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Release by ultraviolet B (u.v.B) radiation of nitric oxide (NO) from human keratinocytes: a potential role for nitric oxide in erythema production

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1 The mechanism of human sunburn is poorly understood but its characteristic features include the development of erythema. In this study we attempted to determine whether human keratinocytes possess a nitric oxide (NO) synthase (NOS), if this enzyme could be activated to release NO following exposure to ultraviolet B (u.v.B) and to define whether this photo-induced response could be involved in the pathogenesis of sunburn erythema.

2 Treatment of human keratinocytes with various doses of u.v.B (290–320 nm) radiation (up to 100 mJ cm^{-2}) resulted in a dose-dependent release of NO and cyclic GMP production that was reduced by N^G-monomethyl-L-arginine (L-NMMA).

3 u.v.B irradiation of keratinocyte cytosol at varying doses (up to 50 mJ cm^{-2}), resulted in a gradual rise in NO production, with a concomitant increase in soluble guanylate cyclase activity (sGC).

4 NOS isolated from the keratinocyte cytosol was constitutively expressed and was dependent on NADPH, Ca^{2+}/calmodulin, tetrahydrobiopterin and flavins.

5 In reconstitution experiments, when purified NOS was added to purified sGC, both isolated from keratinocyte cytosol, a four fold increase in cyclic GMP was observed. The GMP was increased by NO synthesized following u.v.B radiation (up to 20 mJ cm^{-2}) of NOS.

6 In *in vivo* experiments, guinea-pigs were subjected to u.v.B light. A Protection Factor (PF) of 8.71 ± 2.85 was calculated when an emulsified cream formulation containing L-NMMA (2%) was applied to their skin.

7 The present results indicate that u.v.B radiation acts as a potent stimulator of NOS in keratinocytes. NO is lipophilic and may diffuse out of the keratinocytes, activating sGC in endothelial cells and neighbouring smooth muscle cells. This may be a major part of the integrated response of the skin leading to vasodilatation and erythema.

Keywords: Endothelium-derived relaxing factor, EDRF; nitric oxide; NO synthase; soluble guanylate cyclase; ultraviolet B radiation; keratinocyte; erythema; inflammation; vasodilatation

Introduction

Since the description by Furchgott (1988) of endothelium-derived relaxing factor (EDRF), substantial new investigative efforts have been directed to the characterization and identification of the nature of this biological mediator (Lowenstein *et al.*, 1994). Evidence accumulated in recent years suggests that nitric oxide (NO) (or a closely-related compound) represents at least one type (if not the only type) of EDRF, because both NO and EDRF have similar biological and pharmacological properties (Mayer *et al.*, 1989; Busse & Mulsch, 1990). NO is released in sufficient quantities to explain the biological action of EDRF (Ignarro, 1990; Kiechle & Malinski, 1993). Other investigations have provided data suggesting that NO may not account for all the actions of EDRF and that, depending on the vascular bed, and the activator, there may be more than one type of EDRF (Myers *et al.*, 1990). Recent evidence suggests that sulphydryl species can react with oxides of nitrogen under physiological conditions and thereby stabilize EDRF activity. Serum albumin reacts with oxides of nitrogen to form a stable S-nitrosothiol with properties reminiscent of authentic EDRF, supporting the view that protein-associated thiol may participate in the actions and metabolism of EDRF (Keaney *et al.*, 1993).

In many tissues, including vascular endothelium and brain, the basal activity of constitutive nitric oxide synthase (cNOS) rapidly increases in response to activation of specific G-protein-coupled surface receptors. This cNOS is stimulated by Ca^{2+}/calmodulin and the rapid increase in enzyme activity is not dependent on new protein synthesis. By contrast, activation of NOS in macrophages and other leukocytes occurs over several hours in response to specific cytokines and requires new protein synthesis; the inducible NOS (iNOS) is Ca^{2+}-independent (Deliconstantinos *et al.*, 1994; Lowenstein *et al.*, 1994). These regulatory differences possibly reflect the distinct physiological roles for NO in different cell types; in the macrophage, high local concentrations of secreted NO probably play a role in the cell-dependent killing, whereas the primary role of neuronal or endothelial NO appears to be in intracellular signalling (Palmer *et al.*, 1987; Moncada *et al.*, 1991). Purified cNOS contains tightly bound flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). The purified enzyme binds haeme tightly and absorbs at 450 nm following treatment with carbon monoxide, indicating that NOS is a cytochrome P_{450} enzyme (Pufahl & Marletta, 1993). Many of the effects of NO are mediated by guanosine 3':5'-cyclic monophosphate (cyclic GMP), and it appears that NO represents a prevalent inter- and intracellular signal molecule with soluble guanylate cyclase (sGC) as the effector enzyme (Deliconstantinos *et al.*, 1993; Henry *et al.*, 1993).

It has been proposed that direct photon absorption by dermal blood vessels could explain ultraviolet-induced vasodilatation. Photoexcitation of an epidermal chromophore that absorbs the incident energy and then evokes an injurious response culminating in the release of vasoactive agents that

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migrate to the dermal vascular to evoke the vasodilator response has also been reported (Greaves, 1986).

Thus in the present study we decided to investigate whether keratinocytes are directly connected with the skin vasculature, functionally. Here we describe a NOS in human keratinocytes that is constitutively expressed, is Ca^{2+}/calmodulin dependent and when stimulated by u.v.B radiation, causes a concomitant increase in sGC activity. By applying timed and measured doses of u.v.B radiation to a transparent chamber with two compartments separated by a thin teflon membrane, biological samples in one compartment can be stimulated to produce NO which diffuses across the teflon membrane to stimulate other biologically active samples in the opposite compartment. This has proven to be a useful tool in the study of NO-induced interactions between different cell types, which is the subject of much research interest. Our *in vivo* experiments demonstrated, for the first time that u.v.B radiation results in the synthesis of NO by epidermal keratinocytes.

Methods

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% foetal calf serum and essential amino acids, 2 mM L-glutamine, 100 u ml^{-1} penicillin, 100 $\mu g\ ml^{-1}$ streptomycin and 5 $\mu g\ ml^{-1}$ insulin. The cells were grown in a humidified chamber (10% CO_2 and 90% air).

Confluent cells from 10-20 T-75 flasks were harvested by treatment with trypsin 0.25%-Hank's Balanced Salt Solution (HBSS)-EDTA (1 mM) and placed in an incubator until the cells no longer adhered to the flasks (about 5 min). HBSS (pH 7.4) contained (mM): $CaCl_2$ 1.0, KCl 5.3, KH_2PO_4 0.45, $MgSO_4$ 0.5, NaCl 1.25, Na_2HPO_4 0.18, $NaHCO_3$ 4.2 and glucose 5.6. Cells were centrifuged at 5,500 *g* for 10 min and 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. The 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 s (at 5 s intervals) and the resulting cell homogenate was 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 h 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 u.v.B 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 were washed twice with HBSS. Cells ($10^6\ ml^{-1}$) were resuspended in HBSS. Keratinocytes or their cytosol (200 $\mu g\ ml^{-1}$) or purified NOS (1 $\mu g\ ml^{-1}$) were irradiated with various u.v.B 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

The chemical determination of NO is based on the diazotization of sulphanilamide by NO at acidic pH and subsequent oxidation of scopoletin which can be detected fluorophotometrically as previously described (Deliconstantinos *et al.*, 1992). Keratinocytes (10^6 cells per 3 ml of HBSS) were mixed with 100 μl of a reagent consisting of: 20% sulphanilamide in 20% *ortho*-phosphoric acid (H_3PO_4) and 25 μM scopoletin. The NO was monitored as described below. Keratinocyte cytosol (200 $\mu g\ ml^{-1}$) 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 was 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 (Segal, 1986). N^G-monomethyl-L-arginine monoacetate (L-NMMA) (1 mM) dissolved in HBSS, was added where indicated. A stock solution (10 mM) of the calmodulin antagonist, calmidazolium (compound R24571), was prepared in dimethylsulphoxide. One hundred μl of the sulphanilamide–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, in 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 by use of a standard curve constructed with various concentrations of pure NO. Solutions of acidified saline (9 mg ml^{-1} NaCl, 0.1 mM HCl, pH 4.0) were deoxygenated by bubbling with 100% nitrogen for 2 h. NO gas was bubbled into 10 ml of deoxygenated acidified saline at a rate of one bubble s^{-1} for 20 min, while the gas above was flushed away with N_2. The solubility of NO in water is 7.34 cm^3 100 ml^{-1} and, assuming saturation, the maximum concentration of NO in the solution is 3.3 mM (Shikano *et al.*, 1987). Stock solutions of nitrite (NO_2^-) were prepared by dissolving appropriate amounts of sodium nitrite in acid saline to produce a 3.3 mM stock solution (i.e. identical to the estimated stock concentration of the NO saturated solutions). The stock solutions of NO_2^- were prepared in open, ambient air-exposure tubes. The NO and NO_2^- solutions were stored on ice and used within 3 to 4 h. The present fluorophotometric method is highly sensitive with a resolution of less than 5 pmol of NO. The method is about 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 & Snyder (1990). 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^{-1}; leupeptin 10 mg l^{-1}; soybean trypsin inhibitor 10 mg l^{-1}; pepstatin 10 mg l^{-1}; chymostatin 10 mg l^{-1} and phenylmethylsulphonylfluoride 100 mg l^{-1}. The cytosol of keratinocytes, obtained as described above, (12 ml) 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 fritted column which 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.). Protein concentration was measured by the Bradford method (Bradford, 1976) with reagents from Bio-Rad (Richmond, CA, U.S.A.) and bovine serum albumin as a standard.

Assay of cNOS

Purified cNOS activity was measured by monitoring either NO or [^{3}H]-L-citrulline production.

NO determination

Reactions (1 ml sample volumes) were carried out for 5 min at 37°C. Standard reaction mixtures contained: HBSS–EDTA 1 mM, cNOS (1 μg), NADPH 100 μM and various combinations of FAD 5 μM, FMN 5 μM, tetrahydrobiopterin 5 μM and calmodulin 1 μM. L-NMMA (1 mM) was added where indicated. Reactions were terminated by adding 10 u of L-lactic dehydrogenase and 100 μl of Na pyruvate (10 mM). NO determination was carried out by mixing the incubates with 100 μl of the sulphanilamide-scopoletin reagent.

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 cNOS (1 μg). [^{3}H]-L-arginine (100,000 c.p.m.) 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

sGC from keratinocytes 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. sGC was then eluted from the column with 5 ml 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.

Cyclic GMP determination

Concentrations of cyclic GMP were determined by radioimmunoassay after acetylation of the samples with acetic anhydride. 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 or 0.085–1.0 μg purified soluble guanylate cyclase; 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 cyclic GMP was 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 cyclic GMP determination. Cyclic GMP formation was determined with a cyclic GMP assay kit (Amersham).

Description of the incubation chamber

For the determination of NO released by purified NOS, we designed and fabricated a chamber with two compartments of 2.5 cm diameter, from solid rods of clear Plexiglas, which were hollowed out from one end with a machine-lathe to create an identical conical cavity within each of the plexiglas rods. They were then further machined and polished at the open ends, creating a very tight fit between the two conical cavities. A thin square of Teflon sheet (poly-tetrafluorethylene 0.0015 inches in thickness) was sandwiched between the assemblies which were recompressed with the thumbscrews. The two tube-access-parts at either side of the membrane allows biologically active samples and reactive substances to be injected into, withdrawn from, or modified at either side of the membrane during biological reactions (Figure 7, insert).

In vivo *experiments: estimation of protection factor (PF)*

Hartley guinea-pigs weighing 200 to 300 g were used. The day before the experiment hair removal was carried out with cold depilatory wax; talc was lightly applied to the surface of the skin and the animals were left for 24 h. On the following day the animals were lightly anaesthetized with ketamine (100 mg kg^{-1}, i.p.). A formulation containing L-NMMA (2% w/w) in an emulsified cream, 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 u.v.B lamp emitting from 280 nm to 320 nm with a peak at 315 nm was used. The wattage was 5.6 W and an electronic time was used to ensure automatic exposure at precise times. The lamp was placed at right angle to the lower part of the back from which hair had been removed. The test was carried out on 5 guinea-pigs in order to determine: (a) the minimum erythema dose (MED); and (b) the Protection Factor (PF).

Scoring scale of erythema was: 0 = no erythema, 1 = barely visible spots, 2 = obvious erythema, 3 = very visible erythema. MED is the radiation dose that promotes the appearance of a minimum erythema with a score of 1. Animals were exposed for periods of time that are 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 h after application of the formulation and irradiation of animals and the PF was determined. A comparison was made between the control spot MED (cream base only treated skin areas) 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 is then calculated with respect to the control erythema MED (score of 10) 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 three or four independent experiments. Wilcoxon test for unpaired measurements (Wilcoxon rank sum test) was used. The 5% level of statistical significance was used in all experiments.

Materials

[^{3}H]-L-arginine (54 Ci $mmol^{-1}$; 1 Ci = 37 GBq) and cyclic GMP radioimmunoassay kit were obtained from the Radiochemical Centre, 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 and superoxide dismutase (SOD) were obtained from Calbiochem (Switzerland). Other reagents, solvents and salts were of analytical grade and were obtained from Sigma Chemical Co., St. Louis, Mo, U.S.A.

Results

NO and cyclic GMP determination after u.v.B radiation of keratinocytes

Figure 1a demonstrates that radiation of human keratinocytes with u.v.B evoked a dose-dependent increase in detectable NO release. The response occurred with doses of energy known to elicit erythema production in intact human skin (Pentland *et al.*, 1990). The rate of NO formation was decreased by the NOS inhibitor L-NMMA (1 mM). L-NMMA was added 15 min before irradiation to intact keratinocytes to allow it to penetrate into the cells and then complete its inhibitory action on NOS activity. Similarly, u.v.B treatment resulted in a three fold increase in cellular cyclic GMP at a dose of 100 mJ cm^{-2}. L-NMMA (1 mM) also decreased cyclic GMP formation (Figure 1b). Cyclic GMP accumulation provides a sensitive index of NO production because of the ability of NO to activate soluble guanylate cyclase directly (Stamler *et al.*, 1992). Table 1 shows that the production of NO by keratinocyte cytosol in the presence of a saturating concentration of L-arginine (100 μM) (Standard Reaction Mixture; SRM), was 525 ± 65 pmol NO min^{-1} mg^{-1}. This production was dependent on NADPH. In the presence of unsaturated concentrations of NADPH (10 μM and 50 μM) 52% and 61% decreases of NO production, respectively were obtained, when compared to the SRM (control) value ($P<0.02$). Concentrations of NADPH up to 1 mM did not increase the production of NO beyond that seen with 100 μM NADPH (SRM). The amount of NO produced after the addition of 2 mM ATP was the same as that obtained in the absence of ATP ($P>0.05$). Addition of D-arginine (100 μM), L-lysine (100 μM), L-guanidine (100 μM) and L-citrulline (100 μM) caused no statistically significant difference in NO production when compared to SRM from which L-arginine had been omitted ($P>0.05$) and a statistically significant difference when compared to SRM ($P<0.01$). These results suggest that D-arginine, L-lysine, L-guanidine and L-citrulline failed to stimulate enzyme activity, indicating the specificity of the activation to L-arginine. Table 1 also shows that sGC stimulation by L-arginine (100 μM) contained in the SRM was maximal in the presence of exogenous NADPH (100 μM) (6.25 ± 0.65 pmol cyclic GMP min^{-1} mg^{-1}) and concentrations of NADPH up to 1 mM did not increase the sGC activity beyond that seen with 100 μM NADPH. When the specificity of the activation of sGC by L-arginine was examined it was found that D-arginine (100 μM), L-lysine (100 μM), L-guanidine (100 μM) and L-citrulline (100 μM) failed to increase the sGC activity when compared to SRM from which L-arginine had been omitted ($P>0.05$), while a P value of less than 0.01 was obtained when compared to SRM. Table 2 shows that the synthesis of NO by the cytosol was completely Ca^{2+}/calmodulin and tetrahydrobiopterin-dependent. In a Ca^{2+}-free solution containing 1 mM EDTA, the activity of cNOS in the presence of 100 μM NADPH was very low (25.5 ± 2 pmol NO min^{-1} mg^{-1} cytosolic protein). Results from standard incubations without adjustment of the free Ca^{2+} concentration did not differ from those incubations in which the Ca^{2+} concentration was 10 μM or in which calmodulin (1 μM) was added, indicating that standard incubation mixtures contained sufficient Ca^{2+} and calmodulin in the keratinocyte cytosol ($P>0.05$). The calmodulin antagonist R24571 (25 μM) completely inhibited NOS activity.

NO and cyclic GMP determination after u.v.B radiation of keratinocyte cytosol

Figure 2a and b shows NO production and sGC activation after u.v.B radiation of keratinocyte cytosol. The amount of NO production increased four fold after a 50 mJ cm^{-2} dose of u.v.B radiation, accompanied by a four fold increase in the activity of sGC. L-NMMA (1 mM) added to the cell cytosol during the u.v.B radiation, resulted in an approximate 50% reduction of either NO formation or sGC activation. Concentrations of water soluble L-NMMA (up to 5 mM) did not inhibit the production of either NO or cyclic GMP beyond that seen with 1 mM L-NMMA (data not shown). The effectiveness of L-NMMA in partially inhibiting

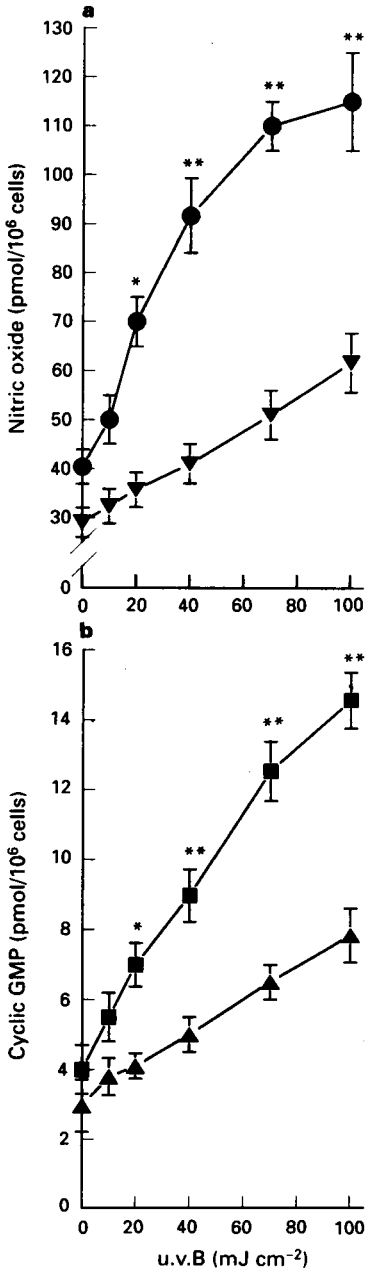

Figure 1 Effect of various doses of u.v.B radiation (290–320 nm) on (a) NO release and (b) cyclic GMP production by human keratinocytes: (●) NO release; (▼) NO release in the presence of L-NMMA (1 mM); (■) cyclic GMP production and (▲) cyclic GMP production in the presence of L-NMMA (1 mM). L-NMMA was added 15 min before the irradiation of the cells. Each point represents the mean ± s.d. of four independent experiments. A statistically significant difference from keratinocytes in the presence of L-NMMA was determined (*$P<0.01$, **$P<0.001$).

the NO and cyclic GMP production by u.v.B-irradiated keratinocytes or their cytosol prompted us to test whether stores of nitrite ions (NO_2^-) could be present in the keratinocytes. The generation of NO by u.v. radiation of NO_2^- in aqueous solutions has been studied (Zafiriou & Mcfarland, 1980; Bauer & Fung, 1993). To test this possibility we inactivated cNOS by boiling the keratinocyte cytosol at 100°C for 3 min. Although L-NMMA (1 mM) inhibited the NOS activity by almost 100%, we decided to denature the enzyme in order to exclude the possibility of an effect of u.v. irradiation on the dissociation of the NOS-L-NMMA complex and to ensure complete inactivation of the enzyme. Following u.v.B radiation (50 mJ cm^{-2}) of the denatured cytosol, the NO production was almost equal (0.78 ± 0.08 nmol NO mg^{-1} protein min^{-1}; $n = 4$) to the non-inhibitable NO production by L-NMMA suggesting the possible existence of stores of NO_2^- ions within the keratinocytes. Furthermore after dialysis (membrane dialysis, Diachema A.G. Switzerland) of the denatured keratinocyte cytosol for 24 h against phosphate buffered saline (PBS) pH 7.4 followed by u.v.B radiation (50 mJ cm^{-2}), the NO production was 0.020 ± 0.008 nmol NO mg^{-1} protein min^{-1} ($n = 4$), supporting the view that NO_2^- stores exist in keratinocytes.

Table 1 Effect of various agents on nitric oxide (NO) and cyclic GMP production by keratinocyte cytosol

Conditions	*NO* (pmol min^{-1} mg^{-1})	*Cyclic GMP* (pmol min^{-1} mg^{-1})
Standard reaction mixture (SRM)	525 ± 65	6.25 ± 0.65
NADPH (10 μM)	252 ± 37*	2.88 ± 0.28*
NADPH (50 μM)	324 ± 42*	4.14 ± 0.45*
NADPH (1 nM)	544 ± 68	6.08 ± 0.61
ATP (2 nM)	532 ± 66	6.42 ± 0.70
L-Arginine omitted	75 ± 8*	0.71 ± 0.05*
D-Arginine (50 μM)	82 ± 12*	0.89 ± 0.06*
D-Arginine (100 μM)	77 ± 7*	0.82 ± 0.05*
L-Lysine (100 μM)	64 ± 6*	0.93 ± 0.07*
L-Guanidine (100 μM)	87 ± 9*	0.78 ± 0.05*
L-Citrulline (100 μM)	71 ± 8*	0.83 ± 0.05*

Keratinocytes were harvested, homogenized and cytosolic fractions were prepared as described in the Methods section. Standard reaction mixture (SRM) contained all compounds except the omitted cofactors or substrates. Enzyme activities were determined as described in the Methods section. Data are mean ± s.d. of triplicate incubations of three independent experiments.
*Statistically significant difference ($P < 0.05$) compared to SRM.

Table 2 Characterization of NO-synthase activity in keratinocyte cytosol

Conditions	*NO* (pmol min^{-1} mg^{-1})
Standard reaction mixture (SRM)	525 ± 65
NADPH omitted	202 ± 31*
Flavins omitted	298 ± 49*
BH_4 omitted	165 ± 24*
Ca^{2+} free	25 ± 2*
Ca^{2+} (10 μM)	512 ± 63
Ca^{2+}/calmodulin (1 μM)	517 ± 67
Ca^{2+}/R24571 (25 μM)	28 ± 2*
Ca^{2+}/calmodulin/R24571	18 ± 2*

Enzyme incubation mixtures contained all compounds expect the omitted cofactor. For changing the Ca^{2+} concentration or testing the effects of calmodulin, EDTA buffers were included (Segal, 1986). Enzyme activities were determined as described in the Methods section. Data are mean ± s.d. of triplicate incubations of three independent experiments.
BH_4: tetrahydrobiopterin.
*Statistically significant difference ($P < 0.05$) compared to SRM.

Citrulline production by keratinocyte cytosol

Figure 3 demonstrates that incubation of the keratinocyte cytosol with 100 μM L-arginine mixed with [^{3}H]-L-arginine (100,000 c.p.m.) and 100 μM NADPH, resulted in the synthesis of [^{3}H]-L-citrulline at the rate of 725 ± 95 pmol mg^{-1}

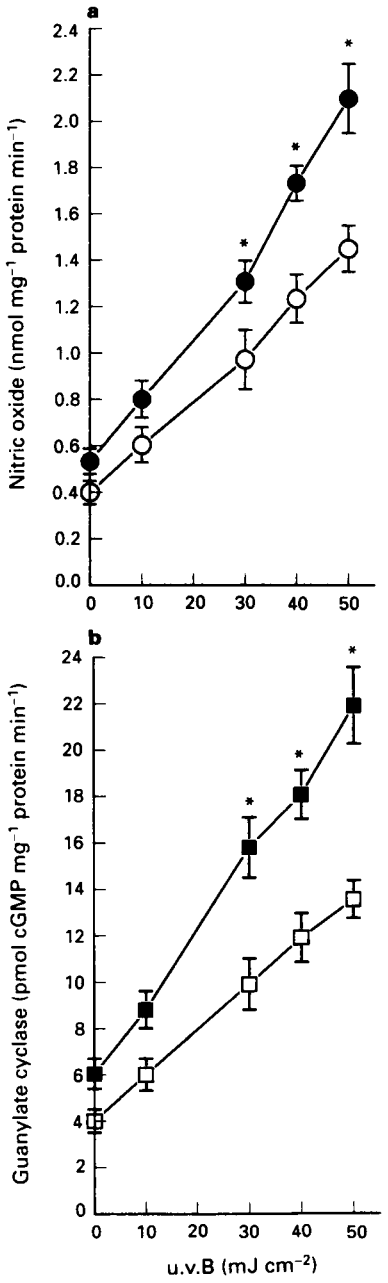

Figure 2 Effect of various doses of u.v.B (290–320 nm) radiation on (a) NO (●) and (b) cyclic GMP (■) production by the cytosol of human keratinocytes. Keratinocyte cytosol (200 μg ml^{-1}) enriched with L-arginine (100 μM); NADPH (100 μM); FAD (5 μM); FMN (5 μM) and tetrahydrobiopterin (5 μM) was incubated at 37°C for 5 min and the reaction was terminated by adding 10 u of L-lactic dehydrogenase and 100 μl of Na^+ pyruvate (10 mM). The effect of L-NMMA (1 mM) on both NO (○) and cyclic GMP (□) production is shown. Each point represents the mean ± s.d. of four independent experiments. A statistically significant difference from the cytosol in the presence of L-NMMA was determined (*$P < 0.02$).

protein min^{-1}. L-Citrulline formation increased four fold after irradiation with 50 mJ cm^{-2} u.v.B. The maximum stimulation of L-citrulline by u.v.B was inhibited by 90% in the presence of L-NMMA (1 mM).

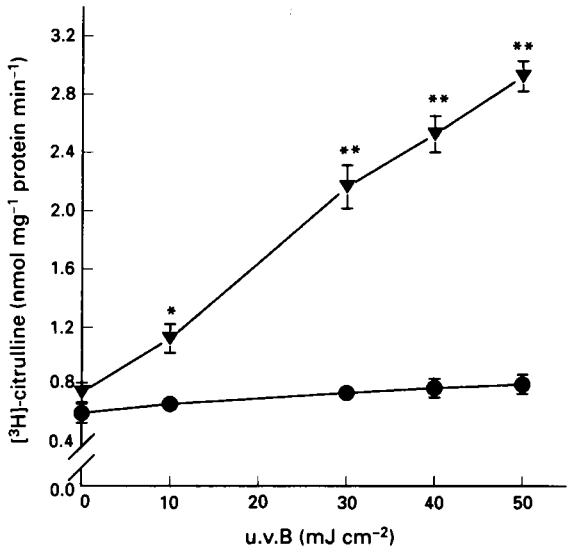

Figure 3 Effect of u.v.B (290–320 nm) radiation on [^{3}H]-L-citrulline (▼) formation by keratinocyte cytosol. The reaction mixture contained: [^{3}H]-L-arginine (100,000 c.p.m.) mixed with cold L-arginine (100 μM), NADPH (100 μM), FAD (5 μM), FMN (5 μM), tetrahydrobiopterin (5 μM), L-NMMA (1 mM) and cytosolic protein (200 μg). L-NMMA (1 mM) (●) inhibited [^{3}H]-L-citrulline formation by approximately 90%. Each point represents the mean ± s.d. of three independent experiments. A statistically significant difference from the cytosol in the presence of L-NMMA was determined (*$P<0.01$, **$P<0.001$).

NO, cyclic GMP and citrulline production by purified NOS

cNOS purified approximately 1000 fold from the keratinocyte cytosol (525 ± 65 pmol mg^{-1} cytosolic protein min^{-1}) showed a specific activity of 575 ± 60 nmol NO mg^{-1} protein min^{-1}. Figure 4 shows the effect of various doses of u.v.B on NO formation by purified cNOS (1 μg) with the appropriate cofactors. u.v.B up to 20 mJ cm^{-2} caused a three fold increase in NO formation. L-NMMA (1 mM) inhibited the enzyme activity by approximately 90%. As sGC is the physiological effector system of L-arginine-derived NO, reconstitution experiments with purified cNOS and sGC were performed. Figure 5 shows that u.v.B radiation increased the activity of purified sGC by four fold, consistent with the increased cNOS activity by the u.v.B irradiation. In the absence of cNOS or L-arginine, u.v.B failed to increase the activity of sGC. Figure 6 (a and b) shows the quantities of NO and L-citrulline detected in samples obtained over a 60 min period following stimulation of purified cNOS by u.v.B radiation (20 mJ cm^{-2}). L-NMMA (1 mM) inhibited both L-citrulline and NO formation by approximately 90%.

NO and cyclic GMP determination with an incubation chamber

Figure 7 shows the amounts of NO detected when purified cNOS and HBSS were placed in two adjacent compartments of a chamber which were separated by a thin teflon membrane (poly-tetrafluoroethylene, 0.0015 inches in thickness, Dupont, Wilmington, Delaware, U.S.A.) permitting only NO diffusion through the membrane. The amounts of NO produced by cNOS with and without u.v.B irradiation, were monitored periodically. A time-dependent increase of NO in the compartment containing HBSS was observed to reach an equilibrium with NO present in the adjacent compartment containing cNOS within 50 min. When purified cNOS was

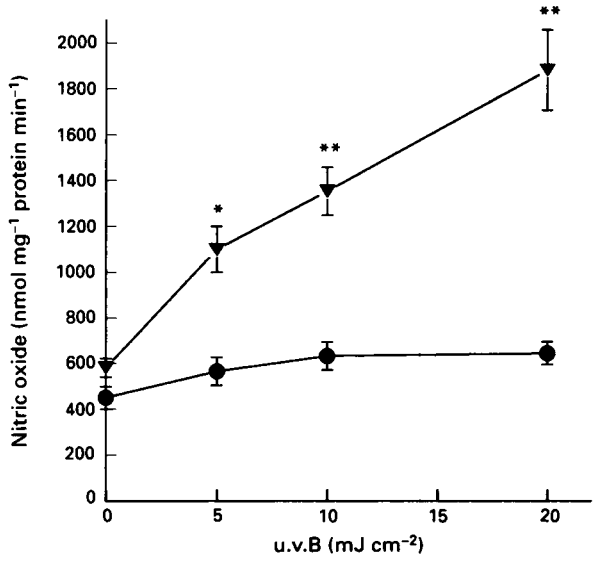

Figure 4 Effect of u.v.B (290–320 nm) radiation on NO production (▼) by purified cNOS isolated from keratinocyte cytosol. The reaction mixture (1 ml) contained HBSS-1 mM EDTA, pH 7.4, 1 μg of purified cNOS, 100 μM NADPH, 100 μM L-arginine, 1 μM calmodulin, 5 μM FAD, 5 μM FMN, 5 μM tetrahydrobiopterin 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 amount of NO produced was determined as described in the Methods section. L-NMMA (●) inhibited NO formation by approximately 90%. Each point represents the mean ± s.d. of four independent experiments. A statistically significant difference from the cNOS in the presence of L-NMMA was determined (*$P<0.01$, **$P<0.001$).

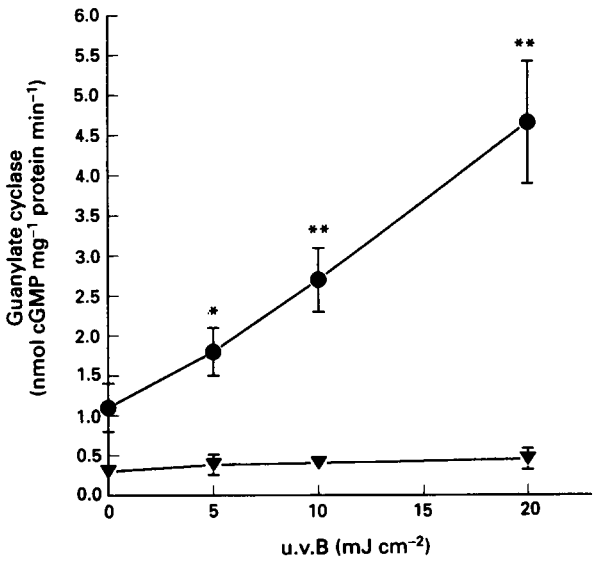

Figure 5 Effect of u.v.B (290–320 nm) radiation on the activity of sGC in the presence (●) and absence (▼) of purified cNOS, in reconstitution experiments consisting of purified sGC (1 μg) and cNOS (1 μg) in 50 mM triethanolamine/HCl buffer pH 7.4, L-arginine (100 μM), NADPH (100 μM), FAD (5 μM), FMN (5 μM), tetrahydrobiopterin (5 μM), Ca^{2+} (10 μM) and calmodulin (1 μM). The appropriate co-factors for the estimation of sGC were used as described in the Methods section. Incubations were carried out at 37°C for 5 min and cyclic GMP formed was determined by radioimmunoassay using a cyclic GMP kit (Amersham). Each point represents the mean ± s.d. of three independent experiments. A statistically significant difference sGC in the absence of cNOS was determined (*$P<0.01$, **$P<0.001$).

subjected to u.v.B irradiation (20 mJ cm^{-2}), the accumulation of NO in the HBSS compartment doubled, as was seen in the absence of u.v.B radiation. Finally, HBSS in the first compartment was replaced by purified sGC for periodic bioassay of NO activity over a 50 min period. In the absence of u.v.B irradiation, we noted that diffusion of NO across the teflon membrane into the sGC compartment resulted in the production of a significant amount of cyclic GMP (1.82 ± 0.12 nmol mg^{-1} protein min^{-1}). When the procedure was repeated with cNOS irradiated with 20 mJ cm^{-2} of u.v.B, a two fold increase in cyclic GMP production was observed, as compared to non-irradiated cNOS.

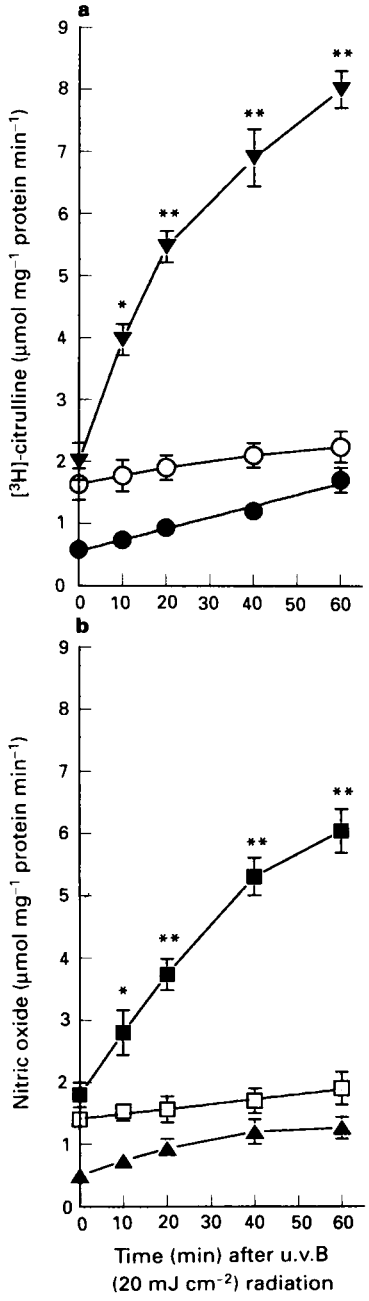

Figure 6 Time course experiments on (a) [^{3}H]-L-citrulline (▼) and (b) NO (■) formation by purified cNOS isolated from keratinocyte cytosol. The reaction mixture was as described in the legends to Figures 3 and 4. Purified cNOS was irradiated with 20 mJ cm^{-2} u.v.B. NO (▲) and [^{3}H]-L-citrulline (●) production by non-irradiated cNOS (control experiments) is also shown. L-NMMA (1 mM) included in the incubation mixture inhibited both NO (□) and [^{3}H]-L-citrulline (○) production by approximately 90%. The data represent the mean ± standard deviation of four independent experiments. At 60 min the difference between the two curves (●) and (○) in (a) and (▲) and (□) in (b) were found to be not statistically significant ($P > 0.05$). A statistically significant difference from cNOS in the presence of L-NMMA was determined (*$P < 0.01$, **$P < 0.001$).

Estimation of protection factor (PF)

In vivo experiments were then conducted where Hartley guinea-pigs were subjected to timed exposure to u.v.B irradiation. Prior to exposure a new formulation containing L-NMMA (2% w/w) in an emulsified cream base (consisting of: phospholipids, glyceryl stearate, glycerol, squalene, acetyl alcohol, acrylium gel, triethanolamine buffer pH 6 in 90% water), was applied to the skin as described in the Methods section. The treated side of the animals was exposed to multiples of the MED in geometric progression 1.2. Treated areas immediately after the application of the cream were exposed at times 18, 21.6, 25.9 and 31.1 min. Skin areas treated with the active ingredient were compared with the cream base-treated adjacent skin areas. According to the FDA recommendations, the PF calculated was 8.71 ± 2.85 ($n = 5$). The PF of the cream base as compared to untreated skin areas was 1.7 ± 0.5.

Discussion

Our present study has shown that u.v.B radiation, which is known to cause erythema in human skin, activates NO release with subsequent cyclic GMP production in keratinocytes (Figure 1). Erythema is a consequence of various inflammatory stimuli, including sunlight. It is thought to result from an increase in blood volume in both the superficial and deep vascular plexi of the dermis. The mechanism of human sunburn is poorly understood but its characteristic features include the development of visible redness following threshold doses of u.v.A and u.v.B, after a latent period of 4–8 h that is maximal at 16–24 h and fades slowly thereafter (Cavallo & Deleo, 1986). Keratinocytes SCC-13, a cell line derived from a human epidermal squamous cell carcinoma, express a constitutive form of the enzyme NOS which converts L-arginine into NO and L-citrulline in the presence of Ca^{2+}/calmodulin; NO is responsible for elevating cyclic GMP levels in keratinocytes or their cytosol (Figures 1 and 2). We confirmed that both NO and cyclic GMP production by keratinocytes or their cytosol were inhibited by L-NMMA, which inhibits the NOS activity (Schmidt *et al.*, 1992). u.v.B radiation used in the present experiments not only stimulated cNOS to produce NO, but also generated NO from NO_2^- in the keratinocyte cytosol, suggesting the existence of NO_2^- stores in keratinocytes. NO production by the purified cNOS can be achieved in a defined system containing L-arginine, NADPH, Ca^{2+}/calmodulin and tetrahydrobiopterin. Exogenous FAD (5 μM) and FMN (5 μM) increased the rate of NO synthesis by purified cNOS, and is consistent with a co-factor role. NADPH provides reducing equivalents to cNOS (Stamler *et al.*, 1992) and NADPH oxidation is coupled to NO synthesis through a FAD-containing flavoprotein and tetrahydrobiopterin (Schmidt *et al.*, 1992).

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 the skin blood vessels. NO release may also restore the 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 (Henry *et al.*, 1993).

Our findings in this study demonstrating that, in a defined reconstitution system consisting of purified cNOS and purified sGC, the formation of cyclic GMP was increased

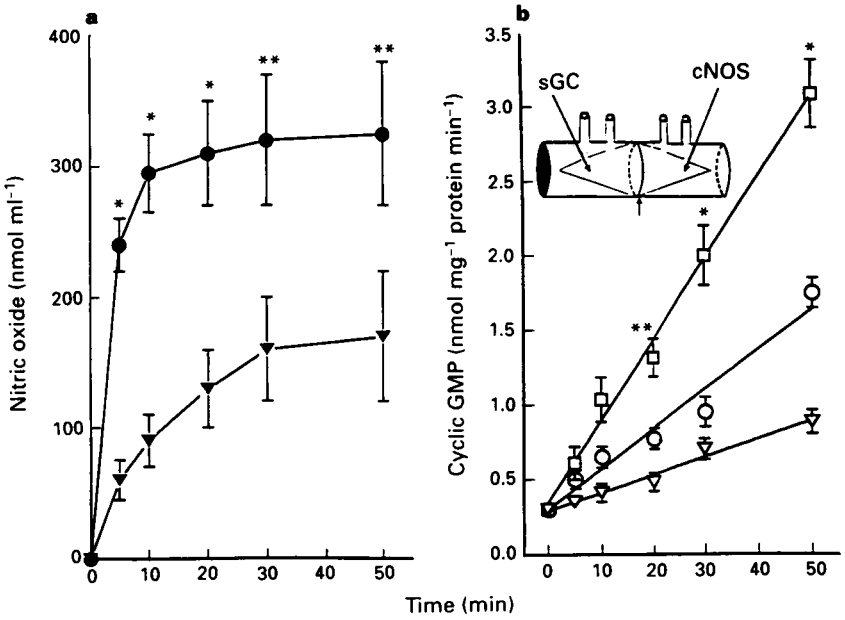

Figure 7 Purified cNOS isolated from keratinocyte cytosol and HBSS was placed in two compartments of a chamber separated by a thin teflon membrane (poly-tetrafluoroethylene 0.0015 inches in thickness), a membrane which permits only NO diffusion. NO was determined in HBSS when cNOS was placed in the opposite compartment (▼). u.v.B radiation (20 mJ cm^{-2}) of cNOS resulted in a two fold increase in the amount of NO that diffused into the HBSS compartment (●). Replacement of HBSS with sGC resulted in a two fold increase of cyclic GMP in the compartment containing sGC (○). When u.v.B (20 mJ cm^{-2}) irradiated purified cNOS was present in the opposite compartment of the chamber a further two fold increase in cyclic GMP production was observed (□). The basal activity of sGC (in the absence of cNOS in the opposite compartment of the chamber) is also illustrated (▽). Each point represents the mean ± s.d. of three independent experiments. A statistically significant difference from non-irradiated cNOS (control) was determined (*$P<0.01$; **$P<0.05$).

after u.v.B radiation, are consistent with the widespread signal transduction system which involves Ca^{2+}/calmodulin-regulated NO formation and activation of sGC (Schmidt *et al.*, 1993). These reconstitution experiments were also verified by placing purified NOS and purified sGC in a special incubation chamber with two compartments separated by a thin teflon membrane through which only NO gas could pass. Cyclic GMP increased approximately two fold, when u.v.B irradiated purified cNOS was placed in the opposite compartment (Figure 7). These experiments clearly suggest that the NO released from u.v.B irradiated keratinocytes is identical to gaseous NO and not to any nitrosothiol compound (Myers *et al.*, 1990) and it is consistent with the observations that glutathione is necessary in endothelial cells for NO synthesis rather than for the NO effect on sGC (Ghigo *et al.*, 1993). In *in vivo* experiments using experimental animals we tried to determine the PF of a cream formulation containing 2% L-NMMA. According to FDA recommendations, the PF calculated was 8.71 ± 2.85.

Prior studies have suggested the role of eicosanoids in the pathogenesis of sunburn since perfusates of radiated erythematous human skin contain increased amounts of vasodilator prostaglandins that can be diminished by pretreatment with nonsteroidal antiinflammatory agents such as aspirin and indomethacin (Snyder, 1976). In this respect, the cyclo-oxygenase enzymes are potential targets for NO because they contain an iron haeme centre at their active sites and indeed the vast majority of the effects mediated by NO are a consequence of its interaction with iron or iron containing enzymes (Salvemini *et al.*, 1993). NO enhances cyclo-oxygenase activity through a mechanism independent of cyclic GMP and in conditions in which both the NOS and cyclo-oxygenase systems are present there is a NO-mediated increase in the produciton of proinflammatory prostaglandins that may result in an exacerbated inflammatory response (Salvemini *et al.*, 1993). It has been shown that primary cultures of human keratinocytes and a mouse keratinocyte cell line respond to γ-interferon and lipopolysaccharide or tumour necrosis factor-α by producing NO at nanomolar concentrations, suggesting the 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 defence during wound healing, and that this may occur because NO, either alone and/or in combination with reactive oxygen intermediates, is toxic (Heck *et al.*, 1992). It was recently published that u.v.B irradiation of rat skin caused delayed onset vasodilatation and by 18 h basal blood flow increased. L-NAME injected locally 17.5 h after u.v.B irradiation abolished the 18 h increase in blood flow (Warren *et al.*, 1993). Our present studies indicating that human keratinocytes possess a constitutive NOS, and that they are capable of releasing NO for prolonged time periods following exposure to u.v. radiation, provide evidence that once NO is released by these cells the gas may continue to be produced and released even after the stimulus is removed (Figure 6). NO acting as EDRF would then diffuse abluminally to smooth muscle cells thereby leading to activation of sGC and enhance the intracellular levels of cyclic GMP that mediates the relaxation response (Deliconstantinos & Villiotou, 1992). Our data also raise the intriguing possibility that NO released by keratinocytes can directly augment sGC activity in smooth muscle cells and bypass the conventional abluminal pathway. Such a phenomenon could provide an explanation for the vasodilatation that accompanies human sunburn erythema and suggests that it may be possible to diminish the risk of the sunburn reaction in man by developing pharmacological agents that diminish epidermal NO production.

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