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Alterations of Nitric Oxide Synthase and Xanthine Oxidase Activities of Human Keratinocytes by Ultraviolet B Radiation

POTENTIAL ROLE FOR PEROXYNITRITE IN SKIN INFLAMMATION

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ABSTRACT. In the present study, we demonstrated that NO synthase (cNOS) and xanthine oxidase (XO) of human keratinocytes can be activated to release NO, superoxide (O_2^-) and peroxynitrite ($ONOO^-$) following exposure to ultraviolet B (UVB) radiation. We defined that this photo induced response may be involved in the pathogenesis of sunburn erythema and inflammation. Treatment of human keratinocytes with UVB (290–320 nm) radiation (up to 200 mJ/cm^2) resulted in a dose-dependent increase in NO and $ONOO^-$ release that was inhibited by N-monomethyl-L-arginine (L-NMMA). NO and $ONOO^-$ release from keratinocytes was accompanied by an increase in intracellular cGMP levels. Treatment of human keratinocyte cytosol with various doses of UVB (up to 100 mJ/cm^2) resulted in an increase in XO activity that was inhibited by oxypurinol. UVB radiation (up to 100 mJ/cm^2) of keratinocytes resulted in a 15-fold increase in S-nitrosothiol formation, which directly increased purified soluble guanylate cyclase (sGC) activity by a mechanism characteristic of release of NO from a carrier molecule. In reconstitution experiments, when UVB-irradiated (20 mJ/cm^2) purified cNOS isolated from keratinocyte cytosol was combined with UVB–irradiated (20 mJ/cm^2) purified XO, a 4-fold increase in $ONOO^-$ production, as compared to nonirradiated enzymes, was observed. $ONOO^-$ synthesized by NO and O_2^- following UVB radiation of cNOS and XO was inhibited by oxypurinol (100 μM). UVB radiation of keratinocyte cytosol resulted in an increase in oxygen free radical production, consistent with the increased production of $ONOO^-$ by UVB-irradiated keratinocyte cytosol. In *in vivo* experiments, when experimental animals were subjected to UVB radiation, a protection factor (PF) of 6.5 ± 1.8 was calculated when an emulsified cream formulation containing nitro-L-arginine (L-NA) (2%) and L-NMMA (2%) was applied to their skin. The present study indicates that UVB radiation acts as a potent stimulator of cNOS and XO activities in human keratinocytes. NO and $ONOO^-$ may exert cytotoxic effects in keratinocytes themselves, as well as in their neighboring endothelial and smooth muscle cells. This may be a major part of the integrated response leading to erythema production and the inflammation process. BIOCHEM PHARMACOL 51;12:1727–1738, 1996.

KEY WORDS. nitric oxide; peroxynitrite; S-nitrosothiols; NO synthase; xanthine oxidase; soluble guanylate cyclase; ultraviolet B radiation; keratinocytes; erythema; inflammation

Major manifestations of cutaneous inflammation are pain, swelling, and redness as a consequence of various inflammatory stimuli, including sunlight, and are thought to result from vasodilation in both the superficial and deep vascular plexi of the dermis. The mechanism of human sunburn is inadequately understood, but its characteristic features include the development of visible redness following threshold doses of UVA and UVB,† after a latent period of 4–8 hr, which is maximal at 16–24 hr and fades slowly thereafter [1, 2].

The cutaneous chromophore responsible for sunburn is best explained by photoexcitation of an epidermal chromophore that absorbs the incident energy and, then, releases vasoactive agents that migrate to the dermal vasculature to evoke the vasodilatory response [3, 4]. Oxygen free radicals are probably involved in the induction of erythema following UV irradiation and methimazole (an oxygen free radical scavenger) may play a useful role in preventing or decreasing erythema [5].

Although pharmacological and chemical evidence indicates that EDRF is identical to NO, other candidates, including S-nitrosocysteine, the dinitrosyl-iron-cystein complex, nitroxyl, and hydroxylamine, have been proposed to

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† *Abbreviations:* L-NMMA, N-monomethyl-L-arginine; D-NMMA, N-monomethyl-D-arginine; L-NA, nitro-L-arginine; sGC, soluble guanylate cyclase; EDRF, endothelium-derived relaxing factor; cNOS, constitutive nitric oxide synthase; NO, nitric oxide; $ONOO^-$ peroxynitrite; HBSS, Hank's balanced salt solution; PF, protection factor; SOD, superoxide dismutase; XO, xanthine oxidase; XD, xanthine dehydrogenase; UVB, ultraviolet B.

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account for the vasorelaxant properties of EDRF [6–8]. NO is produced by NO synthase (NOS) in an unusual reaction that converts arginine and oxygen into citrulline and NO. The mechanism of NO synthesis involves the transfer of electrons between various cofactors, including flavin adenine dinucleotide phosphate (FAD), flavin mononucleotide (FMN), nicotinamide adenine dinucleotide phosphate (NADPH), tetrahydrobiopterin, and heme. Finally, one atom of oxygen binds with the terminal guanidine nitrogen from arginine to form NO [9]. cNOS is a Ca^{2+}/calmodulin-dependent enzyme, but inducible NOS (iNOS) is not dependent on Ca^{2+}/calmodulin [10].

Although there is general agreement that NO is involved in inflammation, the literature is confusing, because both proinflammatory and antiinflammatory properties have been ascribed to NO. Because NO is a vasodilator, sites of NO synthesis should experience increase perfusion and, thus, the heat and redness that partly define inflammation. Indeed, NO appears to increase vascular leakage in several tissues [11, 12]. In inflamed tissue, NO reacts quickly with O_2^-, with the consequent formation of the toxic $ONOO^-$, which promotes lipid and sulfydryl oxidation [13, 14]. $ONOO^-$, on the other hand, activates sGC to produce 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 [15, 16].

The enzyme xanthine oxidase (XO) couples the oxidation of hypoxanthine with the reduction of oxygen, resulting in the production of the O_2^- anion and/or H_2O_2 [17]. In addition to the production of radical moieties within the cell, XO may also contribute to the extracellular pool of oxidants as a result of its constitutive release from endothelial cells [18]. Furthermore, it has been shown that XO-induced inhibition of signal transduction in endothelial cells is a function of H_2O_2-mediated oxidative stress [19]; the finding of inhibition by NO provides an important and novel regulatory function for this molecule [20].

Recent studies from our laboratory have shown that human keratinocytes possess a cNOS that is Ca^{2+}/calmodulin-dependent and when it is stimulated by UVB radiation, causes a concomitant increase in soluble guanylate cyclase in endothelial cells and neighboring smooth muscle cells. This may be a major part of the integrated response of the skin leading to vasodilation and erythema [21]. Furthermore, we have shown that human endothelial cells can be activated to release NO following exposure to UVB radiation, with subsequent activation of soluble guanylate cyclase [22].

In the present study, we demonstrate that human keratinocytes release NO and O_2^-, which react together to form $ONOO^-$. UVB radiation augmented NO and $ONOO^-$ formation in keratinocytes by increasing both cNOS and XO activities. Furthermore, we showed that UVB radiation of keratinocytes caused oxidative stress and formation of S-nitrosothiols that are biologically active and have long chemical half-lives when compared to NO itself. S-nitrosothiols may play a crucial role in the inflammation process.

MATERIALS AND METHODS

Materials

[^{3}H] L-arginine (54 Ci/mmol; 1 Ci = 37 GBq) and the cGMP radioimmunoassay kit were obtained from the Radiochemical Center, Amersham, Bucks, UK; NO (99.99% pure) was obtained from Messer Griesheim (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, D-NMMA, superoxide dismutase (SOD), and xanthine oxidase (XO) were obtained from Calbiochem (Switzerland). Other reagents, solvents, and salts were of analytical grade and were obtained from Sigma.

Cell Culture

Human keratinocytes (derived from an epidermal squamous cell carcinoma SCC-13 cell line) were cultured in a medium consisting of Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum and essential amino acids, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 5 μg/mL insulin. The cells were grown in a humidified chamber (10% CO_2 and 90% air [21].

Confluent cells from 10–20 T-75 flasks were harvested by treatment with trypsin 0.25%-HBSS-EDTA (1 mM) and placed in an incubator until the cells no longer adhered to the flasks (~5 min). HBSS (pH 7.4) contained: $CaCl_2$ (1.0 mM); KCl (5.3 mM); KH_2P0_4 (0.45 mM); $MgS0_4$ (0.5 mM); NaCl (1.25 mM); Na_2HP0_4 (0.18 mM); $NaHCO_3$ (4.2 mM); and glucose (5.6 mM). Cells were centrifuged at 5500 *g* for 10 min, resuspended in HBSS-EDTA (1 mM) medium and, then, counted. Prior to use, the final concentration was 1×10^8–8×10^8 cells per 4 mL. Cell viability throughout the experiments was >95% as judged by Trypan Blue exclusion.

The cells suspended in HBSS-EDTA (1 mM) were homogenized on ice by sonication for 30 sec (at 5-sec intervals) and the resulting cell homogenate used to obtain the cytosolic fraction. The homogenates were centrifuged at 10,000 *g* for 20 min and the supernatant fractions then centrifuged for 1 hr at 100,000 *g* in a Beckman L7 ultracentrifuge. The supernatant fractions of the high-speed spin were employed as cytosolic preparations.

Ultraviolet Irradiation

A fluorescence UVB lamp (VL-6M 1 × 6 wavelength 290–320 nm with a peak at 312 nm Tube, Power 12 W, Vilber Lourmat, France) was used. Culture medium was removed and the keratinocytes washed twice with HBSS. Cells (10^6/

mL) were resuspended in HBSS. Keratinocytes or their cytosol (200 μg/mL) or purified NOS (2 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 lightmeter. After irradiation of the keratinocytes, HBSS was replaced with fresh HBSS. Cell viability was >90% as judged by Trypan Blue exclusion.

Chemical Determination of NO

Determination of NO was performed as previously described [22]. Keratinocytes (10^6 cells per 3 mL of HBSS) were mixed with 100 μL of a reagent consisting of: 20% sulfanilamide in 20% orthophosphoric acid (H_3PO_4) and 25 μM scopoletin. The NO was monitored as described below. Keratinocyte cytosol (200 μg/mL) enriched with L-arginine (100 μM), NADPH (100 μM), FAD (5 μM), FMN (5 μM), and tetrahydrobiopterin (5 μM) was incubated at 37°C for 5 min and the reaction terminated by adding 10 U of L-lactic dehydrogenase and 100 μL of Na^+ pyruvate (10 mM). In some experiments, the free Ca^{2+} concentration was adjusted by Ca^{2+}/EDTA buffer [23]. L-NMMA (1 mM) dissolved in HBSS was added where indicated. One hundred μL of the sulfanilamide-scopoletin reagent was mixed with the incubates and the NO was monitored at room temperature (22°C) with an excitation wavelength of 350 nm and an emission of 460 nm, using an Aminco SPF-500 Fluorescence Spectrophotometer. The fluorescence was monitored continuously in time until the slope of the line could be measured (approx. 8 min). Slope measurements were, then, converted to pmol of NO using a standard curve constructed with various concentrations of pure NO. The solubility of NO in water is 7.34 cm^3/100 mL and, assuming saturation, the maximum concentration of NO in the solution is 3.3 mM [24]. The present fluorophotometric method is highly sensitive, with a resolution of less than 5 pmol of NO. The method is approximately 100 times more sensitive for NO than for NO_2^-.

Purification of cNOS

Purification of cNOS from keratinocyte cytosol was achieved by the method described by Bredt and Snyder [25]. Cells were homogenized by sonication in 5 mL of ice-cold buffer containing: 50 mM Tris HCl pH 7.4; 1 mM EDTA; antipain (10 mg/L); leupeptin (10 mg/L); soybean trypsin inhibitor (10 mg/L); pepstatin (10 mg/L); chymostatin (10 mg/L), and phenylmethylsulphonylfluoride (100 mg/L). The cytosol of keratinocytes (12 ml), obtained as described above, was added to 2 mL of 2′,5′-ADP-agarose equilibrated in a buffer containing: 50 mM Tris HCl (pH 7.4), 1 mM EDTA, and 1 mM dithiothreitol. After a 10-min incubation, the suspension was poured into a fretted column that was washed with 20 mL of 50 mM Tris HCl, containing 1 mM dithiothreitol and 500 mM NaCl, and 50 mL of 50 mM Tris HCl, containing 1 mM dithiothreitol. cNOS was eluted with 8 mL of 50 mM Tris HCl, containing 1 mM dithiothreitol and 10 mM NADPH, and applied to an anion exchange column packed with DEAE-BioGel A (0.5 × 1 cm). After washing the column with 50 mM Tris HCl, containing 1 mM dithiothreitol and 80 mM NaCl, cNOS was eluted with 6 mL of 50 mM Tris HCl, containing 1 mM dithiothreitol and 120 mM NaCl. In some cases, residual Tris HCl, dithiothreitol, and NADPH were removed from purified cNOS by washing with HBSS-EDTA (1 mM) pH 7.4 in a Centricon-30 microconcentrator (Amicon, Danvers, MA, U.S.A.). The specific activity of NOS was 515.8 ± 58.2 nmol NO/mg protein/min. Protein concentration was measured by the Bradford method [26], using reagents from Bio-Rad (Richmond, CA, U.S.A.) and bovine serum albumin as a standard.

L-citrulline Determination

L-citrulline was measured in 50 mM HEPES (pH 7.4) containing the same cofactors as described for NO measurements in the presence of keratinocyte cytosol (200 μg) as previously described [21]. [^{3}H]-L-arginine (100,000 cpm) was mixed with cold L-arginine (100 μM) in a final volume of 200 μL. After incubation for 15 min at 37°C, assays were terminated with 2 mL of 20 mM Dowex AG50W-X8 (Na^+ form), which was eluted with 2 mL of water. [^{3}H]-L-citrulline was measured in a liquid scintillation counter. Blank values were determined in the absence of added enzyme.

Purification of sGC from Keratinocyte Cytosol

sGC from keratinocyte cytosol was purified by GTP-agarose chromatography. Samples of cytosol (10 mg protein) in 12 mL of HBSS containing 10 mM $MnCl_2$ were added to a GTP-agarose column (1.8 × 9 cm) pre-equilibrated with 25 mM Tris HCl buffer pH 7.6, containing 250 mM sucrose and 10 mM $MnCL_2$. After application of the sample, the column was washed with 5-column volumes of equilibration buffer plus 10 mM GTP. The eluted enzyme was immediately concentrated using Centricon-30 microconcentrators and resuspended in 25 mM Tris HCl, pH 7.6 containing 250 mM sucrose.

cGMP Determination

cGMP concentrations were determined by radioimmunoassay after acetylation of the samles with acetic anhydride, as previously described [27]. The reaction mixture contained triethanolamine/HCl (50 mM), creatine phosphate (5 mM), $MgCl_2$ (3 mM), isobutylmethylxanthine (1 mM), creatine kinase (0.6 U), GTP (1 mM), keratinocytes (10^6 cells), or keratinocyte cytosol (200 μg); the total volume was 150 μL. The reactions were initiated by the addition of GTP and samples were incubated for 10 min at 37°C. The incubation medium was aspirated and cGMP extracted by

the addition of ice-cold HCl (0.1 M). After 10 min, the samples were transferred to a new plate, dried, and reconstituted in 5 mM sodium acetate (pH 4.75) for cGMP determination. cGMP formation was determined using a cGMP assay kit (Amersham).

Determination of Peroxynitrite ($ONOO^-$)

$ONOO^-$ was synthesized, titrated, and stored using the method of Ischiropoulos *et al.* [28] as previously described [27]. $ONOO^-$, formed either from keratinocytes or from cNOS plus XO, was measured by luminol-amplified chemiluminescence as described by Radi *et al.* [29]. All the light (photons) emitted was measured using a Berthold AutoLumat LB953 Luminometer. A stock solution (1 mM) of luminol made in DMSO was diluted in HBSS to the final concentration of 10 μM just before use. Luminol was directly injected in the tubes by the Luminometer. Keratinocytes (10^6 cells per 0.5 mL of HBSS) were placed in a vial and stimulated with various doses of UVB. Light emission was recorded by computer interface and reported as the integrated light emission for a total period of 0.05–1 sec. Background chemiluminescence was measured in vials containing the reaction mixture without the cells. The results were calculated at counts per sec (cps). Chemiluminescence responses were, then, converted to pmol of $ONOO^-$ using a standard curve constructed with various concentrations of pure $ONOO^-$. Identical results were obtained when $ONOO^-$ was determined by monitoring the oxidation of scopoletin (25 μM) as previously described [27].

Identification of S-nitrosothiols

S-nitrosothiols were identified by the slow release of NO after treatment with H_2O_2. Luminol reacts with H_2O_2, but the emission is very weak. When luminol (10 μM) and H_2O_2 (500 μM) were present in a reaction vial at high pH, with vigorous stirring the emission became stable. Upon injection of an NO solution, a significant chemiluminescence response was detected. This emission was increased dose-dependently. The chemiluminescence peak was linearly related to NO concentration between 0.1–10 nM of NO. Addition of $NaNO_2$ or $NaNO_3$ produced no emission. When the standard NO solution was bubbled with oxygen (O_2) for 5 min, emission was abolished. SOD (50 U/mL) was added to the chemiluminescence reaction of luminol (10 μM)-H_2O_2 (500 μM) with NO (1 nM). There was no difference in the NO-induced peak of emission in this system in the presence or absence of SOD. Therefore, in this system, O_2^- was not responsible for the chemiluminescence emission. Keratinocytes (10^6 cells/mL of HBSS), irradiated with 100 mJ/cm^2 and HBSS, were placed in two compartments of a chamber separated by a thin teflon membrane (poly-tetrafluoroethylene, 0.0015 inches in thickness, Dupont, Wilmington, DE, U.S.A.) that permits NO diffusion [21]. NO was determined in HBSS when keratinocytes were placed in the opposite compartment. After one hr, the content of the compartment containing both keratinocytes and S-nitrosothiols released from keratinocytes was centrifuged at 5500 *g* for 10 min, and the amount of S-nitrosothiols was detected in the supernatant (0.5 mL) by the slow release of NO in the presence of H_2O_2 (500 μM) and luminol (10 μM). Chemiluminescence was estimated in a Berthold AutoLumat LB953 luminometer. Light emission was recorded for a total period of 10–500 sec. The results were calculated as counts per sec (cps). The amount of NO release by S-nitrosothiols was estimated by use of a standard curve constructed with various amounts of pure NO. In some cases, NO release from S-nitrosothiols formed by UVB-irradiated keratinocytes was identified either by monitoring the conversion of oxymyoglobin to methmyoglobin spectrophotometrically at 540 nm or by detection of nitrite (NO_2^-) formed upon addition of $HgCl_2$, as described by Arnelle and Stamler [30].

Assay of Intracellular XD/XO Activity

Total XD/XO activity was determined by the method of Beckman *et al.* [31]. The assay is based on the conversion of 2-amino-4-hydroxypterine to the fluorescent product isoxanthopterin. Assays for XO + XD and XO were done with and without Methylene Blue, the electron acceptor being O_2 for XO and Methylene Blue for XO + XD. Activities were calculated based on the linear increase in fluorescence due to formation of isoxanthine observed for 2 min. Isoxanthopterin (0.01–0.03 μM) was used as an internal standard. Enzyme activity was expressed as picomoles of isoxanthopterin formed per min per mg protein.

Measurement of O_2^- released from XO by SOD-inhibitable Reduction of Ferricytochrome C

A chamber with two adjacent compartments separated by a thin teflon membrane, as described above, was used, permitting gas diffusion through the membrane. Purified XO (100 mU/mL) was placed in one compartment. Ferricytochrome C (50 μM) and SOD 100 U/mL) in HBSS were placed in the opposite compartment. The amount of O_2^- produced by XO diffused through the teflon membrane and the time-dependent (up to 60 min) increase of O_2^- in the compartment containing ferricytochrome C were determined. The extent of cytochrome C reduction was determined as follows.

The extinction coefficient with light path of 6 mm of ferricytochrome C (reduced)-ferricytochrome C (oxidized) = 12.66

$$\frac{\Delta OD \times 100}{12.66} =$$

nmol O_2^- produced/10 mg protein/60 min

$$\frac{\Delta OD \times 100}{12.66} =$$

nmol O_2^- produced/10^6 cells/60 min

Keratinocyte cytosol O_2^- levels were also measured using lucigenin chemiluminescence according to Munzel *et al.* [32]. Keratinocyte cytosol (1 mg protein/0.5 mL HBSS) was placed in the vials of a Berthold AutoLumat LB953 Luminometer and allowed to equilibrate for 10 min at 37°C. Vials contained 10 μM lucigenin. Chemiluminescence counts were converted to pmol of O_2^- by calibration of chemiluminescence yield with known quantities of X and XO. The specificity of lucigenine chemiluminescence for O_2^- quantitation was estimated in the presence and absence of SOD.

Chemiluminescence Assay for Antioxidant Capacity of Keratinocyte Cytosol

Cytosolic antioxidant capacity was determined by means of a chemiluminescence reaction as described by Whitehead and Thorpe [33]. Sodium perborate in the presence of horseradish peroxidase produces reactive oxygen species, and chemiluminescence is produced when luminol is present. If certain antioxidants are added to the reaction, the light is suppressed and the duration of suppression is proportional to the quantity of the antioxidant added.

Keratinocyte cytosol (10 mg protein) irradiated with 100 mJ/cm^2 was added in a reaction mixture containing sodium perborate (1 mM), luminol (10 μM) and horseradish peroxidase (100 U) in 0.5 mL HBSS pH 7.4. The vial was vigorously stirred and the emission recorded in a Berthold AutoLumat LB953 Luminometer.

In vivo *Experiments.* *Estimation of Protection Factor (PF)*

Guinea pigs weighing 200 to 300 g were used. The day before the experiment, hair was removed using cold depilatory wax; talc was lightly applied to the surface of the skin, and the animals were left for 24 hr. On the following day, the animals were lightly anaesthetized with ketamine (100 mg/kg IP). A formulation containing L-NMMA (2% w/w) and nitro-L-arginine (2%) in an emulsified cream base consisting of phospholipids, glyceryl stearate, glycerol, squalene, cetyl alcohol, acrylium gel, triethanolamine buffer pH 6 in 90% water, was applied topically at a dose of 0.02 mL/cm^2.

A system comprising a fluorescent UVB lamp emitting from 280 nm to 320 nm, with a peak at 315 nm, was used. Power: 5.6 W. An electronic timer was used to ensure automatic exposure at precise times. The lamp was placed at a right angle to the lower part of the back where hair had been removed. The test was carried out on 5 guinea pigs to determine: the minimum erythema dose (MED) and the Protection Factor (PF).

Animals were exposed for periods of time that were multiples of the MED and in arithmetic progression with respect to the MED (18, 21.6, 25.9, and 31.1 min). The erythema was evaluated 6 hr after application of the formulation and irradiation of animals, and the PF was determined. A comparison was made between the control spot MED (skin areas treated only with the cream base) and the spots that had developed for each radiation dose corresponding to times that were multiples of this MED. The sum of scores equal to zero corresponds to 100% protection for the dose studied and the percentage protection was then calculated with respect to the control erythema MED according to the formula:

$$\frac{\text{control erythema-treated erythema}}{\text{control erythema}} \times 100 = \%$$

The upper 100% evaluation corresponds to the coefficient investigated.

$$\text{Protection Factor} = \frac{\text{Exposure duration for minimum erythema in protected skin}}{\text{Exposure duration for minimum erythema in unprotected skin}}$$

Statistical Analysis

All values are expressed as means ± s.d. The data are derived from triplicate incubations of 3 or 4 independent experiments. The Wilcoxon test for unpaired measurements (Wilcoxon rank sum test) was used. The 5% level of statistical significance was used in all experiments.

RESULTS

$ONOO^-$ and NO Determination after UVB Radiation of Human Keratinocytes

Figure 1 shows $ONOO^-$ and NO production by keratinocytes (10^6 cells/mL) irradiated with various doses of UVB (up to 200 mJ/cm^2). NO production by UVB-irradiated keratinocytes follows a biphasic curve. Up to 120 mJ/cm^2, there was a 3-fold increase in NO release as compared to nonirradiated (control) cells. Higher amounts of radiation (up to 200 mJ/cm^2), however, caused an inhibition (approximately 50%) of NO released with respect to the maximal stimulation. In contrast, $ONOO^-$ production by keratinocytes was gradually increased throughout the range of the UVB radiation (40–200 mJ/cm^2) studied. The maximum value obtained (905 ± 72 pmol/10^6 cells) is 9 times higher than that of the control value (nonirradiated cells). The biphasic curve of NO production by UVB-irradiated cells was apparently due to the overproduction of O_2^-, which reacts with NO to form $ONOO^-$. L-NMMA (1 mM) inhibited both $ONOO^-$ and NO released from keratinocytes stimulated with UVB radiation approximately 60% and 50%, respectively, and D-NMMA (1 mM) had no effect, indicating that the inhibition caused by L-NMMA was

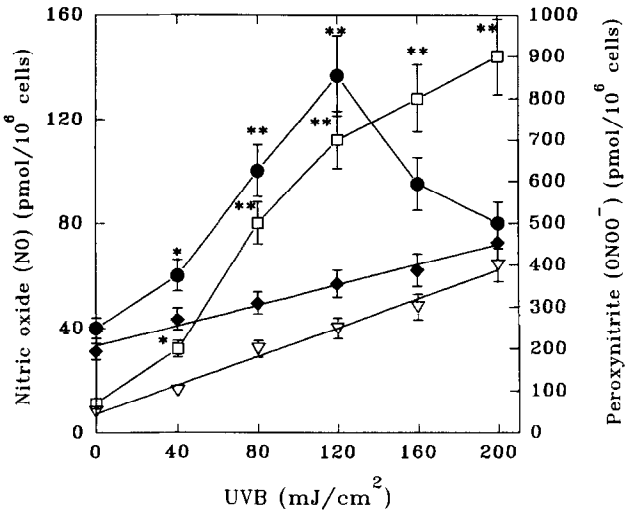

FIG. 1. Effect of various doses of UVB radiation (290–320 nm) on NO release and $ONOO^-$ production by human keratinocytes: (●) NO release; (♦) NO release in the presence of L-NMMA (1 mM); (□) $ONOO^-$ production, and (▽) $ONOO^-$ production in the presence of L-NMMA (1 mM). Each point represents the mean ± SD of 4 independent experiments. A statistically significant difference from keratinocytes in the presence of L-NMMA was determined (*$P < 0.05$, **$P < 0.01$).

stereospecific. Desferrioxamine (500 μM) also inhibited $ONOO^-$ release by UVB-irradiated cells by approximately 90%. Oxypurinol (1 mM) inhibited $ONOO^-$ production by approximately 50%. Results from studies of inhibitors of luminol chemiluminescence elicited by UVB-irradiated keratinocytes showed that SOD (100 U/mL) and urate (1 mM) inhibited chemiluminescence by approximately 70% and 90%, respectively. Aminoacids susceptible to oxidation, that is, cysteine (3 mM), and methionine (1 mM), partially inhibited chemiluminescence. Albumin (0.73 μM), catalase (100 U/mL), and the hydroxyl radical scavenger mannitol (50 mM) and DMSO (50 mM), did not inhibit luminol chemiluminescence. Because authentic $ONOO^-$-mediated luminol chemiluminescence is increased in the presence of $^-HCO_3$ [27], we investigated the effect of $NaCO_3$ on $ONOO^-$-induced chemiluminescence from UVB-irradiated keratinocytes. $NaCO_3$ increased the chemiluminescence response elicited by UVB-irradiated cells approximately 4-fold.

cGMP Determination after UVB Radiation of Keratinocytes

Figure 2 shows that cGMP production was continuously increased by keratinocytes irradiated with various amounts of UVB, reaching a maximum at 200 mJ/cm^2. The fact that NO production declined at 120 mJ/cm^2 showing an approximately 40% inhibition with respect to maximum stimulation at 200 mJ/cm^2 allows us to conclude that $ONOO^-$ can stimulate soluble guanylate cyclase activity. Indeed, cGMP production followed the same pattern as that of $ONOO^-$ release by keratinocytes, suggesting that $ONOO^-$ is capable of increasing soluble guanylate cyclase activity. This was also substantiated when keratinocytes were irradiated in the presence of desferrioxamine (500 μM) and desferrioxamine (500 μM) plus L-NMMA (1 mM). In the presence of desferrioxamine alone, cGMP production by keratinocytes followed a byphasic curve similar to that of NO release, but in the presence of desferrioxamine plus L-NMMA, cGMP was inhibited up to 90%. Furthermore, in the presence of L-NMMA (1 mM) alone, cGMP production was inhibited by approximately 40%. D-NMMA (1 mM) had no effect on cGMP production, but oxypurinol (1 mM) inhibited cGMP production by approximately 40%. These observations support the view that keratinocytes possess NO_2^- stores and that NO_2^- under UVB radiation produces NO [21].

NO and S-nitrosothiol Determination after UVB Radiation of Human Keratinocytes

Figure 3 demonstrates that radiation of human keratinocytes with UVB (100 mJ/cm^2) evoked a time-dependent increase in NO and S-nitrosothiol release. NO was increased 4-fold and S-nitrosothiols 15-fold as compared to nonirradiated (control) cells. The rate of NO and nitrosocompound formation was decreased by the cNOS inhibitor L-NMMA (1 mM) by approximately 50% due to the existence of NO_2^- stores in keratinocytes. L-NMMA was added

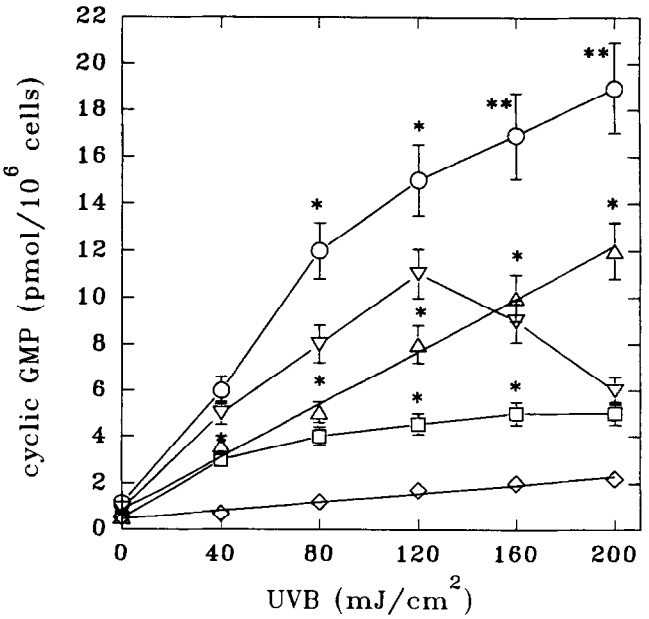

FIG. 2. Effect of various doses of UVB (290–320 nm) radiation on cGMP production by human keratinocyte (○). The effect of L-NMMA (1 mM) (□), desferrioxamine (500 μM) (▽), desferrioxamine (500 μM) plus L-NMMA (1 mM) (◇), and oxypurinol (1 mM) (△) on cGMP production is shown. Each point represents the mean ± SD of 4 independent experiments. A statistically significant difference from keratinocytes in the presence of L-NMMA, desferrioxamine, and oxypurinol was determined (*$P < 0.05$, **$P < 0.01$).

to keratinocytes 15 min before irradiation to allow it to penetrate into the cells and complete its inhibitory action on cNOS activity. Nitrosocompound formation was detected by NO release in the presence of H_2O_2, an oxymyoglobin-NO assay monitoring the loss of characteristic absorbance, and an assay that detects nitrite (NO_2^-) formed from S-nitrosothiols upon addition of $HgCl_2$ *via* Azo Dye formation from sulfanilamide and N-(1-naphthyl)ethylenediamine [30]. By all these methods, the ratio of NO to S-nitrosothiols under physiologic conditions was found to be 1 to 10; in UVB-irradiated human keratinocytes, this ratio was 1 to 50.

Stimulation of sGC by S-nitrosothiols Released from UVB-irradiated Keratinocytes

Figure 4 shows the direct effect of S-nitrosothiols released from UVB-irradiated keratinocytes on purified sGC isolated from keratinocyte cytosol. S-nitrosothiols were obtained after UVB (100 mJ/cm^2) radiation of keratinocytes (10^6 cells/mL) and quantitated by NO release in the presence of H_2O_2. NO released by the cells was neutralized after exposure to the supernatant at 37°C for one hour. It was shown that sGC activity was linearly increased at various amounts of S-nitrosothiols. In the presence of deoxyhemoglobin (Hb) (5 μM), sGC activity was decreased by approximately 65% compared with maximum stimulation obtained at 5 μM of S-nitrosothiols. The remaining 35% increase in sGC activity in the presence of Hb may be due

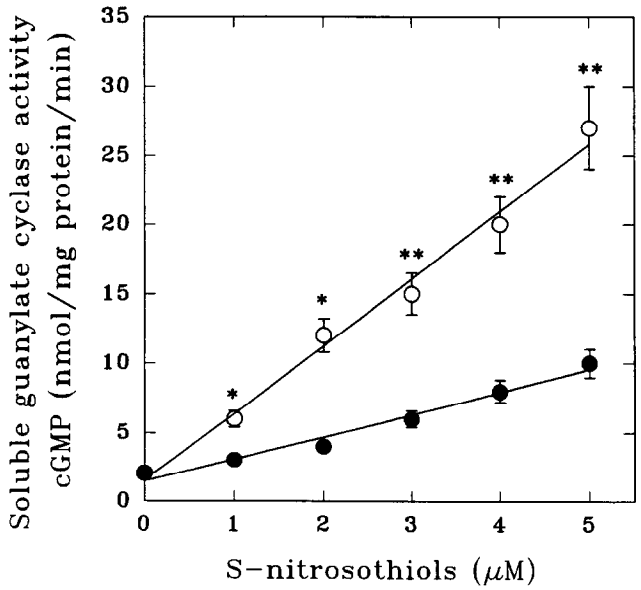

FIG. 4. Effect of S-nitrosothiols on purified sGC activity (○). S-nitrosothiols released by UVB-irradiated (100 mJ/cm^2) keratinocytes were quantitated by the release of NO in the presence of H_2O_2 as described in the text. Hemoglobin (Hb) (5 μM) inhibited sGC activity (●). The data represent the mean ± SD of 4 independent experiments. A statistically significant difference in the presence of Hb was determined. (*$P < 0.05$, **$P < 0.01$).

to a direct effect of S-nitrosothiols on sGC activity or to the presence of other factors stimulating sGC activity and released by the keratinocytes during UVB radiation. The maximum amount of cGMP released in the supernatant from UVB-irradiated keratinocytes was 2.4 ± 0.5 nmol/10^6 cells.

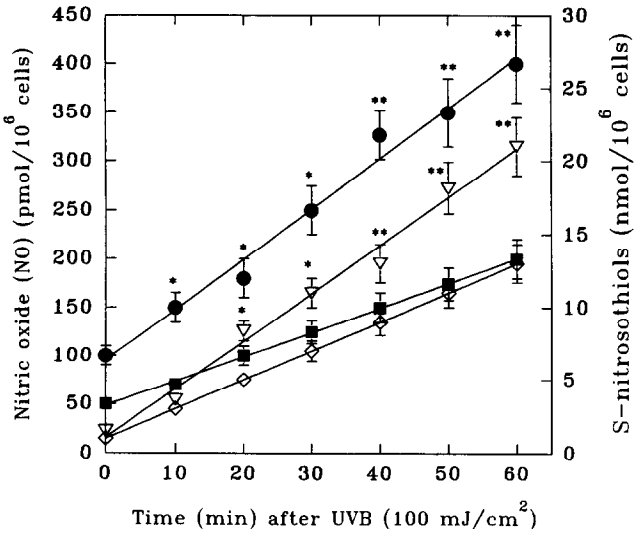

FIG. 3. Time-course experiments on NO (●) and S-nitrosothiol (▽) formation by human keratinocytes irradiated with 100 mJ/cm^2 UVB. S-nitrosothiols were estimated by the release of NO in the presence of H_2O_2 as described in the text. L-NMMA (1 mM) included in the incubation mixture inhibited both NO (■) and S-nitrosothiol (◇) production. The data represent the mean ± SD deviation of 4 independent experiments. A statistically significant difference in the presence of L-NMMA was determined (*$P < 0.05$, **$P < 0.02$).

NO and ONOO$^-$ Determination after UVB Radiation of Keratinocyte Cytosol

Figure 5 shows NO and ONOO$^-$ production after UVB radiation (20–100 mJ/cm^2) of keratinocyte cytosol. NO production was increased 4-fold at 30 mJ/cm^2 dose of UVB radiation; higher amounts of UVB radiation, however (up to 100 mJ/cm^2) showed a decrease with respect to maximum stimulation. ONOO$^-$ production by keratinocyte cytosol was continuously increased up to 10-fold, which was inhibited by approximately 70% in the presence of desferrioxamine (100 μM). When L-NMMA (1 mM) was added to the cell cytosol during UVB radiation, an approximately 60% reduction of NO or ONOO$^-$ formation occurred. Concentrations of the water soluble L-NMMA (up to 5 mM) did not inhibit the production of either NO or ONOO$^-$ beyond that seen with 1 mM L-NMMA. The effectiveness of L-NMMA in partially inhibiting NO and ONOO$^-$ production by UVB-irradiated keratinocytes or their cytosol is due to the existence of stores of NO_2^- in keratinocytes. Oxypurinol (1 mM) inhibited ONOO$^-$ production by approximately 50%.

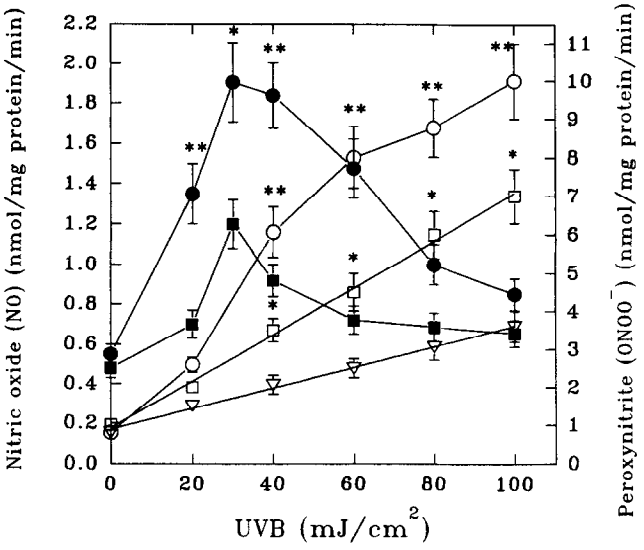

FIG. 5. Effect of UVB (290–320 nm) radiation on NO (●) and $ONOO^-$ (○) production by keratinocyte cytosol. The reaction mixture (1 mL) contained HBSS-1 mM EDTA, pH 7.4, 200 μg of cytosolic protein, 100 μM NADPH, 100 μM L-arginine, 1 μM calmodulin, 5 μM FAD, 5 μM FMN, 5 μM tetrahydrobiopterin, and 1 mM L-NMMA. Incubations were carried out at 37°C for 5 min and the reaction was quenched by the addition of lactic dehydrogenase (10 U) and 100 μL of Na pyruvate (10 mM). The amounts of NO and $ONOO^-$ produced were determined as described in Method. L-NMMA inhibited both NO formation (■) and $ONOO^-$ production (▽). Oxypurinol (1 mM) inhibited $ONOO^-$ by approximately 45% (□). Each point represents the mean ± SD of 4 independent experiments. A statistically significant difference from keratinocyte cytosol in the presence of L-NMMA and oxypurinol was determined (*$P < 0.05$, **$P < 0.01$).

XO Activity and Citrulline Production in UVB-irradiated Keratinocyte Cytosol

Figure 6 shows the effects of various doses of UVB radiation (up to 100 mJ/cm^2) on XO activity and citrulline production by keratinocyte cytosol. UVB evoked a dose-dependent increase in XO activity, which (at 100 mJ/cm^2) was approximately 9-fold higher than that of nonirradiated (control) cytosol. Oxypurinol (1 mM) inhibited XO activity by approximately 90%. Figure 6 also demonstrates that incubation of the keratinocyte cytosol with 100 μM L-arginine mixed with [^{3}H]-L-arginine (100,00 cpm) and 100 μM NADPH resulted in the synthesis of [^{3}H]-L-citrulline. UVB radiation (100 mJ/cm^2) increased [^{3}H]-citrulline formation approximately 6-fold. The maximum stimulation of L-citrulline by UVB was inhibited by 90% in the presence of L-NMMA (1 mM).

NO and O_2^- Production by Purified cNOS and XO

Figure 7 shows the amounts of NO detected when purified cNOS and HBSS were placed in two adjacent compartments of a chamber that were separated by a thin Teflon™ membrane (polytetrafluoroethylene, 0.0015 inches in thickness, Dupont, Wilmington, DE, USA) permitting NO diffusion through the membrane. The amounts of NO produced by cNOS with and without UVB irradiation were monitored periodically. A time-dependent increase in NO in the compartment containing HBSS was observed to reach an equilibrium with NO present in the adjacent compartment containing cNOS within 60 min. When purified cNOS was subjected to UVB irradiation (20 mJ/cm^2), the accumulation of NO in the HBSS compartment tripled, as was seen in the absence of UVB radiation. L-NMMA (100 μM) inhibited NO production by approximately 90%. The experiment was repeated using XO in the first compartment and HBSS in the opposite compartment for periodic assay of O_2^- over a 60-min period. In the absence of UVB irradiation, we noted that diffusion of O_2^- across the Teflon™ membrane into the HBSS compartment resulted in the production of a significant amount of O_2^-. When the procedure was repeated with XO irradiated with 20 mJ/cm^2 of UVB, an approximately 3-fold increase in O_2^- production was observed, as compared to nonirradiated XO. Oxypurinol (100 μM) inhibited O_2^- production by approximately 80%.

Reconstitution Experiments using Purified cNOS and XO

Reconstitution experiments consisting of purified cNOS (2 μg) and XO (10 mU/mL) in HBSS pH 7.4, L-arginine (100

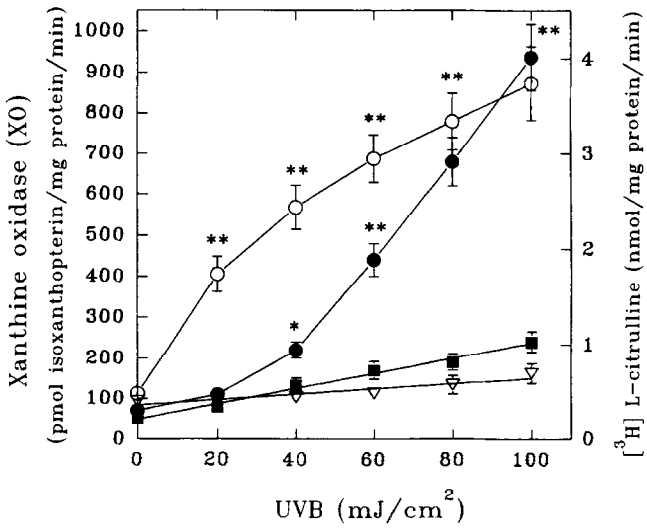

FIG. 6. Effect of UVB (290–320 nm) radiation on XO (●) and [^{3}H]-L-citrulline (○) formation by keratinocyte cytosol. XO activity was determined as described in the experimental section. [^{3}H]-citrulline was determined in a reaction mixture containing of: [^{3}H]-L-arginine (100,000 cpm) mixed with cold L-arginine (100 μM), NADPH (100 μM), FAD (5 μM), FMN (5 μM), tetrahydrobiopterin (5 μM), L-NMMA (1 mM), and cytosolic protein (200 μg). L-NMMA (1 mM) (▽) inhibited [^{3}H]-L-citrulline formation by approximately 80%. Oxypurinol (1 mM) (■) inhibited XO activity by approximately 90%. Each point represents the mean ± SD of 3 independent experiments. A statistically significant difference from the cytosol in the presence of L-NMMA and oxypurinol was determined (*$P < 0.05$, **$P < 0.001$).

μM), NADPH (100 μM), tetrahydropterin (50 μM), calmodulin (10 μM), and xanthine (100 μM) were conducted. cNOS was purified approximately 1000-fold from the keratinocyte cytosol (498.6 ± 49.4 pmol/mg cytosolic protein/min), showing a specific activity of 515.8 ± 58.2 nmol/mg protein/min. Figure 8 shows the effect of various doses of UVB radiation on $ONOO^-$ formation. UVB up to 20 mJ/cm^2 caused an approximately 4-fold increase in $ONOO^-$. In the absence of XO, the formation of NO was increased approximately 3-fold, and $ONOO^-$ was not formed. L-NMMA (100 μM) inhibited both NO and $ONOO^-$ by approximately 90%. In the absence of cNOS or L-arginine, neither NO nor $ONOO^-$ was formed. In the presence of oxypurinol (100 μM), $ONOO^-$ production was inhibited by approximately 65%.

Estimation of the Antioxidant Capacity of Keratinocyte Cytosol

The characteristic spectrum of the chemiluminescence response obtained by UVB-irradiated (100 mJ/cm^2) cytosol was shifted to the right of that obtained by nonirradiated (control) cytosol. The time of the maximum chemiluminescence peak was obtained at 355 ± 17 sec in nonirradiated cytosol and at 605 ± 32 sec in UVB-irradiated cytosol (100 mJ/cm^2). This shift to the right indicates that there was an increase in the oxidative stress in the UVB-irradiated cytosol. It also underlines the fact that there is an inverse relationship between oxidative stress and antioxidant capacity in keratinocytes.

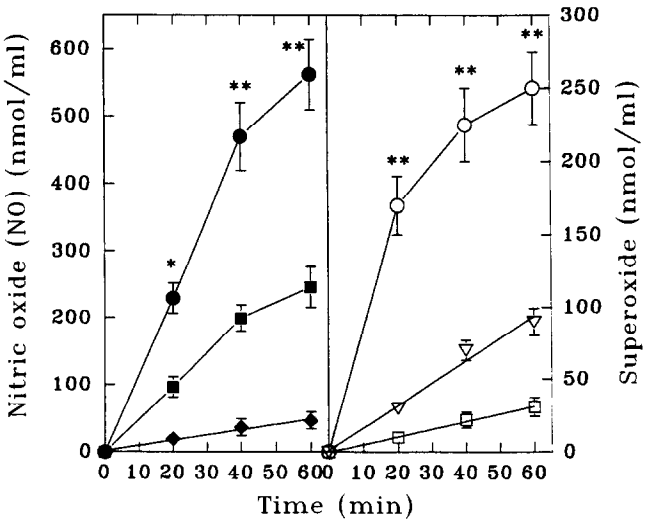

FIG. 7. Purified cNOS isolated from keratinocyte cytosol and HBSS were placed in two compartments of a chamber separated by a thin Teflon™ membrane (poly-tetrafluoroethylene 0.0015 inches in thickness) that permits NO and O_2^- diffusion. NO was determined in HBSS when cNOS was placed in the opposite compartment (■). UVB radiation (20 mJ/cm^2) of cNOS resulted in a 3-fold increase in the amount of NO that diffused into the HBSS compartment (●). Replacement of cNOS with XO resulted in an accumulation of O_2^- in the compartment containing HBSS (▽). When UVB-irradiated (20 mJ/cm^2) purified XO was present in the opposite compartment of the chamber, a 3-fold increase in O_2^- production was observed (○). The effect of L-NMMA (100 μM) on cNOS activity (◆) and that of oxypurinol (100 μM) on XO activity (□) are also shown. Each point represents the mean ± SD of 3 independent experiments. A statistically significant difference from nonirradiated cNOS and XO was determined (*P < 0.05, **P < 0.01).

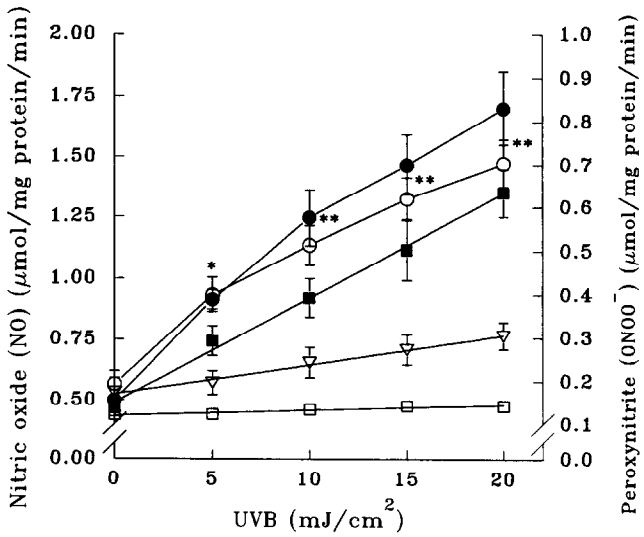

FIG. 8. Effect of UVB (290–320 nm) radiation on $ONOO^-$ production (○) in reconstitution experiments consisting of purified cNOS (2 μg) and XO (10 mU/mL) in HBSS pH 7.4, L-argiine (100 μM), NADPH (100 μM), tetrahydropterin (50 μM), calmodulin (10 μM), and xanthine (100 μM). (●) NO in the absence of XO and (□) $ONOO^-$ in the absence of cNOS are also shown, as well as the effect of oxypurinol (100 μM) on $ONOO^-$ (▽) and on NO (■) production. Each point represents the mean ± SD of 4 independent experiments. A statistically significant difference of $ONOO^-$ production in the presence of oxypurinol was determined (*P < 0.05, **P < 0.001).

Estimation of Protection Factor (PF)

In vivo experiments were conducted where guinea pigs were subjected to timed exposure to UVB irradiation. Prior to exposure, a new formulation containing L-NMMA (2% w/w) and L-NA (2% w/w) in an emulsified cream base (consisting of phospholipids, glyceryl stearate, glycerol, squaline, acetyl alcohol, acrylium gel triethanolamine buffer pH 6 in 90% water) was applied to the skin as described in Methods. The treated side of the animals was exposed to multiples of the MED in geometric progression 1.2. Immediately after application of the cream, treated areas were exposed at times 18, 21.6, 25.9, and 31.1 min. Skin areas treated with the active ingredient were compared with adjacent skin areas treated with the cream base. According to FDA recommendations, the PF calculated was 6.5 ± 1.8 (n = 5). The PF of the cream base as compared to untreated skin areas was 1.7 ± 0.5.

DISCUSSION

In the present study, we demonstrated that UVB (290–320 nm) radiation of human keratinocytes in the presence of extracellular Ca^{2+} stimulated both cNOS and XO activities. We also demonstrated that UVB-irradiated keratinocytes release NO and $ONOO^-$ with a subsequent increase in cGMP synthesis (Figs. 1 and 2). A recent publication suggests that $ONOO^-$ produces coronary vasodilation in dogs; high amounts of $ONOO^-$, however, may be directly damaging to tissues and possibly result in abnormal vasoregulation *in vivo* [16]. In the present study, the chemilugenic substrate luminol was used to evaluate UVB-induced $ONOO^-$ production from human keratinocytes. In our previous studies (15), we found that luminol was not sensitive to oxidation by O_2^-, H_2O_2, or NO alone. Initiation to luminol oxidation appears to require a one-electron oxidation by a potent oxidant such as hydroxyl radical (OH·) or $ONOO^-$. Because OH· radical scavengers did not inhibit luminol-chemiluminescence from UVB-irradiated keratinocytes, a potent oxidant other than OH· radical must be formed to initiate luminol oxidation.

We documented that UVB radiation of human keratinocytes increased S-nitrosothiol formation, thus, changing the molar ratio of NO to S-nitrosothiols from 1 to 10 (under normal conditions) to 1 to 50 (Fig. 3). This change in molar ratio is apparently due to the longer half-lives of S-nitrosothiols compared to NO itself. Thus, our demonstration of S-nitrosylation of biological sulfydryl groups with NO represents critically needed evidence in favor of the role of S-nitrosothiols in the biochemical mechanism of endogenously derived NO.

Our present data suggest that: (a) NO can react with thiol groups to form S-nitrosothiols; (b) this reaction occurs under physiologic conditions; (c) these compounds are biologically active; (d) S-nitrosothiols have long chemical half-lives compared to NO itself; and (e) the biochemical mechanism of action of S-nitrosothiols through activation of sGC. It has been shown that S-nitrosothiols act as NO^+, NO·, and NO^- donors under physiologic conditions and that biological activities of nitrosothiols may be associated with a heterolytic as well as homolytic mechanism of decomposition [30]. In our previous studies, we showed that UVA irradiation of squamous carcinoma cells resulted in the formation of S-nitrosothiols and hydroxylamine (NH_2OH), indicative of nitroxyl (NO^-) release [8]. Because free sulfydryl groups can react with oxidized forms of NO, this explains the paradoxical effect of thiols because, at low concentrations, act as inactivators and at high concentrations, via S-nitrosation, as carriers for NO [7]. In the present study, we showed that, when sGC isolated from keratinocytes is exposed to various amounts of S-nitrosothiols, an increased activity of the enzyme occurs by a mechanism characteristic of NO release from a "carrier molecule" (Fig. 4).

The potent $ONOO^-$ produced by UVB-irradiated keratinocyte cytosol was dependent on the enhanced production of both NO and O_2^- (Fig. 5). Inhibition of UVB-irradiated keratinocyte cytosol-mediated luminol chemiluminescence by desferrioxamine and L-cysteine further supports $ONOO^-$ as the major oxidant species. Additionally, the efficient inhibition of UVB-irradiated keratinocyte cytosol-mediated luminol chemiluminescence by urate is consistent with $ONOO^-$ being the oxidant species. Urate inhibits $ONOO^-$-mediated dihydrorhodamine 123 oxidation [34], and [$^-HCO_3$] has been shown to augment $ONOO^-$-mediated luminol chemiluminescence[35].

Our findings in this study demonstrating that UVB radiation activates both XO and cNOS (Fig. 6) were also verified in experiments consisting of purified cNOS and purified XO in the presence of appropriate cofactors and substrates. The increased formation of both NO and O_2^- after UVB radiation (Fig. 7) is consistent with the widespread signal transduction system that involves Ca^{2+}/calmodulin-regulated NO and $ONOO^-$ formation and activation of sGC [36, 37]. These results were also verified by reconstitution experiments consisting of purified cNOS and purified XO in the presence of the appropriate cofactors and substrates. $ONOO^-$ formation was increased after UVB radiation of cNOS and XO, consistent with the simultaneous generation of NO and O_2^-, which react together to form $ONOO^-$ (Fig. 8).

The physiological role of the constitutive forms of the NOS in keratinocytes may be the production of small quantities of NO that are important in maintaining the vascular tone of skin blood vessels. NO release may also restore normal blood flow in extreme cases of vasoconstriction of the cutaneous microvasculature. Alternatively, NO may act as an autacoid in keratinocytes, inhibiting ribonucleotide reductase as well as mitochondrial respiration and DNA synthesis, events that control cellular proliferation [38]. It has been shown that primary cultures of human keratinocytes and a mouse keratinocyte cell line respond to γ-interferon and lipopolysaccharide or tumor necrosis factor-α by producing NO at nanomolar concentrations, suggesting expression of the inducible type of NOS in response to inflammatory stimuli. It is proposed that NO produced by keratinocytes may function in nonspecific host defense during wound healing, and that this may occur because NO, either alone and/or in combination with reactive oxygen intermediates, is toxic [39]. The visible redness following UVB irradiation, after a latent period of 16 to 24 hr may be a result of UVB-induced oxygen radicals that activate nuclear factor kB. This activation may be a novel positive feedback loop in which NO might activate transcription factors that, in turn, could increase NO synthesis by causing additional cNOS expression [40]. A recent study has shown that UVB irradiation of rat skin caused delayed onset vasodilatation and, by 18 hr, basal blood flow had increased. L-NAME injected locally 17.5 hr after UVB irradiation abolished the 18 hr increase in blood flow [41]. In the present study, in *in vivo* experiments in animals, we tried to determine the PF of a cream formulation containing L-NA

(2% w/w) and L-NMMA (2% w/w). According to International Standardization, the PF calculated was 6.5 ± 1.8.

Our recent studies indicating that human keratinocytes possess a constitutive NOS, and that they are capable of releasing NO for prolonged time periods following exposure to UVB radiation, provide evidence that after NO is released by these cells, the gas may continue to be produced and released even after the stimulus is removed [21]. NO acting as EDRF would, then, diffuse abluminally to endothelial cells, thereby leading to activation of sGC, in turn, enhancing the intracellular levels of cyclic GMP that mediate the relaxation response and NO production by the same cells with a positive feedback mechanism [42].

Our present data suggest that $ONOO^-$ formation by human keratinocytes during UVB radiation may exert cytotoxic effects in the cells where it is produced. $ONOO^-$ may also diffuse out of the keratinocytes and exert cytotoxic effects in the neighboring endothelial and smooth muscle cells, resulting in the inflammation that accompanies human sunburn reactions. It may be possible to diminish the risk of the sunburn reaction in humans by developing pharmacological agents that either diminish NO production or scavenge $ONOO^-$ formed in the skin.

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