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Up-regulation of Interleukin-1β-Stimulated Interleukin-8 in Human Keratinocytes by Nitric Oxide

Penelope J. Andrew, Hanna Harant† and Ivan J. D. Lindley‡*
NOVARTIS FORSCHUNGSINSTITUT, DEPARTMENT OF CELLULAR AND MOLECULAR BIOLOGY, VIENNA, AUSTRIA

ABSTRACT. Nitric oxide (NO) is able to regulate the expression of a number of inflammatory mediators. In this study, the effect of NO on the expression of the chemokine interleukin-8 (IL-8) by primary human keratinocytes and the lines KB and HaCaT was examined. Incubation with the NO donor *S*-nitroso-*N*-acetylpenicillamine (SNAP) for 24 hr increased IL-8 protein only in HaCaT cells, partly due to the presence of constitutive interleukin-1 (IL-1). However, in combination with IL-1β, SNAP enhanced both IL-8 mRNA and protein in all three cell types. Transfection of cells with an IL-8 promoter reporter gene construct showed that the effect of NO was at least partly due to transcriptional activation. Despite small variations in the response to NO by the three cell types, these results demonstrate that NO can up-regulate IL-1β-stimulated IL-8 expression in human keratinocytes. This study provides a regulatory mechanism which may be important in the context of skin inflammation, and supports the role of NO as an inflammatory mediator in the skin. BIOCHEM PHARMACOL **57**;12:1423–1429, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. nitric oxide; interleukin-8; cytokines; keratinocytes

Cytokine production by keratinocytes is of major importance in both normal keratinocyte function and in the regulation of immune and inflammatory processes in disease. Among the cytokines produced by keratinocytes is the chemokine IL-8§, whose expression by these cells can be up-regulated by IL-1 and TNF-α [1]. IL-8 is now recognised as having a wide range of functions, and is constitutively released from both proliferating and quiescent keratinocytes [2]. It can act as an autocrine growth factor [3], as well as being chemotactic for keratinocytes [4]. Aberrant expression of IL-8 is associated with psoriasis [5], and roles have been proposed for IL-8 in some of the common features of the disease, including keratinocyte hyperproliferation [3], the angiogenic response [6], neutrophil infiltration [7] and overexpression of iNOS [8, 9]. Regulation of IL-8 expression is complex, with cytokines such as IL-1 and TNF-α not only stimulating transcription but also stabilising the mRNA, leading to a large, usually transient increase in IL-8 production.

In a previous study, we showed that the reactive radical NO up-regulated the expression of TNF-α-stimulated IL-8 in a human melanoma cell line, via a transcriptional mechanism involving the DNA binding site for the transcription factor NF-κB [10]. A number of other target genes have been shown to be regulated by NO, with the outcome depending on both the cell type and stimulus used. Examples of up-regulation include interleukin-4 in T lymphocytes [11] and IL-1β-stimulated TNF-α in monocytes [12], although when lipopolysaccharide was used as the co-stimulus, the opposite effect was seen. NO also up-regulated IL-8 expression in an endothelial cell line, ECV304 [13] although in primary endothelial cells, NO down-regulated the cytokine-stimulated expression of IL-8, VCAM-1 [14], and macrophage-colony stimulating factor [15], via inhibition of NF-κB. Clearly, the effect of NO on expression of target genes is dependent upon both cell type-specific mechanisms and upon the stimulus, and it is therefore important to demonstrate the effect of NO on the gene of interest in the relevant cell type.

NO is produced by the enzyme NOS, three main isoforms of which are expressed in numerous cell types. The inducible isoform of NOS has been shown to be overexpressed in psoriatic lesions containing large amounts of IL-8 [16]. The co-localisation of IL-8 receptor and iNOS mRNA, coupled with the induction of iNOS by IL-8 and interferon-γ, prompted Bruch-Gerharz *et al.* to propose that keratinocyte-derived IL-8 forms an autocrine loop for iNOS induction

* Current address: Institut für Pharmakologie und Toxikologie, Karl-Franzens-Universität Graz, Universitätsplatz 2, A-8010 Graz, Austria.

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‡ Corresponding author: Dr. Ivan Lindley, Novartis Forschungsinstitut, Department of Cellular and Molecular Biology, Brunner Strasse 59, A-1235 Vienna, Austria. Tel. +43 1 86634 328; FAX +43 1 86634 518; E-mail: ivan.lindley@pharma.novartis.com

§ *Abbreviations:* IL-1, interleukin-1; IL-1ra, interleukin-1 receptor antagonist, 1°HK, primary human keratinocytes; SNAP, *S*-nitroso-*N*-acetylpenicillamine; TNF-α, tumor necrosis factor alpha; IL-8, interleukin-8; NO, nitric oxide; iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor κB; and NOS, nitric oxide synthase.

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[8]. The production of both IL-8 [17] and NO [18] is also up-regulated in response to UV stimulation. Although considerable evidence therefore already exists for the co-regulated production of the two molecules in keratinocytes, it is not yet known whether NO can directly affect the expression of IL-8 by these cells.

One of the most favoured keratinocyte-like cell lines is the spontaneously transformed HaCaT line [19] because of the morphologic and phenotypic similarity to 1°HK, coupled with ease of culture. Another line which has commonly been used as a model for transformed keratinocytes [20] is the epidermoid carcinoma line KB, although these cells do not morphologically resemble 1°HK in culture. These two keratinocyte-like cell lines, as well as 1°HK, were used in this study with the aim of defining a role for NO in the regulation of keratinocyte-derived IL-8. Expression was analysed at the mRNA and protein levels, and at the level of transcription, using an IL-8 promoter-driven reporter gene assay. Results show that NO released by an NO donor enhances IL-1β-stimulated IL-8 expression, in a similar manner to the up-regulation of TNF-α-stimulated IL-8 in a melanoma cell line [10], but in contrast to the down-regulation of IL-1α-stimulated IL-8 in endothelial cells [14]. In addition, variations in the response to NO by the three keratinocyte cell types tested are discussed.

MATERIALS AND METHODS

Materials

Recombinant human IL-1β was provided by P. Ramage, Novartis, Basel. A 300 mM stock solution of the NO donor SNAP (Affiniti) was prepared immediately before use in methanol. Recombinant human IL-1 receptor antagonist was a kind gift from the Rheumatology Dept., Kaiser Franz Josef Hospital, Vienna, and was used at a concentration of 100 ng/mL. KB cells were purchased from ATCC (Rockville, MD). HaCaT cells were provided by Dr. N. Fusenig (DKFZ, Heidelberg). 1°HK from breast tissue were provided by I. Schuster, Novartis Forschungsinstitut, Vienna.

Cell Culture

KB cells were cultured in RPMI 1640 medium/10% fetal bovine serum, HaCaT cells in Dulbecco's modified Eagle's medium/10% fetal bovine serum, and 1°HK in Keratinocyte Growth Medium (Clonetics), supplemented with glutamine and antibiotics. 1°HK were used in passage 2 or 3.

IL-8 ELISA

Cells were grown to confluence in 96-well plates (Nunc) before overnight treatment with 100–300 μM SNAP and/or 100 ng/mL IL-1β in 300 μL medium (0.1% methanol). The supernatants were analysed for secreted IL-8 by a double ligand sandwich ELISA [21].

IL-8 Promoter Reporter Gene Assay

The IL-8 promoter reporter gene plasmid containing the luciferase reporter gene construct under the control of nucleotides −1470 to +40 relative to the transcription start site of the *IL-8* gene has been described previously [10]. KB cells (5×10^6) were electroporated in a Bio-Rad Gene Pulser at 250 μF, 400 V with 5 μg of plasmid DNA, and were then plated into 48-well plates (Costar) at 8×10^4 cells/well. HaCaT cells (2.5×10^6) were plated in a 10-cm dish and incubated overnight before transfection with 5 μg plasmid DNA in 250 μg/mL *O*-diethylaminoethyl-dextran/0.4 mM chloroquine for 1 hr, followed by a 2-min incubation with 15% glycerol. After a further overnight incubation, cells were trypsinised and replated as for KB. Cells were allowed to adhere for at least 6 hr before overnight treatment with 100–300 μM SNAP and/or 100 ng/mL IL-1β in 300 μL medium (0.1% methanol). The cells were lysed in lysis buffer (Promega) and analysed for luciferase activity in a Labsystems Luminoskan luminometer using the Promega Luciferase Assay System.

Northern Analysis

Cells were cultured in 6-well plates until confluent and were then treated with 100 ng/mL IL-1β, in the absence or presence of 100–300 μM SNAP. After the indicated incubation period, cells were lysed in TRIzol reagent (GIBCO BRL) and total RNA was prepared according to the manufacturer's instructions. Northern analysis was performed as described previously [22]. Blots were quantified using a phosphorimager (Packard Instant Imager) and normalised relative to the glyceraldehyde-3-phosphate dehydrogenase signal.

Statistical Analysis

Two or three independent experiments were performed, each using triplicate wells. The data were analysed using one-way analysis of variance, and are expressed as means ± SE. Differences were considered to be statistically significant when $P < 0.05$, and significance is indicated in the figures by $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

RESULTS

SNAP-enhanced Expression of IL-1β-stimulated IL-8 Protein

Cells were treated with the NO donor SNAP in the presence or absence of IL-1β, and secreted IL-8 protein was measured after 24 hr (Fig. 1). There were marked differences in both the constitutive and IL-1β-stimulated IL-8 levels among the three cell types. However, in all three cell types, IL-8 levels in the presence of IL-1β were enhanced by the addition of SNAP. Experiments using a second NO donor, *S*-nitroso-L-glutathione, were performed in KB and HaCaT cells and produced similar results (data not shown),

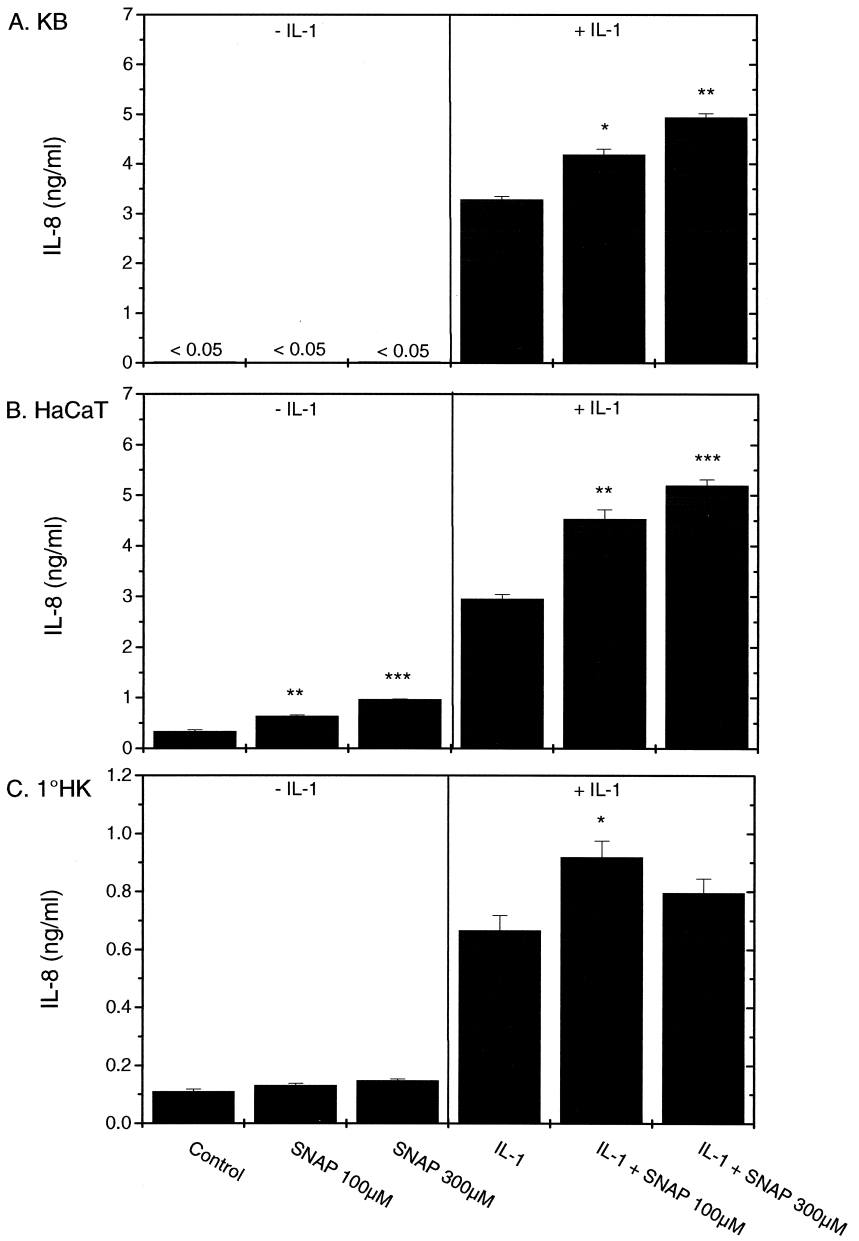

FIG. 1. SNAP enhances the expression of IL-1β-stimulated IL-8 protein. Cells were treated as indicated for 24 hr and the supernatants were analysed for secreted IL-8. IL-1β was used at a concentration of 100 ng/mL. Data are expressed as the means ± SE of triplicate wells (N = 3). *P < 0.05, **P < 0.01, and ***P < 0.001 compared to the control without SNAP.

indicating that NO was mediating the observed effects. In addition, *N*-acetyl-penicillamine, the unnitrosylated parent compound of SNAP, had no effect on IL-8 levels in KB cells, in either the presence (3.29 ± 0.05 vs. 2.83 ± 0.49) or absence (below detection) of IL-1β. In the absence of IL-1β, SNAP had no significant effect except in HaCaT cells, where IL-8 levels were up-regulated. Since keratinocytes can express IL-1α and IL-1β constitutively [23], IL-1 receptor antagonist (IL-1ra) was used to determine whether constitutive IL-1 expression could be involved in the effect of SNAP alone in HaCaT cells. SNAP-stimulated IL-8 expression was reduced to control levels by IL-1ra (Table 1), confirming the involvement of endogenous IL-1. The

TABLE 1. Expression of IL-8 protein by HaCaT cells in the presence and absence of IL-1ra

Treatment	IL-8 (ng/mL)	(% Control)
Control	0.655 ± 0.105	(100 ± 16%)
IL-1ra	0.470 ± 0.087	(72 ± 13%)
SNAP	0.976 ± 0.024	(149 ± 4%)
SNAP + IL-1ra	0.674 ± 0.053	(103 ± 8%)

Confluent HaCaT cells were incubated in the presence or absence of 100 ng/mL IL-1ra for 1 hr, prior to the addition of 300 μM SNAP. After a 24-hr incubation, supernatants were analysed for secreted IL-8. Data are expressed as the means ± SD of triplicate wells (N = 3).

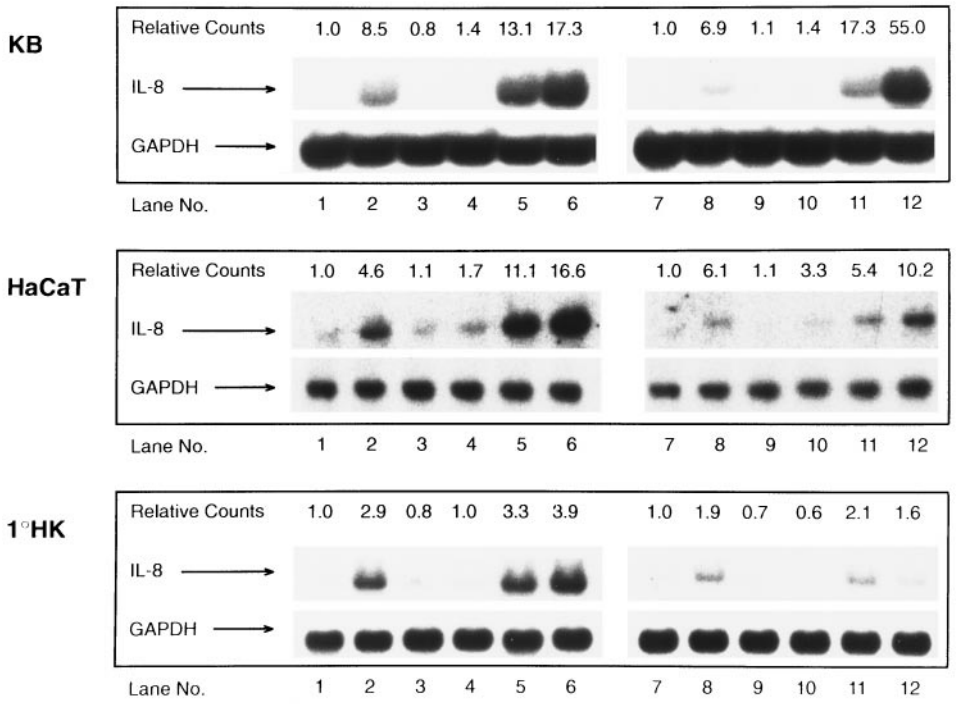

FIG. 2. SNAP enhances the levels of IL-1β-stimulated IL-8 mRNA. Cells were incubated for 6 hr (lanes 1–6) or 24 hrs (lanes 7–12) before lysis for analysis of mRNA. Lanes: 1 and 7, control; 2 and 8, IL-1β 100 ng/mL; 3 and 9, SNAP 100 μM; 4 and 10, SNAP 300 μM; 5 and 11, IL-1β + SNAP 100 μM; 6 and 12, IL-1β + SNAP 300 μM. Relative counts represent band intensity relative to the unstimulated control after normalisation to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data are representative of three similar experiments.

constitutive production of IL-8 was also reduced by IL-1ra, although the difference was not statistically significant.

This analysis of IL-8 protein expression by the three keratinocyte cell types showed that, regardless of the absolute levels of constitutive and cytokine-stimulated IL-8, NO released from an NO donor was able to enhance the amount of IL-1β-stimulated IL-8 protein secreted into the medium.

Levels of IL-8 mRNA in KB, HaCaT, and 1°HK Are Enhanced by SNAP, with a Prolonged Effect Seen Only in KB Cells

Northern blot analysis was performed on RNA isolated from KB, HaCaT, and 1°HK after 6- and 24-hr incubations (Fig. 2). IL-8 mRNA levels at 6 hr in the presence and absence of IL-1β alone largely reflected the amount of secreted IL-8 protein, and in all cases, mRNA levels were reduced after 24 hr. At 6 hr, IL-1β-stimulated IL-8 mRNA levels were dose-dependently enhanced in the presence of SNAP in all three cell types, although the overall increases seen in KB and HaCaT cells compared to unstimulated cells were greater than that seen in 1°HK (17.3- and 16.6-fold vs 3.9-fold). At 24 hr, the effect of NO was markedly enhanced in KB and HaCaT cells, whereas in 1°HK, IL-8 mRNA levels were returning to control levels. SNAP alone increased IL-8 mRNA at 24 hr only in HaCaT cells, an observation which parallels the protein expression data in Fig. 1. Thus, the increase in IL-8 protein secretion was reflected in an up-regulation in the level of mRNA. In HaCaT cells and 1°HK, this increase in mRNA was transient, whereas in KB cells, the mRNA up-regulation was prolonged.

SNAP-enhanced IL-8 Promoter Activity

Reporter gene experiments were performed in KB and HaCaT cells to investigate the mechanism by which NO was exerting its stimulatory effect. Transfections were not performed on 1°HK due to limited cell numbers. Cells were transiently transfected with a luciferase reporter gene construct under the control of nucleotides −1470 to +40 relative to the transcription start site of the *IL-8* gene. Both unstimulated and IL-1β-stimulated luciferase activities were increased by addition of SNAP (Fig. 3), suggesting that the effect of NO on IL-1β-stimulated IL-8 expression at the level of both mRNA and protein was at least partly due to an increase in transcriptional activity.

DISCUSSION

NO has been shown to regulate the expression of IL-8 in several different cell types, but although IL-8 and NO are intensively studied molecules which have been implicated in the regulation of inflammation and other processes in

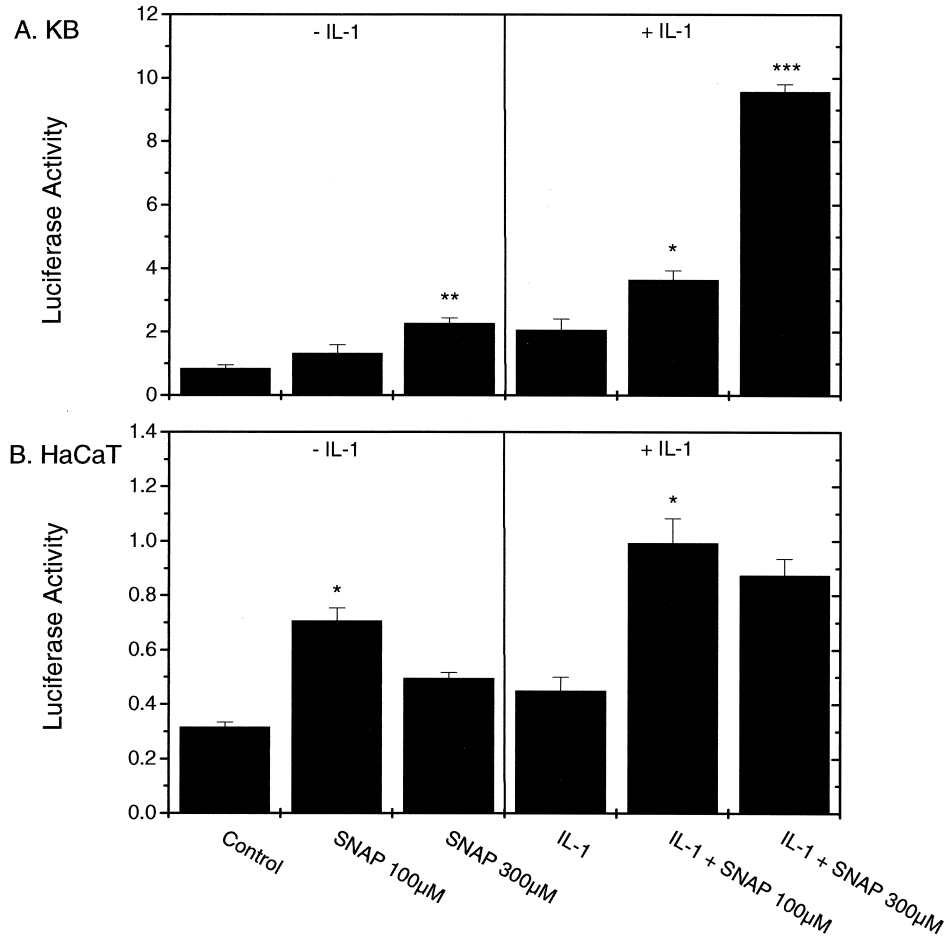

FIG. 3. SNAP up-regulates IL-8 promoter activity in KB and HaCaT cells. Cells transiently transfected with the IL-8 promoter reporter gene construct were treated as indicated for 24 hr before lysis for analysis of luciferase activity. IL-1β was used at a concentration of 100 ng/mL. Data are expressed as the means ± SE of triplicate wells (N = 2). *P < 0.05, **P < 0.01, and ***P < 0.001 compared to the control without SNAP.

the skin, no study has yet shown an effect in keratinocytes. In this study, we have demonstrated that NO up-regulates IL-1β-stimulated IL-8 expression in two keratinocyte-like cell lines as well as in 1°HK. The NO-mediated increases in IL-8 are similar in magnitude to the amounts of IL-8 which have been shown to increase proliferation of HaCaT cells by more than 50% [3], and are therefore likely to be of biological significance. These results parallel our previous observation that NO synergises with TNF-α to upregulate IL-8 in a melanoma cell line [10], but contrast with the down-regulation of IL-1α-stimulated IL-8 in endothelial cells [14], despite the use of similar NO donor concentrations and incubation times. These contrasting results emphasise the cell type-specific nature of the signal transduction pathways which govern the response to NO. Although all three keratinocyte-like cell types used in this study exhibited broadly the same response to NO, differences were found. SNAP alone had no effect in either KB cells or 1°HK, but did induce significant increases in IL-8 expression in HaCaT cells. This difference is likely due to the presence of constitutively produced IL-1, since treatment with IL-1ra reduced the SNAP-induced IL-8 expression to control levels. The effect of SNAP on IL-8 mRNA levels in KB cells was markedly enhanced at 24 hr compared to 6 hr. This increase was not completely reflected in the level of protein, suggesting that SNAP, while enhancing mRNA levels, may be having an inhibitory effect at the posttranscriptional or posttranslational level. The KB cell line therefore appears to possess additional NO-responsive pathways which lead to prolonged and excessive IL-8 mRNA production, although the corresponding expression of protein is prevented in the presence of NO. The reporter gene assay results in this study show clearly that SNAP increases the transcriptional activity of the IL-1β-stimulated IL-8 promoter. The small increase caused by SNAP alone, which was not observed at the mRNA or protein levels in untransfected KB cells, may be due to the presence of small amounts of IL-1 induced by the transfection procedure. Alternatively, it could be attributed to a purely transcriptional effect of SNAP alone in the absence of any posttranscriptional effects on mRNA stability, as described above. Since the involvement of additional posttranscriptional

effects in the presence of IL-1β on IL-8 expression was not excluded, the reason for the discrepancy between mRNA level and protein remains unclear, but could be due to inhibition of IL-8 secretion, or to a block in translation of mRNA, as occurs when IL-1β and TNF-α are transcriptionally up-regulated by TGFβ but at the same time translationally inhibited [24].

The mechanism by which NO increases the expression of inflammatory molecules such as IL-8 has been shown to involve the transcription factor NF-κB [10, 14]. The molecular mechanism has not been shown directly, although it is well known that reactive oxygen intermediates activate the transcription factor [25]. NO donors can also inhibit NF-κB activity by inhibiting both proteolytic degradation of IκB [26], and binding of the factor to DNA [27]. These contrasting results emphasize the cell type-specific dependence of NF-κB regulation. The NF-κB site in the IL-8 promoter is essential for activation by cytokines [28], and we have previously shown that mutation of the site abolishes activation by NO donors [10]. It is therefore likely that NF-κB is also involved in mediating the effects of NO observed in this study in keratinocytes.

Previous reports have shown that NO can affect gene expression in an autocrine manner [10, 13]. Keratinocytes are able to express all three isoforms of NOS [18, 29, 30], depending on the differentiation state of the cell and, in the case of iNOS, upon cytokine stimulation [31, 32]. However, NOS inhibitors were unable to inhibit cytokine-stimulated IL-8 expression in the cells used in this study (data not shown), indicating that endogenously produced NO is not involved. The *in vivo* source of NO may not be the keratinocytes themselves, but a neighbouring or infiltrating cell type. Possible sources include Langerhans cells [33] and monocytes [34], which can be induced to express iNOS upon lipopolysaccharide stimulation and CD23 ligation, respectively.

The demonstration that NO can up-regulate IL-8 expression in keratinocytes, as well as in melanoma cells, may have implications for the understanding of angiogenesis, since, while IL-8 is considered to be a proangiogenic molecule [35] both in a psoriatic setting [6] and in cancer [36], NO can be either angiogenic or angiostatic, a phenomenon which probably depends on the concentrations involved [37]. Any disruption of the balance between pro- and antiangiogenic factors is likely to contribute to a pathogenic alteration. Thus, the modulation of IL-8 expression by NO may be relevant to the regulation of angiogenesis not only in psoriasis but also in wound healing and tumor growth.

The results presented here show that NO can synergise with IL-1β to up-regulate the expression of IL-8 by keratinocytes. Reciprocal up-regulation of the expression of IL-8 and NO could lead *in vivo* to an exacerbation of the inflammatory response, and may contribute to the development of chronic inflammation.

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