a medium consisting of Hanks balanced salt solution (HBSS), pH 7.4, containing 200 mM mannitol, 0.1 mM dithiothreitol, 44 mg/l PMSF and 50 μg of membrane protein. Protein concentration was measured by the Bradford method [20] with reagents from Bio-Rad and BSA as a standard.

Ultraviolet irradiation

A fluorescent UVB lamp (VL-6M 1x6, wavelength 290–320 nm with a peak at 312 nm, power 12 W; Vilber Lourmat, Marne La Vallée, France) was used. Keratinocyte plasma membranes (50 μg/ml) and/or Lubrol-PX solubilized plasma membranes (50 μg/ml), or keratinocyte microsomes (200 μg/ml) were irradiated with various UVB doses delivered within 2 min. Radiation doses were controlled by altering the distance between the radiation source and the samples and were measured with an IL-200 light meter.

Assay of particulate NO-synthase activity

Determination of NO

NO synthase activity in keratinocyte plasma membranes was measured by monitoring either NO and/or L-[^{3}H]citrulline production as previously described [21,22]. Reactions (1 ml sample volumes) were conducted for 10 min at 37 °C. Standard reaction mixtures contained HBSS, 1 mM EDTA, plasma membranes (50 μg protein) and/or microsomal membranes (200 μg protein), 100 μM L-arginine, 100 μM NADPH, 5 μM FAD, 5 μM FMN, 5 μM tetrahydrobiopterin and 1 μM calmodulin. NO determination was performed by mixing the incubated mixtures with 100 μl of a reagent consisting of 25 μM scopoletin and 20 % (w/v) sulphanilamide in 20 % (v/v) H_3PO_4. The present method estimates both NO and NO_2^-, but the rate of scopoletin oxidation by NO is much faster than by NO_2^-. The reaction in the presence of NO is terminated within 1–2 min, whereas in the presence of NO_2^- it is terminated in 6–8 min. In time-course experiments of NO production (within 60 min) shown in Figure 3(A), NO_2^- ions are formed from the oxidation of NO (half-life less than 1 s in aerobic solutions), and therefore both NO and NO_2^- are measured. Therefore the results are given in arbitrary units. For the assay of the allosteric inhibition of the plasma membrane NO synthase by Mn^{2+}, the reaction mixture contained increasing amounts of $MnCl_2$ as indicated in Figure 9.

Determination of L-citrulline

L-Citrulline was measured in 50 mM Hepes buffer, pH 7.4, containing the same cofactors as described for NO measurements in the presence of keratinocyte plasma membranes (50 μg of protein). L-[^{3}H]arginine (100000 c.p.m.) was mixed with unlabelled L-arginine (100 μM) in a final volume of 200 μl, and L-[^{3}H]citrulline was measured in a liquid-scintillation counter as previously described [19].

Determination of superoxide anion (O_2^-)

The production of O_2^- by keratinocyte microsomes (50 μg of protein) was measured by monitoring cytochrome *c* (10 μM) reduction spectrophotometrically at 550 nm for 10 min at 37 °C. A molar absorption coefficient of 21000 $M^{-1} \cdot cm^{-1}$ was used to calculate the rate of O_2^- production, and corrected for cytochrome *c* reduction not inhibited by SOD (100 units/ml).

Determination of $ONOO^-$

$ONOO^-$ was synthesized, titrated and stored as previously described [23,24]. The formation of $ONOO^-$ by keratinocyte microsomes (200 μg of protein per 0.5 ml of HBSS), in the presence of luminol (10 μM), was measured by luminol-amplified chemiluminescence with a Berthold AutoLumat LB953 Luminometer. The results were calculated as c.p.s. Chemiluminescence responses were then converted to nmoles of $ONOO^-$ by using a standard curve constructed with various concentrations of pure $ONOO^-$.

Purification of sGC, and cGMP determination

sGC from human keratinocytes was purified by GTP-agarose chomatography, and cGMP was determined by radioimmmunoassay after acetylation of the samples with acetic anhydride with a cGMP assay kit (Amersham) as previously described [19].

Steady-state fluorescence polarization studies

Lipid fluidity was assessed by the steady-state fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) by the method of Shinitzky and Barenholz [25]. The polarization of fluorescence was expressed as the fluorescence anisotropy (r), and the anisotropy parameter $[(r_0/r)-1]^{-1}$ was calculated from the value of $r_0 = 0.365$ (r_0 is the fluorescence anisotrophy value in the absence of any rotational motion of the probe) for DPH as previously described [26]. The temperature dependence of $[(r_0/r)-1]^{-1}$ was determined over the range 4–40 °C. Keratinocyte plasma membranes were warmed initially to 40 °C and the fluorescence polarization was estimated every 1–2 °C as the suspension cooled slowly to 4 °C. Plots of $\log[(r_0/r)-1]^{-1}$ against $1/T$ were constructed to detect thermotropic transition temperatures [27].

Statistical analysis

Three or four independent experiments were performed. All samples were assayed in duplicate or in triplicate. The partial correlation coefficient (r) for the straight lines were above 0.98. Means and S.D. values were calculated. Comparisons between two groups were performed by the use of a standard Student t

test. A value of $P < 0.05$ was considered significant. When two different curves were constructed with the same membrane preparation under the same experimental conditions, the individual points and the slopes obtained showed a maximum variability of 5%. For different membrane preparations the maximum variability was less than 10%.

RESULTS

Determination of NO and L-citrulline after UVB irradiation of keratinocyte plasma membranes

The curves representing the changes in particulate NO synthase activity of keratinocytes at various doses of UVB are shown in Figures 1 and 2. NO and L-[^{3}H]citrulline production showed a 3-fold increase on irradiation at 20 mJ/cm^2. In the presence of 100 μM (L-NMMA) the production of both NO and L-[^{3}H]citrulline was inhibited at all UVB radiation doses used. Figures 1 and 2 also demonstrate that Lubrol-PX-solubilized membranes irradiated with various doses of UVB resulted in an approx. 2-fold increase in both NO and L-[^{3}H]citrulline production at 20 mJ/cm^2; these were inhibited by approx. 90% in the presence of 100 μM L-NMMA. D-Arginine (100 μM) failed to stimulate the enzyme activity, indicating the specificity of the activation by L-arginine. The enzyme activity was also Ca^{2+}/calmodulin- and tetrahydrobiopterin-dependent. In a Ca^{2+}-free solution containing 1 mM EDTA the activity of NO synthase was very low (280 ± 45 pmol NO/min per mg of protein).

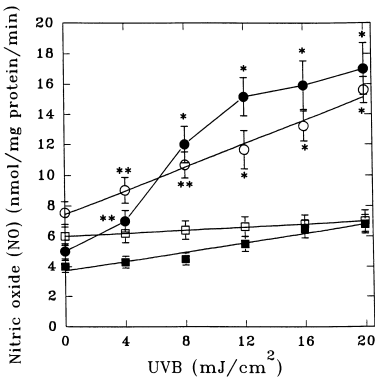

Figure 1 NO release following UVB irradiation of keratinocyte plasma membranes

Symbols: ●, effect of UVB radiation on nitric oxide (NO) production by particulate (membrane-bound) NO synthase of human keratinocyte plasma membranes; ■, NO production in the presence of L-NMMA (100 μM); ○, NO production by Lubrol-PX-solubilized particulate NO synthase; □, NO production by UVB-irradiated Lubrol-PX-solubilized NO synthase in the presence of L-NMMA (100 μM). Points and bars represent means ± S.D. for four different experiments. A statistically significant difference from the plasma membranes in the presence of L-NMMA was determined (*$P < 0.01$, **$P < 0.05$).

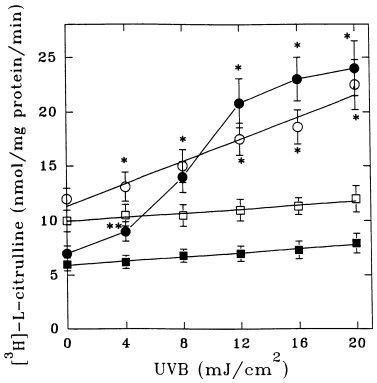

Figure 2 L-Citrulline production following UVB irradiation of keratinocyte plasma membranes

Effect of UVB radiation on L-[^{3}H]citrulline production by particulate (membrane-bound) NO synthase of human keratinocyte plasma membranes (●) and in the presence of L-NMMA (100 μM) (■); L-[^{3}H]citrulline production by UVB-irradiated Lubrol-PX-solubilized particulate NO synthase (○) and in the presence of L-NMMA (100 μM) (□). Points and bars represent means ± S.D. for four different experiments. A statistically significant difference from the plasma membranes in the presence of L-NMMA was determined (*$P < 0.01$, **$P < 0.05$).

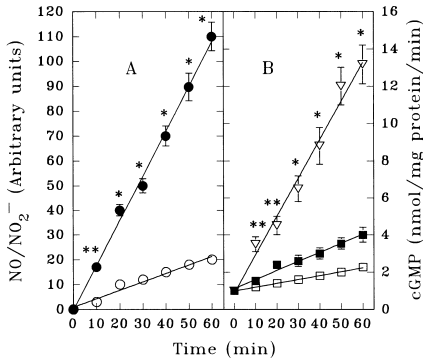

Figure 3 NO release following UVB irradiation of keratinocyte plasma membranes, accompanied by analogous changes in sGC activity

Keratinocyte plasma membranes and HBSS were placed in two adjacent compartments of a chamber separated by a thin Teflon membrane [0.0015 inches (38 μm) thick], a membrane that permits only NO diffusion. (**A**) NO/NO$_2^-$ were determined in HBSS when plasma membranes (50 μg) with the appropriate cofactors were placed in the opposite compartment (○), and in UVB-irradiated (20 mJ/cm^2) plasma membranes (●). (**B**) Replacement of HBSS with sGC resulted in a doubling of cGMP in the compartment containing sGC (■) compared with the basal sGC activity (□). When plasma membranes were irradiated with UVB (20 mJ/cm^2) an approx. 4-fold increase in cGMP production was observed (▽). Each point represents the mean ± S.D. for three independent experiments. A statistically significant difference from UVB-irradiated plasma membranes was determined (*$P < 0.01$, **$P < 0.05$).

NO and cGMP determination with an incubation chamber

Figure 3 shows the amounts of NO/NO$_2^-$ and cGMP produced when plasma membranes (50 μg) were placed with the appropriate substrates and cofactors and HBSS in two adjacent compartments of a chamber separated by a thin Teflon membrane [0.0015 inches (38 μm) thick; Dupont, Wilmington, DE, U.S.A.) permitting NO diffusion through the membrane. A time-dependent increase in NO/NO$_2^-$ in the compartment containing HBSS was observed to reach equilibrium within 60 min with the NO/NO$_2^-$ present in the compartment containing plasma membranes. When plasma membranes were irradiated with UVB at 20 mJ/cm^2 the accumulation of NO/NO$_2^-$ in the HBSS compartment was increased approx. 5-fold, as was seen with control plasma membranes not irradiated with UVB. Finally, HBSS in the first compartment was replaced by purified sGC, isolated from keratinocyte cytosol, for a bioassay of NO over a 60 min period. In control plasma membranes we noted that the diffusion of NO across the Teflon membrane into the sGC compartment resulted in the production of a significant amount of cGMP (3.95 ± 0.45 nmol/min per mg of protein). When the procedure was repeated with plasma membranes irradiated with UVB at

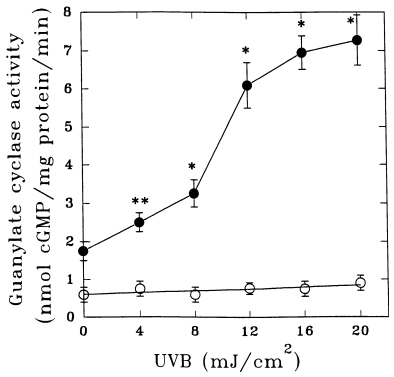

Figure 4 Effect of UVB radiation on the activity of sGC in the presence (●) or absence (○) of particulate NO synthase

Each point represent the mean ± S.D. for four independent experiments. A statistically significant difference between sGC in the presence and in the absence of particulate NO synthase was determined (*$P < 0.01$, **$P < 0.05$).

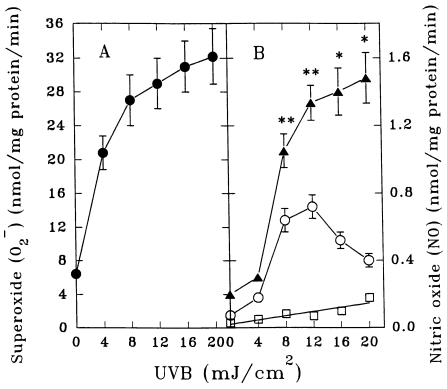

Figure 5 NO and superoxide (O_2^-) release following UVB irradiation of keratinocyte microsomal membranes

(**A**) Effect of various doses of UVB radiation on superoxide (O_2^-) production by particulate NO synthase of keratinocyte microsomal membranes(●). (**B**) Effect of various doses of UVB radiation on nitric oxide (NO) production by particulate NO synthase of keratinocyte microsomal membranes (○); NO production in the presence of L-NMMA (100 μM) (□) and SOD (100 U/ml) (▲) by microsomal membranes. Each point represents the mean ± S.D. for three independent experiments. A statistically significant difference from membranes in the presence of SOD was determined (*$P < 0.01$, **$P < 0.05$).

20 mJ/cm² an approx. 4-fold increase in cGMP production was observed compared with non-irradiated membranes.

NO and cGMP production by particulate NO synthase (reconstitution experiments)

As sGC is the physiological effector system of L-arginine-derived NO, reconstitution experiments with particulate NO synthase and sGC purified from keratinocyte cytosol were performed. The reaction mixture consisted of 50 μg of keratinocyte plasma membrane, 1 μg of purified sGC in 50 mM triethanolamine/HCl buffer, pH 7.4, 100 μM L-arginine, 100 μM NADPH, 5 μM FAD, 5 μM FMN, 5 μM tetrahydrobiopterin, 10 μM Ca^{2+} and 1 μM calmodulin. The appropriate cofactors for the estimation of sGC were used as described in the Materials and methods section. Figure 4 shows that UVB irradiation increased the activity of purified sGC by approx. 4-fold, consistent with the increased

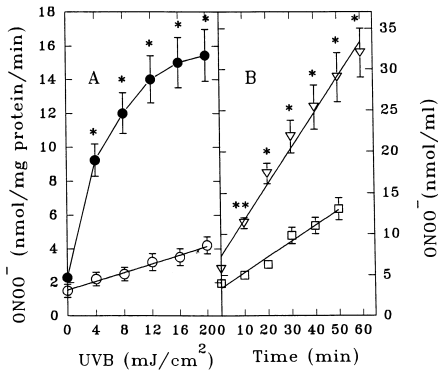

Figure 6 Peroxynitrite ($ONOO^-$) production following UVB irradiation of keratinocyte microsomal membranes

(**A**) Effect of various doses of UVB radiation on $ONOO^-$ production by particulate NO synthase of keratinocyte microsomal membranes (●), and $ONOO^-$ production in the presence of L-NMMA (100 μM) (○). (**B**) Time-dependence of $ONOO^-$ production by keratinocyte microsomal membranes irradiated with UVB (20 mJ/cm²) (▽). The scavenging effect of desferrioxamine (100 μM) on $ONOO^-$ production by microsomes is also shown (□). Each point represents the mean ± S.D. for three independent experiments. A statistically significant difference from UVB-irradiated microsomes in the presence of L-NMMA and desferrioxamine was determined (*$P < 0.001$, **$P < 0.01$).

particulate NO synthase activity by UVB radiation. In the absence of particulate NO synthase or L-arginine, UVB failed to increase the activity of sGC.

NO and O_2^- production by keratinocyte microsomal membranes

The effect of UVB radiation on NO and O_2^- production by microsomal membranes is shown in Figure 5. NO production followed a biphasic curve, whereas O_2^- increased gradually up to approx. 5-fold at various amounts of UVB. Low doses of radiation (up to 12 mJ/cm²) increased approx. 7-fold the NO production; however, high doses (up to 20 mJ/cm²) decreased NO production (by approx. 50 %) with respect to the maximal stimulation. In experiments when SOD (100 units/ml) was included in the incubation mixture, the NO production by microsomes at various doses of UVB radiation increased gradually up to 4-fold at 20 mJ/cm² compared with microsomes in the absence of SOD. L-NMMA (100 μM) decreased the enzyme activity by approx. 90 %.

$ONOO^-$ production by keratinocyte microsomal membranes

Figure 6(A) shows the effect of various doses of UVB radiation (up to 20 mJ/cm²) on $ONOO^-$ production by keratinocyte microsomal membranes. $ONOO^-$ formation was increased approx. 8-fold at 20 mJ/cm². In the presence of 100 μM L-NMMA, $ONOO^-$ formation was decreased by 90 % at various doses of UVB radiation. Figure 6(B) shows a time-dependent increase of $ONOO^-$ formation by microsomes irradiated with 20 mJ/cm² of UVB. An approx. 6-fold increase of $ONOO^-$ formation within 60 min in UVB-irradiated microsomes was observed compared with non-irradiated (control) microsomes. Desferrioxamine (100 μM) decreased $ONOO^-$ formation by approx. 70 %.

Temperature dependence of keratinocyte particulate (plasma membrane-bound) NO synthase

The temperature dependence of NO synthase activity in plasma membranes irradiated with UVB at 20 mJ/cm² is shown in

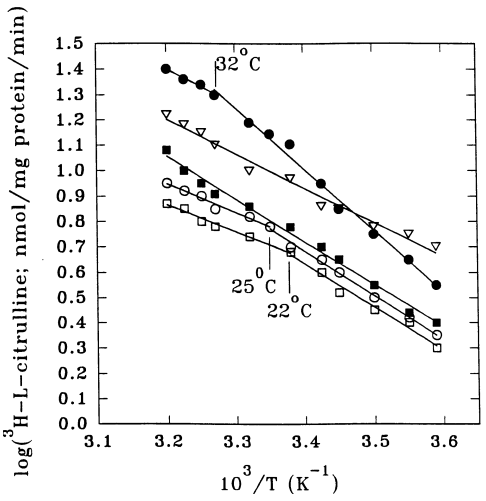

Figure 7 Arrhenius plots of particulate NO synthase activity from UVB-irradiated (20 mJ/cm²) plasma membranes (●), in UVB-irradiated plasma membranes in the presence of L-NMMA (100 μM) (○), in non-irradiated (control) plasma membranes (□), in Lubrol-PX-solubilized membranes (■) and in UVB-irradiated Lubrol-PX-solubilized membranes (▽)

Each point represents the average for duplicate determinations from a typical experiment that was repeated three times.

Figure 7. Arrhenius plots of plasma membrane NO synthase activity (nmol of L-[^{3}H]citrulline/min per mg of protein) showed a transition temperature at 21.9±1.6 °C. Plasma membranes irradiated with 20 mJ/cm² showed an elevation of the break point to 32.2±2.4 °C, approx. 10 °C above that of non-irradiated (control) membranes, consistent with a decrease in the fluidity of the lipid microenvironment of the enzyme. UVB-irradiated plasma membranes in the presence of L-NMMA (100 μM) showed a break point at 24.5±1.9 °C, suggesting that NO caused the decrease in the fluidity in the lipid microenvironment of the enzyme. Figure 7 also shows that (1) the break point (transition temperature) for NO synthase was abolished and a linear relation was obtained after solubilization of the enzyme with the non-anionic detergent Lubrol-PX, and (2) Lubrol-PX increased the enzyme activity by approx. 50%. UVB irradiation (20 mJ/cm²) of the solubilized enzyme increased its activity by approx. 50%, whereas the Arrhenius plot showed a linear relation at all the range of temperatures used.

Temperature dependence of fluorescence anisotropy of keratinocyte plasma membranes

The effects of temperature on the fluorescence anisotropy parameter, $[(r_0/r)-1]^{-1}$, of DPH in plasma membranes are illustrated by representative Arrhenius plots in Figure 8. A thermotropic transition temperature (separation phase) was observed at 23.5±1.8 °C in non-irradiated plasma membranes. In irradiated membranes the thermotropic transition temperature was elevated to 34.2±2.4 °C. This upward shift, which corresponds to an increase of 10–11 °C in the break point temperature, is consistent with a general decrease in the fluidity of the plasma membrane [28]. In the presence of L-NMMA (100 μM) and/or desferrioxamine (100 μM), UVB irradiation (20 mJ/cm²) failed to increase the thermotropic transition temperature (26.8±2.0 for L-NMMA, and 25.5±1.8 for desferrioxamine). Desferrioxamine acts as a scavenger of NO. Non-irradiated (control) membranes treated either with L-NMMA (100 μM) or

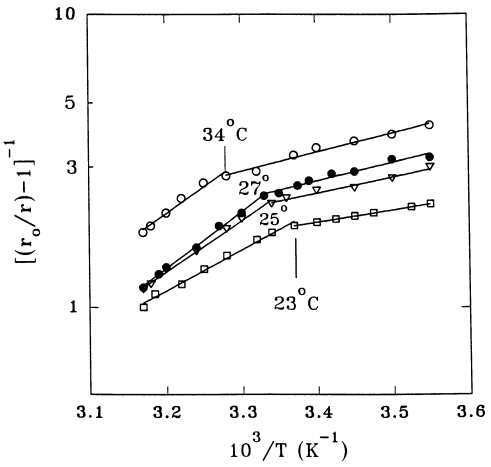

Figure 8 Temperature dependence of the fluorescence anisotropy of DPH in control plasma membranes (□), in UVB-irradiated plasma membranes (○), and in L-NMMA-treated (●) and desferrioxamine-treated (▽) plasma membranes that had been irradiated with UVB (20 mJ/cm²)

This experiment was representative of three that were performed.

with desferrioxamine (100 μM) showed no changes in membrane fluidity.

Co-operative inhibition of the keratinocyte plasma membrane-bound (particulate) NO synthase by Mn^{2+} ions

Figure 9 shows the curves obtained when the relative rates of enzymic activity were plotted against different concentrations of

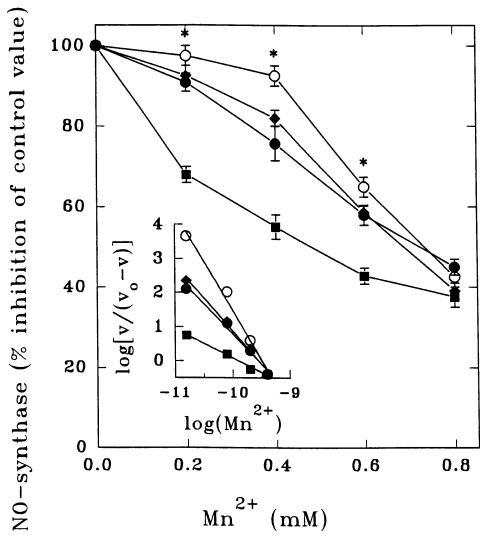

Figure 9 Allosteric inhibition of particulate NO synthase activity by Mn^{2+} ions following UVB irradiation of keratinocyte plasma membranes

Effect of Mn^{2+} on the reaction rate of NO synthase (nmol NO/min per mg of protein) for UVB-irradiated (○), UVB-irradiated in the presence of L-NMMA (100 μM) (◆), untreated (control) (●) and Lubrol-PX-solubilized plasma membranes (■). The insert shows Hill plots of the same data. The corresponding Hill coefficients are 2.45±0.22 (○), 1.85±0.17 (◆), 1.75±0.15 (●) and 0.90±0.08 (■); v is the reaction velocity and v_0 is the rate of the reaction in the absence of Mn^{2+}. Each point represents the mean±S.D. for three independent experiments (*$P < 0.05$).

Mn^{2+} in control plasma membranes and in membranes irradiated at 20 mJ/cm². The Hill coefficient (slopes) for the control (non-irradiated) plasma membranes was 1.75 ± 0.15, indicating the presence of co-operativity; in plasma membranes irradiated at 20 mJ/cm², the Hill coefficient was increased to 2.45 ± 0.22, suggesting a higher co-operativity of the enzyme; this is consistent with a decrease in the fluidity of the annular lipids of the enzyme. When plasma membranes were irradiated at 20 mJ/cm² in the presence of L-NMMA (100 μM), the Hill coefficient (1.85 ± 0.17) showed no statistically significant difference ($P > 0.05$) from that of non-irradiated plasma membranes. In contrast, in Lubrol-PX-solubilized irradiated plasma membranes, the Hill coefficient was decreased to 0.90 ± 0.08, suggesting a loss of co-operativity of the enzyme, consistent with an increase in the fluidity of the annular lipids of the enzyme [29,30].

DISCUSSION

In the present study we have demonstrated that human keratinocytes possess a Ca^{2+}/calmodulin-dependent particulate (membrane-bound) NO synthase that can be activated directly by UVB (290–320 nm) radiation to produce NO and L-citrulline. UVB-irradiated keratinocyte membranes produced both O_2^- and NO, which react together to form the noxious oxidant $ONOO^-$, which decreased membrane fluidity with a subsequent increase in particulate NO synthase activity (positive feedback mechanism).

The curves representing the changes of NO synthase activity in intact plasma membranes and in Lubrol-PX-solubilized plasma membranes irradiated with various doses of UVB indicate a direct stimulatory effect of UVB radiation on particulate NO synthase activity. L-NMMA inhibited both NO and L-citrulline by approx. 90% (Figures 1 and 2). Particulate NO synthase is capable of releasing NO for prolonged periods after exposure to UVB radiation, providing evidence that once NO is released by the membranes, the gas can continue to be produced and released even after the stimulus has been removed (Figure 3). These experiments also showed that the NO released from keratinocyte plasma membranes is identical with gaseous NO and not to any nitrosothiol compound [31].

Our findings in this study demonstrate that in a defined reconstitution system consisting of purified sGC and particulate NO synthase, the formation of cGMP was increased after UVB irradiation (Figure 4); this is consistent with the widespread signal transduction system that involves Ca^{2+}/calmodulin-regulated NO formation and activation of sGC [32]. UVB radiation caused an increase in both NO and O_2^- production by the keratinocyte microsomes; these products react together to form $ONOO^-$ (Figures 5 and 6). O_2^- might be produced by microsomes in redox reactions of mixed-function oxidases through cytochrome P_{450} [33]. In Figure 5(B) the decline in the curve showing NO production by microsomal membranes at high doses of UVB radiation was apparently due to neutralization of NO by O_2^- to form $ONOO^-$. This was supported by the fact that in the presence of SOD the production of NO followed a gradual increase up to a UVB dose rate of 20 mJ/cm².

UVB irradiation of microsomal membranes resulted in an 8-fold increase in $ONOO^-$ production (Figure 6A). In time-dependent experiments (within 60 min) a 6-fold increase in the $ONOO^-$ production by UVB irradiation (20 mJ/cm²) of keratinocyte microsomes compared with non-irradiated (control) microsomes was observed (Figure 6B). In previous studies we have shown that $ONOO^-$ increased cGMP synthesis in cultured human keratinocytes [13]. Furthermore it has recently been suggested (18) that $ONOO^-$ produces coronary vasorelaxation in dogs and that the mechanism of O_2^- inactivation of NO is by converting it to a shorter-lived and less potent vasorelaxant species. High concentrations of $ONOO^-$, or a prolonged exposure to a lower steady-state concentration of $ONOO^-$, however, might be directly damaging to tissues and possibly result in abnormal vasoregulation *in vivo*.

Previous reports indicate that $ONOO^-$ initiates lipid peroxidation and causes oxidation of plasma lipoproteins [34]. In this study $ONOO^-$ released within the keratinocytes modulated their lipid and protein components, thus changing their lipid fluidity. The characteristic temperatures ('break points') of Arrhenius plots of the fluorescence anisotropy of DPH and/or particulate NO synthase activity were employed as measures of changes in membrane fluidity. In Figure 7, Arrhenius plots of plasma membrane NO synthase activity exhibited a break points at approx. 22 °C. UVB radiation increased this to approx. 32 °C and this is matched by a similar effect on thermotropic transition temperatures estimated by the DPH fluorescence anisotropy (approx. 23 °C and approx. 34 °C respectively; Figure 8). The thermotropic separation phase temperature of UVB-irradiated plasma membranes was restored to that of non-irradiated (control) plasma membranes (approx. 25 ° C) when these membranes were treated with L-NMMA and/or desferrioxamine, a scavenger of NO [35]. These findings clearly indicate that (1) UVB-induced changes in plasma membrane fluidity are mainly due to NO production, and (2) plasma membrane NO synthase activity is governed by both the bulk lipid fluidity and the fluidity of its annular lipids. Moreover UVB radiation of the Lubrol-PX-solubilized NO synthase resulted in an approx. 50% increase in the enzyme's activity, abolishing the biphasic nature of the Arrhenius plot for the enzyme and establishing a linear relation. These results suggest that UVB radiation exerts a direct stimulatory effect on the protein molecule and an indirect stimulatory effect on the the membrane-bound enzyme through changes to its lipid microenvironment.

In the present study further evidence that UVB radiation affects the fluidity of the lipid microenvironment of the plasma membrane NO synthase was obtained from alterations in the co-operativity behaviour of this enzyme [2,27–29]. As shown in Figure 9, the Hill coefficient for the allosteric inhibition of NO synthase by Mn^{2+} was increased in UVB-irradiated keratinocyte plasma membranes, indicating a higher co-operativity of the enzyme, consistent with a decrease in the fluidity of the annular lipids of the enzyme. However, in the presence of L-NMMA, UVB radiation failed to decrease the fluidity of the annular lipids of the enzyme, indicating that NO and/or $ONOO^-$ were responsible for the UVB-induced changes to the keratinocyte plasma membrane lipid microenvironment [36].

In the light of all these experiments relating to changes in the membrane fluidity the following positive feedback mechanism for the control of the NO synthase activity after UVB irradiation can be suggested: UVB radiation directly activates NO synthase to produce NO. In the next step NO decreases membrane fluidity, thus resulting in the maintenance of high NO synthase activity levels and continued production of NO. This mechanism explains the production of NO even after the removal of the UVB stimulation.

The physiological role of the particulate form of NO synthase in keratinocytes might be the production of small quantities of NO that are important in maintaining the vascular tone of the blood vessels of the skin. NO might also act as an autacoid in keratinocytes, inhibiting ribonucleotide reductase as well as mitochondrial respiration and DNA synthesis, events that control cellular proliferation [32]. It has been proposed that NO produced by keratinocytes might function in non-specific host defence during wound healing, and that this might occur because

NO, either alone and/or in combination with reactive oxygen intermediates, is toxic [37]. It has also been shown that UVB irradiation of rat skin caused delayed-onset vasodilation and an increase in basal blood flow. Nitro-L-arginine methyl ester injected locally 17.5 h after UVB irradiation abolished the 18 h increase in blood flow [38]. Our previous studies *in vivo* showed that when guinea pigs were subjected to UVB irradiation, an erythema protection factor of 8.71 was calculated when an emulsified cream formulation containing L-NMMA (2%, w/v) was applied to their skin [19].

In conclusion, the present studies demonstrate that UVB irradiation of keratinocytes changed particulate (membrane-bound) NO synthase activity, resulting in deviations from normal skin function. Furthermore UVB radiation causes an imbalance in keratinocyte-derived NO and $O_2^{\cdot-}$. $ONOO^-$ formation by human keratinocytes during UVB radiation might exert cytotoxic effects in the cells in which it is produced. $ONOO^-$ might also diffuse out of the keratinocytes and exert cytotoxic effects in the neighbouring endothelial and smooth-muscle cells, resulting in the erythema and inflammation that accompany human sunburn reactions.

This work was supported by funds from the Greek Ministry of Research and Technology (92 SYN17) and from the Medical School, University of Athens.

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Received 27 November 1995/29 August 1996; accepted 30 August 1996