Biphasic Effect of Exogenous Nitric Oxide on Proliferation and Differentiation in Skin Derived Keratinocytes but Not Fibroblasts

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Nitric oxide (NO) is known to exert cytotoxic and cytostatic effects in various cells and tissues. Although NO formation in human skin has been convincingly demonstrated, little is known about the NO-mediated effects in skin physiology and pathology. Here, we investigate the influence of NO on proliferation, differentiation, and apoptosis of primary cultures of normal human keratinocytes and fibroblasts. Four different NO donors at concentrations ranging from 0.01 to 5 mM were added every 12 h or 24 h to primary cultures of human keratinocytes and fibroblasts, and cells cultured for up to 3 d in the presence of these compounds. Cultures were examined for necrosis or apoptosis using trypan blue exclusion and *in situ* nick-translation. Cultures were also screened for the expression of the proliferation marker Ki67 and for an increase in cell numbers using neutral red staining. In addition, keratinocytes were stained for cytokeratin 6 expression to assess differentiation. We find that both keratinocytes and fibroblasts are highly resistant towards necrosis- or apoptosis-inducing effects of NO. In both cell types NO modulates cell growth, albeit in a cell-type specific pattern: cytostasis becomes significant in fibroblasts at concentrations of $\geqslant$ 0.25 mM of the NO donor. In keratinocytes a biphasic effect is found with increased proliferation at low concentrations ranging from 0.01 to 0.25 mM and cytostasis at concentrations of $\geqslant$ 0.5 mM. Conversely, expression of cytokeratin 6 is decreased at the lower NO donor concentrations and increased at higher concentrations as an indication of induction of differentiation at higher NO concentrations. Collectively, our results demonstrate that NO modulates proliferation and differentiation in human skin cells, a finding that will help to explain the pathophysiology of human skin diseases. Moreover, these findings suggest that NO generation in human skin diseases is not directly associated with local cell destruction, in contrast to findings in several other human diseases. ***Key word: human skin cells. J Invest Dermatol 111:286–291, 1998***

Nitric oxide (NO) is known to act cytostatic and cytotoxic in various cells and tissues. NO is produced by an enzyme family of nitric oxide synthases (NOS) consisting of three isoforms, the neuronal constitutive (ncNOS), the endothelial constitutive (ecNOS), and the inducible isoform (iNOS) (Kröncke *et al*, 1995a). Expression of both the constitutive as well as the inducible isoform has been shown in several cells resident to the skin, including keratinocytes and fibroblasts (Bruch-Gerharz *et al*, 1996, 1997; Du *et al*, 1997). To examine the effects of NO on cell proliferation, differentiation, and apoptosis in keratinocytes and fibroblasts, cells were incubated in the presence of four different NO donors and examined for proliferation or differentiation by immunostaining with the Ki67 antigen or cytokeratin 6. Furthermore, NO-mediated apoptotic or necrotic effects were investigated using *in situ* nick-translation or trypan blue exclusion.

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Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; NP, nitroprusside.

Altered states of proliferation and differentiation are known to play an important role in various hyperproliferative diseases such as psoriasis. In the case of human skin diseases associated with epidermal hyperproliferation, several different morphologic and biochemical alterations have been observed. For instance, induction of cytokeratins 6 (K6) and 16 (K16) combined with the reduction of cytokeratins 1 (K1) and 10 (K10) are significant differences to normal epidermis (Weiss *et al*, 1984; McGuire *et al*, 1984). Thus, in normal human epidermis as well as during wound healing processes K6 and K16 are expressed transiently. These cytokeratins are constitutively expressed at low levels (Fuchs and Green, 1978; Tyner and Fuchs, 1986).

An increasing number of investigations have shown local cytotoxic effects of NO when generated at higher concentrations via the activation of the inducible NOS (Butler and Williams, 1993; Förstermann *et al*, 1994), and it has been convincingly demonstrated that NO causes local organ destruction in autoimmune diseases (Kolb and Kolb-Bachofen, 1992).

The use of chemical NO donors is ideally suited to addressing the question of NO-mediated effects on skin cells. Chemistry has provided several molecules that generate NO after intracellular enzymatic degradation or spontaneous release in aqueous solutions with varying kinetics of decay (Kröncke *et al*, 1993). The use of these agents thus allows the activities of NOS enzymes to be experimentally mimicked and the effects of intracellular or extracellular NO generation *in vitro* to be studied. We have used this experimental tool to examine the effect of NO on

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keratinocyte viability, proliferation, and differentiation and compared these with dermal fibroblasts treated identically.

MATERIALS AND METHODS

Cells Primary cultures of human keratinocytes and fibroblasts were derived from breast reduction material and also from foreskin material. Cells were grown in 100 mm cell culture dishes (Greiner, Frickenhausen, Germany) under standard culture conditions. Keratinocytes were maintained in serum-free keratinocyte medium (Gibco Life Technologies, Eggenstein, Germany) supplemented with penicillin-streptomycin (4 ml per 500 ml). Primary human fibroblasts were cultured in RPMI 1640 (PAA Laboratories, Linz, Austria) supplemented with 10% fetal calf serum otherwise under the same conditions. Cells were passaged every 4–5 d. Experiments were performed on eight-chamber or 16-chamber glass slides (Nunc LabTec, Wiesbaden, Germany) in the presence or absence of the NO donors nitroprusside (NP), heterocyclic sydnonimine derivative (SIN-1), S-nitrosothiol-N-acetyl-penicillamine (SNAP), or a polyamine/NO-complex [DETA/NO: 1-Hydroxy-2-oxo-3,3-bis (3-aminoethyl)-1-triazene]. NO donor concentrations ranged from 0.01 mM to 5 mM. At various time periods medium was removed and slides were dried and processed for *in situ* nick-translation or immunostaining procedure. Using 24 h, 2 × 24 h, 4 × 12 h, or 3 × 24 h incubation periods, medium was removed after 24 h or after 12 h and fresh medium with or without NO donor was added.

Trypan blue exclusion Trypan blue dye exclusion was assessed and quantitated after a 5 min incubation and the number of cells excluding the dye was expressed as a percentage counted from several randomly chosen areas of each well.

***In situ* nick-translation** For detection of DNA strand breaks cells were fixed in acetone for 10 min. Endogenous peroxidase activity was blocked by incubating slides in methanol plus 0.3% hydrogen peroxide for 30 min. The *in situ* nick-translation mixture contained 3 μM biotin-dUTP, 5 U DNA polymerase I per 100 μl, 3 μM of each dGTP, dATP, dCTP, 50 mM Tris-HCL, pH 7.5, 5 mM $MgCl_2$, and 0.1 mM dithiothreitol. The reaction was performed at room temperature for 20 min. The slides were then washed in phosphate buffered saline and processed for immunocytochemical detection of biotin-labeled UTP by peroxidase-labeled avidin, followed by the enzyme reaction using diaminobenzidine as substrate.

Neutral red staining Neutral red solution in phosphate buffered saline (Sigma, Deisenhofen, Germany) was added to each well to give a final concentration of 0.03% and incubated for 90 min at 37°C in the dark. Medium was removed, and cells were washed two times with phosphate buffered saline and dried. After dissolving in 100 μl isopropanol plus 1% 1 M HCl, the absorption of the probes was measured at 530 nm in an enzyme linked immunosorbent assay microplate reader (Multiskan Plus MK 2, Helsinki, Finland).

Immunostaining procedure Cells were stained with either the anti-Ki67 monoclonal antibody (Gerdes *et al*, 1984) from DAKO (Glostrup, Denmark) or the anti-cytokeratin 6 monoclonal antibody (Demirkesen *et al*, 1995) from Boehringer Ingelheim (Heidelberg, Germany). Labeling procedures were performed by using the DAKO LSAB Kit (Peroxidase) following the manufacturer's instructions. As negative controls, we used isotype-matched irrelevant monoclonal antibodies. After fixation with acetone at room temperature for 10 min, slides were covered either with the primary antibody (Ki67, cytokeratin 6) or with the control antibody for 30 min at room temperature. Cells were then incubated with a link-antibody and streptavidin peroxidase for 30 min each at room temperature, and substrate-chromogen-solution using diaminobenzidine as substrate was applied for 5 min at room temperature. Finally, slides were counterstained with hematoxylin and eosin.

NO donor incubations Nitroprusside was purchased from Merck (Darmstadt, Germany), SIN-1 was a gift from Cassella-Hoechst (Frankfurt am Main, Germany), DETA/NO was kindly provided by Olaf Grapenthin (Institute of Pharmaceutial Chemistry, Heinrich-Heine-University, Duesseldorf, Germany), and SNAP was synthesized and characterized as described previously (Fehsel *et al*, 1996) and used at the concentrations indicated. Stock solutions were always freshly prepared and immediately used. Controls consisted of $DETA/NO_{-NO}$ and $SNAP_{-NO}$ at the highest relevant concentrations. These were obtained by prolonged incubation at 37°C of DETA/NO or SNAP yielding totally denitrosylated substances plus reaction end products.

Statistical analysis Statistical comparisons were performed with the paired t Student's test. Significance refers to $p < 0.05$ or $p < 0.001$.

RESULTS

Resistance of primary human keratinocytes and fibroblasts against the cytotoxic effects of NO NO at higher concentrations that are generated by the inducible NOS is generally assumed to have cytotoxic or cytostatic effects (Kröncke *et al*, 1994). As the relative sensitivity of human skin cells towards NO is not known, we initially analyzed whether NO induces cell death in primary cultures of human keratinocytes or fibroblasts. We used trypan blue exclusion to search for necrosis and *in situ* nick-translation to detect DNA strand breaks as a hallmark for apoptosis. Both keratinocytes and fibroblasts were incubated with the NO donors SIN-1 or SNAP, and keratinocytes were additionally treated with NP or DETA/NO at concentrations ranging from 0.5 mM to 5 mM for various periods of time. With both cell types, neither DNA strand breaks nor cellular lysis were significant at NO donor concentrations up to 5 mM. Only incubation with SIN-1, which simultaneously generates oxigen radicals, led to significant cell death (35.6% dead keratinocytes and 20.4% dead fibroblasts at 5.0 mM and 24 h) (**Fig 1**) and DNA damage (53.9% and 32.2% strand breaks in keratinocytes and fibroblasts at 5.0 mM and 24 h) (**Fig 2*a*, *b***). A low, but significant cell death was found (25–30% of cultured keratinocytes or fibroblasts; data not shown) only at the highest and nonphysiologic concentrations of the different NO donors NP, DETA/NO, or SNAP (5.0 mM) and with a prolonged incubation period (48 h). These results suggest that both keratinocytes and fibroblasts express a relative resistance toward the cytotoxic effects of NO.

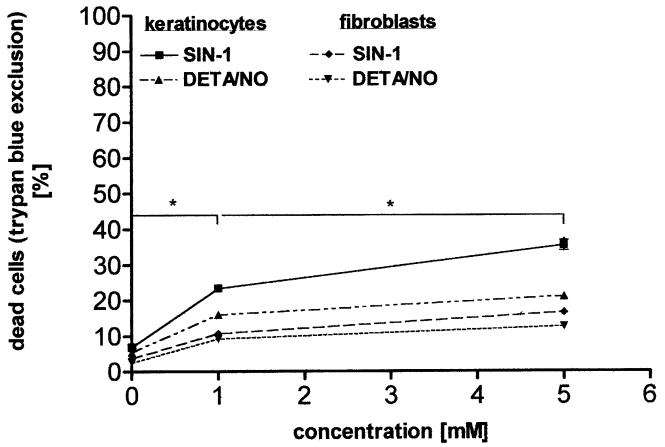

Figure 1. Keratinocytes and fibroblasts are relatively resistant towards the cytotoxicity of NO. Effect of SIN-1 or DETA/NO on cell viability of primary human keratinocytes or fibroblasts after a 24 h incubation period. Cells were examined with trypan blue exclusion and expressed as a percentage of dead cells. Each point represents the mean ± SD of four independent experiments. Significant differences were found with SIN-1 treatments only (*$p < 0.05$ for SIN-1 only between 0 and 1 mM and between 1 and 5 mM).

Proliferation or growth arrest depending on different concentrations of exogenous NO Primary human keratinocytes or fibroblasts were incubated with various concentrations of DETA/NO that, due to its half-time ($t_{1/2}$ = 7 h), allows for studying effects of long-term NO exposure. Cells were cultured for a total of 48 h with addition of the NO donor every 24 h or every 12 h. Keratinocytes maintained at concentrations of 0.01 mM to 0.25 mM of DETA/NO, exhibited increased positive immunostaining for Ki67 as an indication for increasing proliferation (**Fig 3*a***). The same effect was found with SNAP, albeit at higher concentrations (**Fig 3*b***), in accordance with the fact that DETA/NO releases 2 mol of NO per mol of polyamine complex, whereas SNAP releases NO in a 1:1 ratio. With higher NO donor concentrations the reverse effect was found, i.e., Ki67 labeling decreased as an indication for an antiproliferative activity. By measuring cell numbers via neutral red staining we could confirm this biphasic effect on cell proliferation (**Fig 4**). These effects were found with three different donors of cells and two different skin regions that cells were derived from. Interestingly, with dermal fibroblasts cultured under the identical conditions we found a different type of response: here, a proliferation enhancing effect could not be demonstrated. Even at the lowest concentrations a significant decrease of Ki67 expression and proliferation was found with both methods

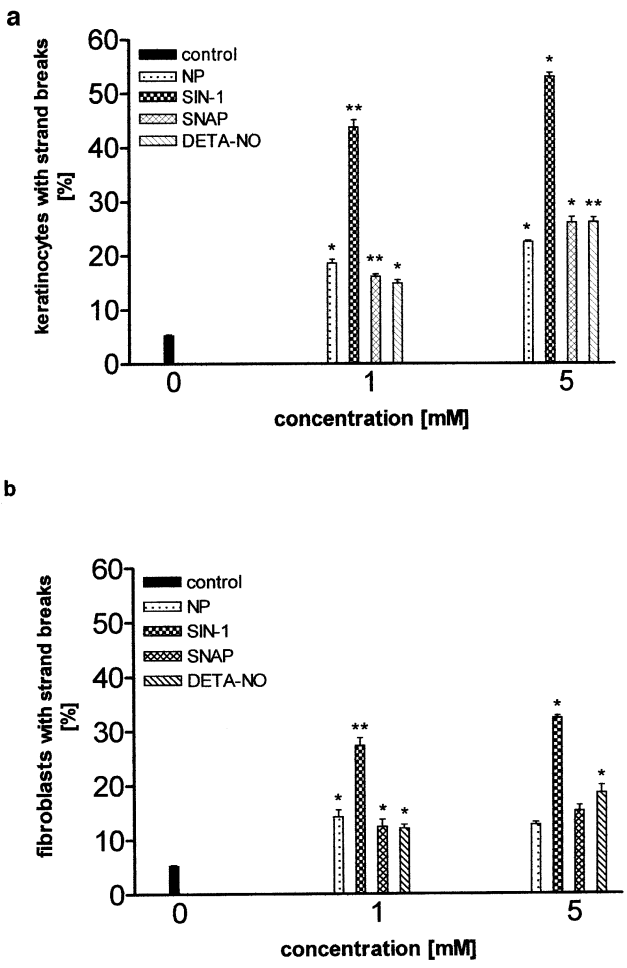

Figure 2. Even at nonphysiologic concentrations NO does not significantly induce apoptosis in keratinocytes and fibroblasts. Strand breaks induced by NP, SIN-1, SNAP, or DETA/NO in primary human keratinocytes (*a*) or fibroblasts (*b*) after a 24 h incubation period were visualized by *in situ* nick-translation. Each bar represents the mean ± SD of four independent experiments. Significance of differences in NO-mediated apoptosis was determined by comparing 1 mM with untreated controls or 5 mM with 1 mM (*$p < 0.05$, **$p < 0.001$).

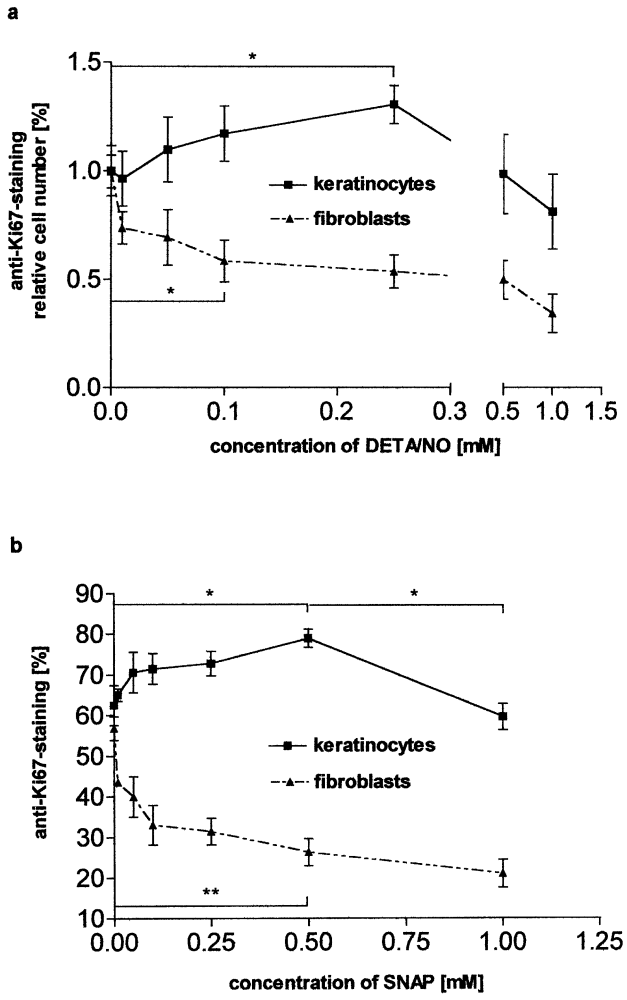

Figure 3. NO-modulated proliferation is different in keratinocytes as compared with fibroblasts. Effect of DETA/NO after 2 × 24 h incubation periods on the proliferation of primary human keratinocytes or fibroblasts as revealed with anti-Ki67 immunostaining. Each point represents the mean ± SD of seven independent experiments. The percentages of positive cells in controls differed with donors and medium batches between 30% and 68%. Statistically significant differences are as indicated. This experiment was repeated using a different NO donor (SNAP) to confirm the NO-mediated effects. Each point represents the mean ± SD of four different independent experiments (*$p < 0.05$, **$p < 0.001$).

(**Figs 3*a*, *b*, 4**). **Figure 5** shows the Ki67 expression in both keratinocytes and fibroblasts depending on the different concentrations of DETA/NO.

Depression of differentiation at low concentrations of NO donors and increased differentiation at higher NO concentrations We further investigated the effects of exogenous NO on keratinocyte differentiation by studying the expression of cytokeratin 6. By immunocytochemistry, we determined the percentage of cells with weak or strong staining as an indicator for ongoing differentiation. Cells were incubated with DETA/NO for 2 × 24 h as described before. In primary keratinocytes exposed to low NO donor concentrations up to 0.05 mM DETA/NO, a slight but not significant increase of cells staining weakly for anti-cytokeratin 6 was observed in all experiments. At concentrations ranging from 0.05 mM up to 1.0 mM DETA/NO, the expression pattern changed drastically with the majority of cells exhibiting strong staining as an indication for ongoing differentiation of keratinocytes (**Figs 6*a*, *b*, 7**).

The results found with DETA/NO as NO donor could again be reproduced with SNAP at incubation periods of 2 × 24 h (**Fig 8*a***) or 4 × 12 h (data not shown).

We also analyzed whether the use of DETA/NO for longer periods of time would alter the differentiation pattern, but findings were essentially the same (**Fig 8*b***).

DISCUSSION

There is a multitude of effects and functions for NO described so far, one of which is cytotoxicity leading to the destruction of bacteria and parasites but also of cells and tissues, thereby contributing to unspecific immunity and inflammation (Corbett *et al*, 1996; Brenner *et al*, 1997). Moreover, several reports have demonstrated the potential of NO to influence the proliferation and differentiation cascade in several mammalian cells (Nüssler and Billiar, 1993; Saunders and Jetten, 1994; Arany *et al*, 1996). Because the relative sensitivity of human skin cells towards NO is not yet known, we investigated whether NO mediates cytotoxicity or influences proliferation and differentiation in cultures of primary human keratinocytes and fibroblasts.

In a first set of experiments, we analyzed whether NO induces cell death in these cell cultures. In human skin, physiologic cell death can result from apoptosis (Albina *et al*, 1993; Kaneo *et al*, 1995), but the exact mechanism of cell death may vary from one type of cell to another. Moreover, morphologic evidence exists that apoptosis also contributes to the pathogenesis of several human skin diseases, although knowledge

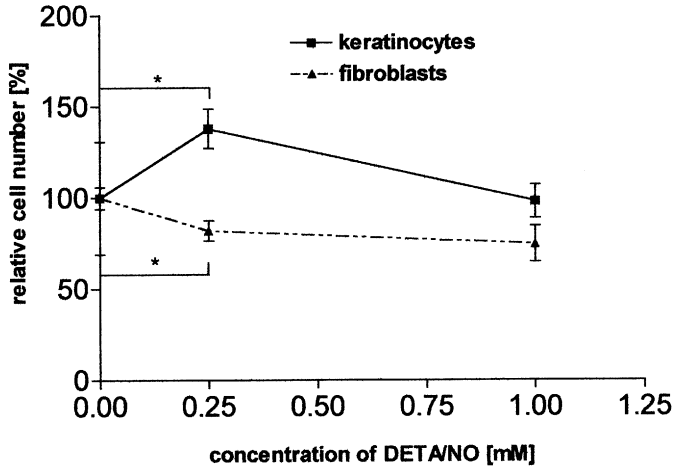

Figure 4. NO-modulated cell proliferation is also seen by cell counts in the cultures at the end of incubations. Effects of DETA/NO after 2 × 24 h incubation periods on the proliferation of primary human keratinocytes or fibroblasts as revealed with the neutral red staining. Each point represents the mean ± SD of seven independent experiments controls (*$p < 0.05$).

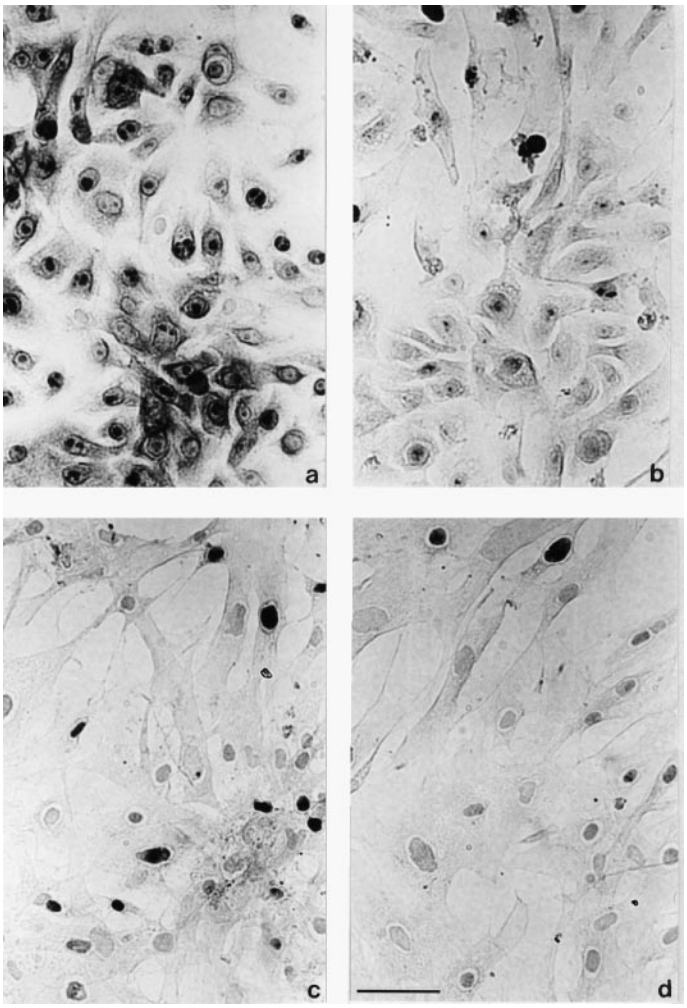

Figure 5. NO modulates cell proliferation in cell cultures of keratinocytes and fibroblasts. Ki67 expression in primary cultures after 2 × 24 h incubation periods with low concentrations (0.1 mM, ***a***, ***c***) or high concentrations (1.0 mM, ***b***, ***d***) of DETA/NO: keratinocytes (*a*, *b*) show high labeling frequency at low concentrations and reduced incidence at high NO donor concentrations. Fibroblasts (*c*, *d*) exhibit reduced labeling even at low concentrations and a further reduction at high concentrations of NO donor; *scale bar*, 50 μm.

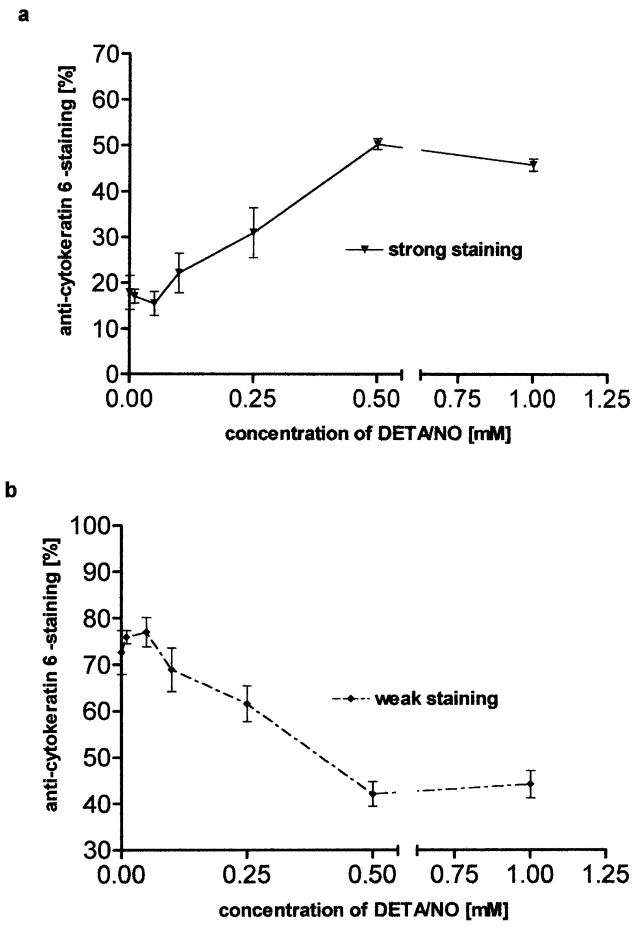

Figure 6. NO modulates keratinocyte differentiation in a biphasic pattern. Effects of DETA/NO after 2 × 24 h incubation periods on cell differentiation of primary human keratinocytes: cells immunostained with anti-cytokeratin 6 were counted for weak (*b*) or strong (*a*) staining. Percentages of strongly or weakly stained cells in controls differed with donors and medium batches between 15% and 20% or between 71% and 75%. Each point represents the mean ± SD of seven independent experiments. Significances of differences in strong (*a*) or weak (*b*) cell staining were determined with concentrations of 0.5 mM as compared with untreated controls (*$p < 0.05$).

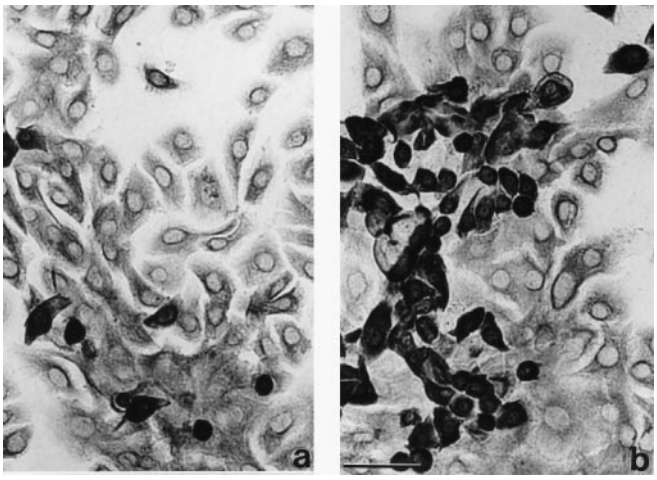

Figure 7. Micrographs of NO-exposed keratinocyte cultures show the concentration-dependent effects on differentiation. Cytokeratin 6 expression in keratinocytes is related to concentrations of DETA/NO: (*a*) small fraction of strongly stained cells at a concentration of 0.05 mM; (*b*) numerous darkly stained cells at a concentration of 1 mM; *scale bar*, 50 μm.

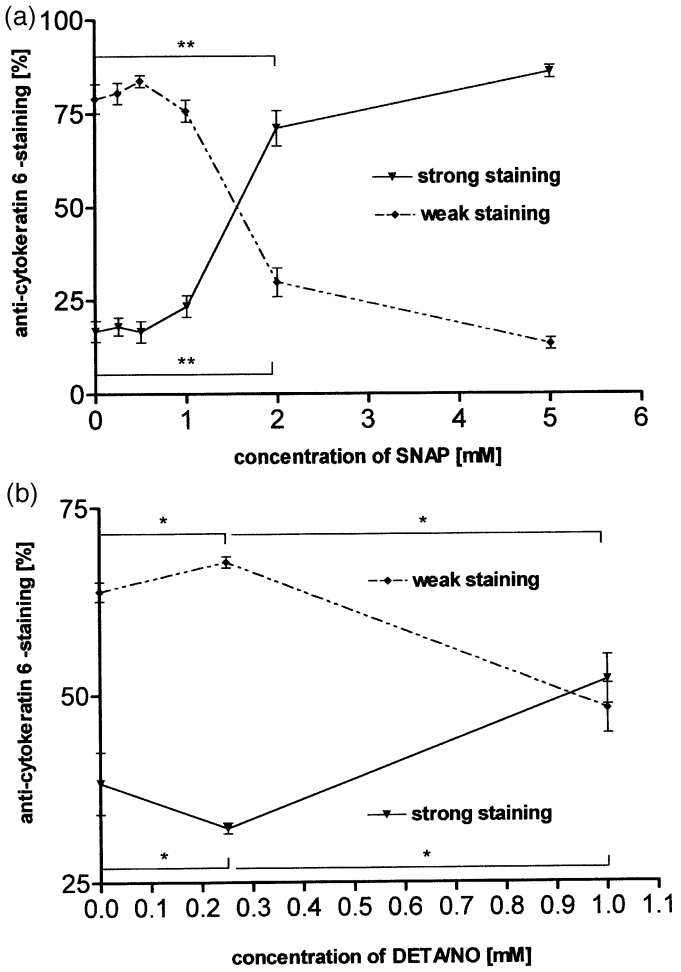

Figure 8. Use of different NO donors or prolongation of NO exposure do not alter the effects on keratinocyte differentiation. Effects of SNAP (*a*) or DETA/NO (*b*) after incubation periods of 2 × 24 h (*a*) or 3 × 24 h (*b*) on cell differentiation of primary human keratinocytes: cells immunostained with anti-cytokeratin 6 were counted for weak or strong staining. Each point represents the mean ± SD of four independent experiments. Statistically significant differences in differentiation were determined with SNAP (*a*) at concentrations of 2.0 mM as compared with untreated controls or with DETA/NO (*b*) at concentrations of 0.25 mM as compared with untreated controls and at concentrations of 1.0 mM as compared with 0.25 mM (*$p < 0.05$, **$p < 0.001$). ◆, Weak staining; ▲, strong staining.

about potential inducers of apoptosis and about molecular and structural alterations in human skin is scarce (Paus *et al*, 1993).

One result of our investigations was that epidermal human keratinocytes and fibroblasts are resistant towards cytotoxic or apoptosis-inducing effects of exogenous NO. Of all NO donors used, only SIN-1 resulted in significant cell death at physiologically relevant concentrations. It is likely that this is due to oxigen free radicals produced simultaneously with NO during the decay of SIN-1 but not with any of the other NO donors used here. These findings suggest that NO generation in human skin diseases is not directly associated with local cell destruction, in contrast to findings in several other human diseases.

When studying the effect of NO on skin cell growth, however, we found a unique and apparently cell type-specific regulatory activity: keratinocytes show an increasing proliferation and a decreasing differentiation when maintained at low NO concentrations, whereas the pattern of proliferation and differentiation changes into the opposite pattern when cultured at higher concentrations. This biphasic activity of NO on the proliferation and the differentiation cascade is readily seen, although keratinocytes are maintained under proliferative culture conditions as is reflected by the relatively high expression of Ki67 in the absence of NO donors. Interestingly, skin fibroblasts did not reveal this biphasic type of response, but exhibited a significant growth arrest even at the lowest concentrations of NO donors used. For these experiments cells were exposed to repetitive doses of the polyamine/NO-complex DETA/NO that decays in a first order kinetic. Accordingly, 2 mM of DETA/NO will generate 6.6 μM of NO per minute (Berendji *et al*, 1997). In the immediate vicinity of a cell monolayer that enzymatically generates NO, a steady-state concentration of about 4–5 μM of NO has been recently calculated (Laurent *et al*, 1996). Therefore, DETA/NO concentrations of up to 2 mM as used here can be considered as near physiologic and comparable NO releases are indeed seen with live cells.

Both cell growth and differentiation regulating activities of NO have been described previously. In primary human keratinocytes it was found that endogenous NO synthesis correlates with onset of differentiation (Arany *et al*, 1996).

Two other studies examined the effects of NO donors or iNOS-generated NO in fibroblasts (Du *et al*, 1997; Gansauge *et al*, 1997), both describing an increased cell proliferation with single doses of NP or SNAP or GSNO. Results are not comparable, mostly because we studied effects of long-term exposure with repeated addition of the NO donors DETA/NO or SNAP.

After a 24 h incubation period of fibroblasts with single doses of NO donors like SNAP ($t_{1/2} \approx 4$ h) or GSNO ($t_{1/2} \approx 70$ min), some of the measured effects may reflect cellular responses after a release from NO-mediated blockade, whereas blocking is maintained with constant NO release from iNOS or from repetitive doses of DETA/NO ($t_{1/2} \approx 7$ h). In addition, the use of NP argues against a difference between NO generated exogenously *versus* NO produced within cells, as this agent will generate NO only after enzymatic breakdown within cells (for a review see Kröncke *et al*, 1995b).

In summary, our results demonstrate an important role for NO in human skin homeostasis, exerting both increased proliferation as well as the onset of differentiation, depending on local NO concentrations and cell type.

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