

The Inflammatory and Cytotoxic Effects of a Nitric Oxide Releasing Cream on Normal Skin

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We describe the pro-inflammatory and cytotoxic effects of nitric oxide *in vivo* in human skin. Nitrite and ascorbic acid were mixed on the skin of 12 normal volunteers, three times daily, to release nitric oxide. Exposure to nitric oxide was varied by randomizing the concentration of nitrite and duration of application. Nitric oxide treated skin showed significant increases in cells expressing CD3, CD4, CD8, CD68, neutrophil elastase, ICAM-1, VCAM-1, nitrosotyrosine, p53, and apoptotic cells compared with skin treated with ascorbic acid alone. There was no significant increase in mast cells. Following application of nitric oxide there were significantly fewer CD1a positive Langerhans cells in the epidermis. These appeared to lose dendritic morphology

and migrate from the epidermis. There was no significant difference in staining for Ki-67, a marker related to proliferating cell nuclear antigen, between active and control skin but staining was greater after exposure to higher dose nitric oxide than the low dose. Apoptosis, cytotoxicity, and p53 staining were relatively greater after 48 h exposure than after 24 h. These results suggest that nitric oxide is pro-inflammatory and is toxic to DNA, leading to the accumulation of p53 and subsequent apoptosis. High-dose nitric oxide paradoxically led to a smaller increase in macrophages and T cells than low dose suggesting an immunosuppressive effect of higher levels. **Key words:** acidified nitrite/adhesion molecules/apoptosis/p53. *J Invest Dermatol* 113:392-397, 1999

Nitric oxide (NO) is a locally synthesized messenger with many physiologic and pathologic activities. It is synthesized from arginine by isoforms of NO synthase (NOS) enzymes. The inducible isoform of NO synthase (iNOS) is induced by lipopolysaccharide products of bacterial cell walls or by cytokines, notably tumor necrosis factor α , interferon- γ (Moncada, 1992), interleukin (IL)-1 β (Moncada, 1992), IL-6 (Sawada *et al*, 1997), IL-8 (Bruch-Gerharz *et al*, 1996), and granulocyte-macrophage colony-stimulating factor (Laskin *et al*, 1996). Unlike the pmol quantities of NO produced by constitutive endothelial and neuronal NOS, iNOS produces nanomolar concentrations (Anggard, 1994) which are involved in the immune response to pathogens (Moncada, 1992), in tumor cell necrosis and in apoptosis (Messmer *et al*, 1994).

Increased iNOS expression has been found in the skin in psoriasis (Sirso *et al*, 1996; Ormerod *et al*, 1998), atopic dermatitis (Rowe *et al*, 1997), irritant and allergic contact dermatitis (Ormerod *et al*, 1997a), lupus erythematosus,¹ sunburn-induced erythema,² and NO contributes to mustard oil induced neurogenic vasodilation (Lippe *et al*, 1993), and carrageenin-induced edema (Ianaro *et al*, 1994). NO can potentiate Th₁ cell responses in carrageenin-induced inflammation, where increases in IL-1, IL-2, IL-6, and interferon- γ are inhibited by the L-arginine analog NG-monomethyl L-arginine (Ianaro, 1994). NO specifically induced the DR marker B7-2 on murine macrophages but not other class 2 IA

markers suggesting NO to be a costimulatory molecule in antigen presentation.³ In one study, growth was inhibited by the activation of NOS in keratinocyte cultures (Heck *et al*, 1992). NO, however, also promoted wound healing and keratinocyte proliferation after ultraviolet B exposure (Benrath *et al*, 1995), inhibition of cNOS has also inhibited keratinocyte proliferation (MacNeil *et al*, 1993). NO promotes keratinocyte proliferation via the heme oxygenase pathway (Clark *et al*, 1997). NO may also contribute to angiogenesis (Papapetropoulos *et al*, 1997b; Noiri *et al*, 1998). Tumor necrosis factor- α and platelet-activating factor promote endothelial cell chemotaxis by an NO-dependent pathway (Montrucchio *et al*, 1997). NO also mediates the endothelial cell mitogenic effect of vascular endothelial growth factor (Morbidelli *et al*, 1996) and transforming growth factor- β 1-induced capillary organization (Papapetropoulos *et al*, 1997a).

The cytotoxic effects of NO depend on combination of NO with superoxide to form peroxynitrite (ONOO⁻). Nitration of tyrosine, tryptophan, and guanidine and oxidation of sulfhydryl groups and thioethers can occur via newly liberated NO⁺ and NO₂⁺ intermediates. Oxidation of sulfhydryl groups leads to dysfunction of enzymes in the mitochondrial respiratory chain, notably aconitase and glyceraldehyde-3-phosphate dehydrogenase. Lipid

¹Kuhn A, Fehsal P, Lehman P, Ruzicka T, Kolb-Bachofen V: Expression of inducible nitric oxide synthase (iNOS) in human epidermis of lupus erythematosus. *J Invest Dermatol* 107:504, 1996 (abstr.)

²Kuhn A, Fehsel K, Lehman P, Ruzicka T, Kolb-Bachofen V: UVA and UVB irradiation of normal skin induces time restricted expression of inducible nitric oxide synthase in keratinocytes. *J Invest Dermatol* 109:446, 1997 (abstr.)

³Qureshi AA, Lerner EA: Nitric oxide induced B7-2 expression in murine macrophages. *J Invest Dermatol* 108:577, 1997 (abstr.)

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Abbreviations: iNOS, inducible nitric oxide synthase; NO, nitric oxide.

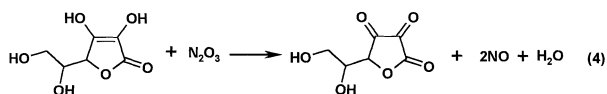
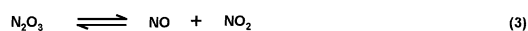
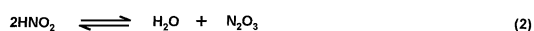
oxidation, can lead to disruption and dysfunction of cell membranes. Peroxynitrite can react directly with nucleic acids prompting DNA strand breakage (Szabo, 1996a; Zingarelli *et al*, 1996). This leads to the induction of wild-type p53 as shown in cultured cells (Messmer, 1994; Forrester *et al*, 1996; Ho *et al*, 1996), which in turn leads to apoptosis (Messmer, 1994; Brune *et al*, 1995).

NO also has important immunosuppressive properties. It can autoregulate IL-1 and IL-6 production from macrophages (Persoons *et al*, 1996), IL-2 production, and Th₁ proliferation (Taylor-Robinson, 1997), and inhibit expression of the adhesion molecules VCAM-1, ICAM-1 (Takahashi *et al*, 1996), E-selectin (De Caterina *et al*, 1995), and P-selectin (Kanwar and Kubes, 1995). In some models NO suppresses lymphocyte proliferation (Moilanen and Vapaatalo, 1995; Schwacha and Eisenstein, 1997; Sciorati *et al*, 1997) and function, especially Th₁ cells (Liew, 1995) and natural killer cell activity has also been inhibited (Ito *et al*, 1996). NO also downregulates the expression of major histocompatibility complex class II antigens (Sicher *et al*, 1995). NO may be partly responsible for the immunosuppression observed following ultraviolet light, possibly through interference with dendritic cell function (Gillardon *et al*, 1995).

To examine the pro-inflammatory and anti-inflammatory effects of NO *in vivo* we examined the effects of NO on normal human skin.

MATERIALS AND METHODS

To deliver NO to the skin we used an acidified nitrite as NO donor (Feelish and Stamler, 1996) substituting ascorbic acid to reduce sodium nitrite to NO (Williams, 1988).



This leads to nitrous acid (1), which rapidly dissociates to form dinitrogen trioxide (2). With most acids the dinitrogen trioxide dissociates into NO and NO₂ (3) but the key chemical transformation is reduction of the nitrous anhydride (N₂O₃) to NO with concurrent oxidation of ascorbic acid to dehydroascorbic acid, as shown in equation 4. The evolution of pure NO from ascorbic acid and nitrite reactions has been established *in vitro* using the chemiluminescence meter's (Thermo Environmental Instruments, Franklin, MA) ability to detect NO and NO₂ separately (Fig 1).

In a pilot study of three subjects, reapplication of 0.5% sodium nitrite with 2% ascorbic acid, three times daily over 48 h produced mild skin inflammation whereas 5% nitrite with 2% ascorbic acid produced marked inflammation. In the subjects treated with 5% nitrite, we measured NO production by chemiluminescence (using the method of Ormerod *et al*, 1997b) and blood flow by laser Doppler. Erythema, increased blood flow, and NO production lasted for 60 min and were of a similar magnitude to that in a psoriatic plaque. Based on these findings, 12 normal volunteers with no current or recent history of skin disease and taking no medication were randomized to receive either low-dose (0.5% nitrite) or high-dose (5% nitrite) NO to their skin and to receive this for either 24 h (three applications) or 48 h (six applications), see Table I. Two per cent wt/wt ascorbic acid in aqueous cream was applied to a control site and an active site, nitrite containing cream being added only to the active site. Both sites were then occluded with adhesive plastic and the procedure repeated every

8 h. The creams were made up freshly on the day of the experiment and kept refrigerated in view of the instability of ascorbic acid.

The last application of the cream was made 5 h before the assessment of the response and biopsy to allow the immediate vasodilatory effects of NO to subside. At each site a spring thickness gauge (Mitotoyu, Tokyo, Japan) was used to measure skin thickness, a reflectance erythema meter measured erythema, and 4 mm punch biopsies were taken. The protocol was approved by the Joint Ethical Committee of Grampian Health Board and the University of Aberdeen.

Immunohistochemistry was performed using a streptavidin-biotin method and diaminobenzidine detection. Mouse anti-human monoclonal antibodies used in immunocytochemistry were CD1a antibodies (1:5, Becton Dickinson) for detection of Langerhans cells; CD3 antibody (1:20, Becton Dickinson); CD4 antibody (1:150, Becton Dickinson, Oxford, U.K.) for T helper cells; CD8 antibodies (1:20 Scottish Antibody Production Unit) for cytotoxic/suppressor cells; CD54 antibody (1:100, Serotec, Oxford, U.K.) for ICAM-1; CD68 antibody (1:10, Dako, Ely, Cambs, U.K.) for macrophages; CD106 antibody (1:100 Serotec) for VCAM-1; Ki67 antibody 91:400 (Immunotech, Luton, U.K.) associated with proliferating cell nuclear antigen in proliferating cells; p53 antibody (1:50, Novacastra, Newcastle on Tyne, U.K.) for wild-type p53; neutrophil elastase antibody (1:100, Dako) for neutrophils and a rabbit anti-human polyclonal antibody for nitrosotyrosine antibody (1:100, Upstate, Waltham, MA) for nitrosylated tyrosine. ApoptTag Plus *in situ* nick end labeling detection kit (Oncor, Gaithersburg, MD) to identify apoptotic cells. DNA fragmentation leads to nucleosome 3' OH DNA ends. These are catalytically extended *in situ* using a terminal deoxynucleotidyl transferase to add digoxigenin-nucleotide residues. Anti-digoxigenin antibody peroxidase conjugate is then used to label apoptotic nuclei. All staining was performed on cryostat sections of snap frozen cryopreserved specimens except apoptosis and neutrophil elastase which were performed on formalin-fixed, paraffin-embedded material. Appropriate controls, including secondary antibody alone and omission of primary antibody were negative. No nonimmune rabbit serum was available for the polyclonal nitrosotyrosine antibody, but other controls were negative.

Staining was quantitated by computerized image analysis. Briefly, the image of the section was captured on a purpose-built computer (Seescan, Cambridge, U.K.) a blinded observer selects a threshold which identifies the cells as positively stained and places a standard measuring frame on the test area of the image. A computer algorithm then counts the positively stained cells within that area. The data were analyzed by Wilcoxon's test for paired samples and Kruskal-Wallis' test for nonparametric analysis of variance in the multiple independent samples analyzed for effects of dose and duration. Cell counting was not possible where stains were diffuse and where morphology of cells was dendritic. In these cases the blinded observer rated staining on a scale of 0-4 including intermediate 0.5 steps.

RESULTS

The reflectance erythema measurement of the NO-treated sites was 32.25 ± 5.46 (mean \pm SD) significantly higher than the control sites 18.08 ± 5.81 ($p = 0.0022$, Wilcoxon's). Skin-fold thickness was 5.04 ± 0.75 mm in the NO-treated patches, which was significantly greater than that of control skin 3.25 ± 0.54 ($p = 0.0022$, Wilcoxon's). These measures were not significantly influenced by dose or duration of exposure, except there was a trend for greater skin fold thickness in the high dose group ($5.4 \text{ mm} \pm 0.21$ vs $4.7 \text{ mm} \pm 0.32$) ($p = 0.075$).

NO-treated skin showed significant increases in cells expressing CD3, CD4, CD8, CD68, neutrophil elastase, ICAM-1, VCAM-1, nitrosotyrosine, p53, and cells undergoing apoptosis compared with control-treated skin (Table I). Apoptag-positive cells were few in number compared with those showing cloudy swelling. There were significantly fewer CD1a-positive cells in the epidermis (Table I) and these could be seen rounding up and dropping out of the epidermis (Fig 2A). Ki-67 staining, a marker related to proliferating cell nuclear antigen did not differ significantly between active and control sites.

Histology of all NO-treated sites showed a significant increase in edema, endothelial swelling, cloudy swelling of keratinocytes, and a mixed infiltrate of lymphocytes and neutrophils. These changes were quantitated on a 0-4 ordinal scale and were similar in low dose, high dose, short exposure, and long exposure. The number of mast cells seen in Azure A stained sections was similar in control- and NO-treated skin (Fig 3J). A cytotoxic effect was

Table I. Subjects were randomly allocated to low or high nitrite and to 24 or 48 h treatment. The resulting disposition of patients is shown

Duration	0.5% nitrite	5% nitrite
24 h	2	4
48 h	4	2

seen in all keratinocytes that manifested as cloudy swelling (**Fig 3G**). When extensive this led to the formation of bullae high in the epidermis filled with acute inflammatory cells and cells that have undergone cytotoxic changes with constriction of the nucleus and cloudy swelling of clear cytoplasm around them (**Fig 3H**). Only a minority of these degenerate cells had undergone apoptosis as judged by staining with "Apoptag" (**Fig 3D**). Within the epidermis, there was also an increase in apoptotic cells. Apoptotic cells were also detected in the dermis, particularly around adnexal structures. Positive nitrosotyrosine staining around sebaceous glands suggests that NO was preferentially absorbed through follicles (**Fig 3B**).

The following effects of dose were noted (**Table III**). CD4-positive cells were fewer in the high-dose (280 ± 188 cells per $\text{mm}^2 \pm \text{SD}$) than the low-dose group (936 ± 444 , $p = 0.02$), and CD68-positive cells were less (379 ± 150 per mm^2) with high dose than with low dose (916 ± 628 , $p = 0.04$ Kruskal-Wallis).

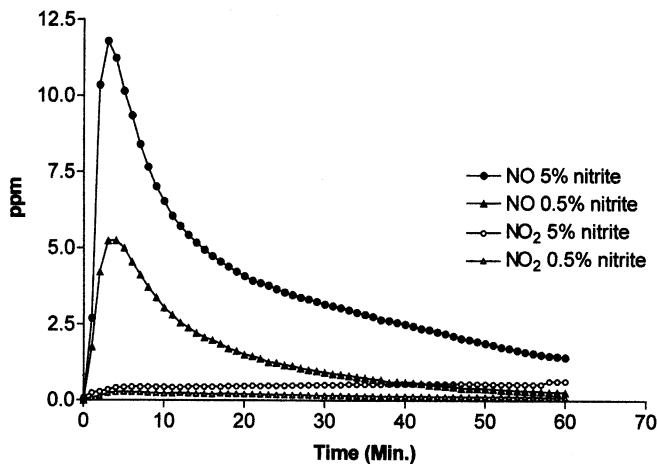


Figure 1. Time course of evolution of NO and NO₂. The time course of evolution of NO and NO₂ after the mixing of 50 μl of nitrite cream (0.5% or 5%) and 50 μl ascorbic acid cream (2%) in a 0.5 liter vessel detected by chemiluminescence.

There was a significant increase in Ki-67-positive proliferating keratinocytes with high dose (152 ± 84 cells per $\text{mm}^2 \pm \text{SD}$) compared with low dose (73 ± 19 , $p = 0.01$, Kruskal-Wallis), although there was no difference in Ki-67-positive cells between the control and NO treatments.

"Apoptag" staining was significantly greater after 48 h exposure to NO (14 ± 6 cells per 3 mm section) than after 24 h (3.5 ± 4.4 , $p < 0.005$, Kruskal-Wallis). CD4-positive cells also increased more at 48 h (824 ± 463 cells per mm^2) than at 12 h (286 ± 463 , $p = 0.05$, Kruskal-Wallis). The difference for p53 (335 ± 300 at 48 h compared with 70 ± 112 at 24 h) was not quite significant ($p = 0.07$) (**Table III**). Cloudy swelling tended to be greater in the longer duration treatment (mean score 2.5 ± 0.5 at 48 h *vs* 1.7 ± 0.79 at 24 h) but was not statistically significant ($p = 0.07$). Staining for p53 was noted in the basal layer of the epidermis, increased with time and correlated with nitrosotyrosine staining which followed a similar distribution ($r = 0.62$, $p < 0.0001$).

DISCUSSION

The combination of ascorbic acid and nitrite on the skin causes the release of pure NO. The dose of NO released under the conditions described was pro-inflammatory. Some cells had wide variability as indicated (**Table II**), reflecting wide variability in the response. The small numbers and introduction of variables of time and dose may explain this but statistical tests take this variation into account. The unusual histologic features of the inflammation provide important clues to the pro-inflammatory role of NO when generated at high concentrations by iNOS, for example in psoriasis or in sunburn. Fifteen minutes after application of the acidified nitrite to normal skin, blood flow was similar to that measured in a psoriatic plaque (laser Doppler measurements, data not shown). Peak NO production, 5 min after application of the creams, measured by the chemiluminescence method described elsewhere (Ormerod, 1998) (data not shown), was up to 10 times greater than that measured epicutaneously in psoriasis (Ormerod, 1997b). Not all the NO released in the skin, however, would be measured at the surface and proportionately less of the NO applied to the skin would diffuse into the living epidermis/dermis, and the plateau

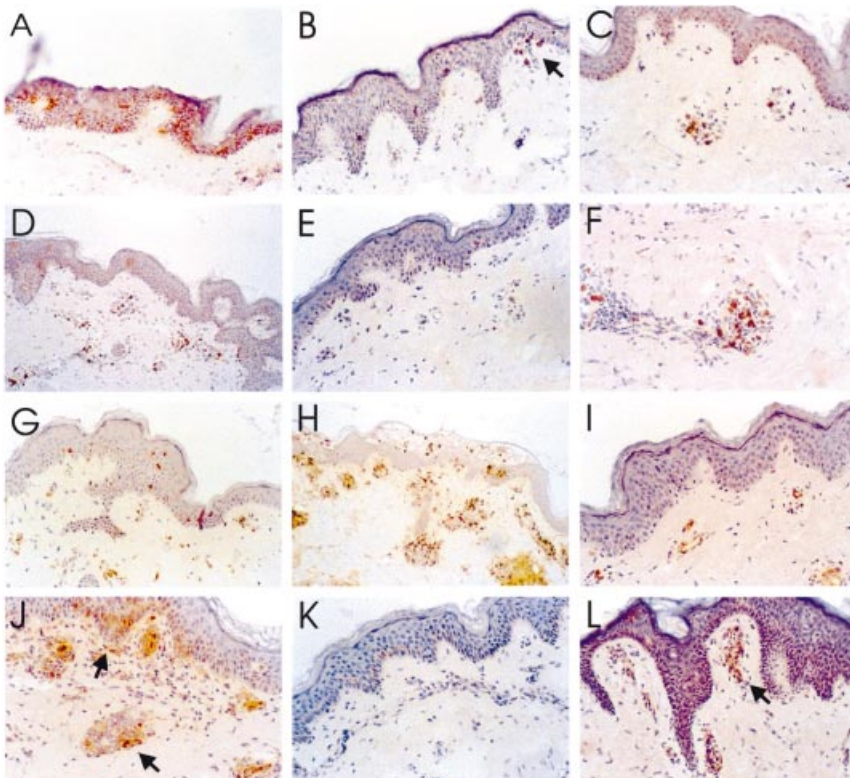


Figure 2. Immunocytochemistry results. h, high dose; l, low dose; 1 = 1 d, 2 = 2 d after application. (A) Control (2), Langerhans cells normally distributed with dendritic morphology. (B) NO treated (h2) Langerhans cells are fewer showing loss of dendricity and apparently migrating from the epidermis (arrow). (C) Control CD8 (2). (D) NO treated CD8 (h2) showing increased numbers around vessels and some epidermotropism. (E) Control CD4 (1). (F) NO treated CD4 (h1) showing increased numbers around vessels. (G) Control CD 68 (macrophages) (1). (H) NO treated CD68 (h1) showing marked increase in macrophages around appendages and in the epidermis, including some subcorneal pustules. (I) Control ICAM-1 (2). (J) NO treated ICAM-1 (h2) showing upregulation of ICAM-1 in endothelial cells and keratinocytes. (K) Control VCAM-1 not expressed (1). (L) NO treated showing upregulation of VCAM-1 (h1) confined to endothelium.

phase of NO release is close to that we found in severe psoriasis. Physiologic iNOS production would be continuous. In this *in vivo* model it is not easy to say if the inflammatory effects are secondary to cytotoxic tissue injury or are NO specific. If the inflammation were secondary to cell damage we would expect cytotoxicity to be greater in the shorter treatment and inflammation to be greater in the longer treatment group. The opposite was found, however, cytotoxic changes increased with time, whereas the inflammatory changes did not. We are not aware of any other studies of the direct effects of NO on intact viable organs *in vivo*.

When NO combines with superoxide to form peroxynitrite (ONOO⁻) it initiates DNA nicking and leads to DNA cleavage (Szabo, 1996a,1996b). Although *in situ* nick end labeling was significantly increased, only a few cells in the epidermis and dermis were labeled. Recently it has been shown that keratinocytes are

extremely resistant to the cytotoxic effects of NO but are more likely to be killed if the NO is combined with superoxide (Schuy *et al*, 1997). Nitrosylation of tyrosine is a marker of peroxynitrite activity (Szabo, 1996b). Staining for this was superficial, epidermal and not found at sites of macrophage and neutrophil activity suggesting that the peroxynitrite formation is not important for the inflammatory response we observed. Nitrosotyrosine staining was, however, seen in proximity to the basal layer where p53 was maximal so peroxynitrite is likely to be involved in DNA damage in these cells. It seems likely that NO contributes to cytokine and ultraviolet-induced apoptosis in the epidermis.

The increase in T helper cells and macrophages was greater in the low-dose subjects and suggests that at lower doses NO can be pro-inflammatory but at higher doses becomes cytotoxic to the immunocompetent cells and begins to exert an inhibitory effect.

Figure 3. Further immunocytochemistry results.

Abbreviations as in Fig 2. (A) Control stained for nitrosated tyrosine (2). (B) NO treated showing marked nitrosation of tyrosine (h2) in stratum corneum/hair follicle and increased staining in the basal layer (arrow). (C) Control skin stained with *in situ* nick end labeling (Apoptag) for apoptosis (2). (D) NO-treated skin stained for apoptosis (h2), whereas some of the cells showing cloudy swelling showed increased uptake, only those with apoptotic morphology (three indicated by arrow) were counted. (E) Control stained for wild-type p53 (2). (F) NO treated showing increase in p53 (h2) in the basal and suprabasal keratinocytes. (G) Hematoxylin/eosin stain showing numerous keratinocytes exhibiting cloudy swelling of the nuclei (arrow) (h2). These exceed apoptosis and were not associated with increased p53 suggesting nonspecific and reversible cytotoxicity. (H) NO treated, hematoxylin and eosin showing regular histology (h2) of the inflammation with perivascular inflammatory response modest spongiosis, civatte body formation and subcorneal pustules containing apoptotic inflammatory cells (neutrophils and macrophages). (I) Control stained with azure A for mast cells stained violet (arrow) (2). (J) NO treated stained for mast cells showing no increase in number (arrow) (h2). (K) Control stained for neutrophil elastase (1). (L) NO treated showing increased neutrophils (h1) in the dermis epidermis and in subcorneal pustules.

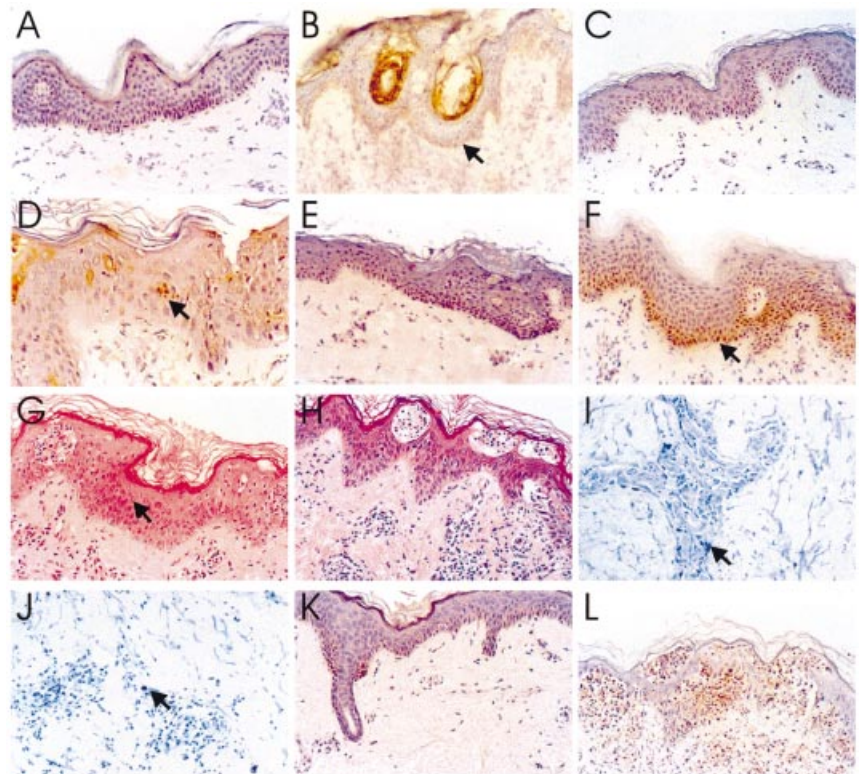


Table II. The results of quantitation of immunostaining, for all 12 subjects, showing differences in control and NO-treated skin^a

	Nitric oxide		Control		Significance Wilcoxon's
	Mean	SD	Mean	SD	
Apoptag	12.5	10.1	0.41	1.16	0.0033
Ki67	6.82	3.88	6.62	2.854	0.67
CD3	574.7	396.3	216.1	122.1	0.0186
CD4	608.2	458.2	176.3	149.9	0.0125
CD8	275.7	193.1	122.1	106.1	0.0284
CD68	673.1	542.4	301.4	361.3	0.0044
p53	214.4	266.4	22.08	53.8	0.0029
Neutrophils	569.4	385.9	71	113.1	0.043
ICAM-1	705.9	704.5	201.9	160.9	0.0209
CD1a ^b	1	—	3.5	—	0.022
Mast cells ^b	0	—	1	—	0.0537
Nitrosotyrosine ^b	4	—	1	—	0.043
VCAM-1 ^b	1.5	—	0	—	0.0357

^aFor continuous measurements (computerized cell counting) showing the mean and standard deviation.

^bWhere cell counting was difficult, e.g., more diffuse staining/dendritic cells, staining was graded subjectively on a scale of 0–4 and quoted values are medians. Ki-67 was counted in the epidermis and apoptag-positive cells counted per standard section through a 3 mm punch biopsy. All other counts were done by computerized image analysis on a fixed standard measuring frame and are expressed as cells per mm².

Table III. Differences in the 12 actively treated sites found^d

Cell marker	Dose		Duration	
	0.5% nitrite	5% nitrite	24 h	48 h
Apoptag	10.5 (7.3)	7.2 (8.2)	3.5 (4.4)	14 (6)****
Ki67	4.6 (1.1)	9.6 (4.5)****	5.9 (0.3)	7.3 (4.8)
CD3	43.2 (26.7)	28.6 (22.5)	33.7 (21.1)	38.1 (29.9)
CD4	58.5 (25.9)	17.5 (11.0)***	17.8 (12.1)	51.4 (29)**
CD8	15.9 (12.4)	18.9 (13.2)	11.8 (6.3)	19.9 (13.8)
CD68	57.3 (40.2)	23.8 (9.1)**	28.7 (6.6)	53.2 (43.9)
p53	11.7 (10.6)	15.5 (23.3)	4.38 (6.6)	20.9 (19.2)*
Neutrophils	32.1 (15.7)	39.1 (31.7)	36.4 (24.6)	34.8 (25.9)
ICAM-1	35.5 (18.7)	55.8 (66.5)	22.9 (6.7)	54.7 (51.7)
CD1a**	20.1 (13.9)	22.9 (25.2)	28.1 (22.9)	16.9 (13.9)
Nitrosotyrosine ^d	3	4	3	4
VCAM-1 ^d	1	2	1.5	1

^dDose and duration indicating cells per mm² (SD), except nitrosotyrosine and VCAM-1 which are on a scale of 0–4.

*p = 0.07, **p < 0.05, ***p < 0.02, ****p < 0.01.

This has been seen in other biologic systems and may especially be true of Th₁ cells (Liew, 1995; Taylor-Robinson, 1997). iNOS mutant mice develop significantly stronger Th₁ responses with increased susceptibility to leishmania infections and decreased inflammatory responses to *Leishmania* (Wei *et al*, 1995). In the human NO inhibited interferon- γ , IL-2, IL-5, IL-10, and IL-4 by anti-CD3 activated T cells (Bauer *et al*, 1997). An autoimmune inflammatory model in mice showed attenuation of vasculitis but not of glomerulonephritis or arthritis after iNOS knockout (Gilkesson *et al*, 1997). Another mechanism facilitating infiltration with leukocytes following NO may be the activation of metalloproteinases (Murrell *et al*, 1995) which break down basal laminae, collagen, and cell matrix assisting cell migration and angiogenesis. The paradoxical suppression of macrophages and T cells in our higher dose subjects may reflect the immunosuppressive effects of higher doses of NO for these cells.

In contrast to our *in vivo* findings of marked induction of ICAM-1 and moderate increase in VCAM-1 expression, NO has been reported to inhibit expression of the adhesion cell molecules VCAM-1 (De Caterina, 1995; Shin *et al*, 1996), ICAM-1, E-selectin, and P-selectin (Kanwar, 1995), although this has been challenged by Cartwright *et al* (1997) who found no effect of NO. Also NO induced E-selectin and VCAM-1 expression in endothelial cells activated by polyinosinic-polycytidylic acid to mimic viral infection (Faruqi *et al*, 1997). The contradiction may relate to intermediate cytokine activation or may be a concentration-dependent phenomenon.

The pattern of inflammation was unusual in showing a marked infiltrate of macrophages after only 24 h. We suggest that NO released from activated macrophages specifically recruits more macrophages to kill a pathogen or tumor cell. The only feature that was unchanged by NO was the number of mast cells.

The ability of NO to promote keratinocyte proliferation after ultraviolet B exposure (Benrath, 1995) has been previously described. Inhibition of cNOS inhibited keratinocyte proliferation (MacNeil, 1993). This is of particular interest in the context of the contribution NO makes to the pathology of psoriasis. Although we did not find a significant increase in Ki-67-positive proliferating cells compared with control, the relative increase in the high-dose group may indicate an influence of NO at higher doses promoting keratinocyte proliferation and wound repair. The NO-treated skin shows features in common with several clinical situations. The cytotoxic changes, p53, and apoptotic changes and migration of Langerhans cells suggest that NO mediates these aspects of sunburn and the development of sunburn cells. The subcorneal pustule formation seen with NO are not unlike those in pustular psoriasis. The upregulation of ICAM-1, VCAM-1 T cell infiltrate, and Langerhans cell changes following NO suggest that NO contributes to these aspects of psoriasis. The response of Langerhans cells to NO was striking, and as these cells are known to produce NO we

speculate that NO is an important signaling molecule in their activation. The pro-inflammatory role of NO we demonstrated *in vivo* suggests a potential for disease modification by inhibitors of iNOS and for immunopotentiality by NO donors in the treatment of skin infections.

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