Control of growth and differentiation of normal human epithelial cells through the manipulation of reactive nitrogen species

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In this work, we addressed the issue of whether exogenous NO and $ONOO^-$ (peroxynitrite) are able to alter growth, viability and/or differentiation of normal epithelial cells using cultured normal human keratinocytes as a model. 3-Morpholino-sydnonimine (SIN-1), a donor of both NO and $O_2^{-\bullet}$, leading to the production of $ONOO^-$, dose-dependently inhibited growth of human keratinocytes without loss of viability. This inhibitory effect was lowered when SIN-1 was transformed into a pure NO donor by scavenging $O_2^{-\bullet}$ with superoxide dismutase/catalase. Finally, scavenging NO release from SIN-1 with carboxy-1H-imidazol-1-yloxy,2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-3-oxide (PTIO) resulted in a loss of the inhibitory effect of SIN-1. Together these findings suggest that both $ONOO^-$ and NO exert a growth inhibitory effect on human keratinocytes without cytotoxicity. Further support for this conclusion came from the treatment of human keratinocytes with the $NO^\bullet$ donor propanamine 3-(2-hydroxy-2-nitroso-1-propyl hydrazino) or with authentic peroxynitrite. Moreover, only SIN-1 or peroxynitrite, and not NO, was able to trigger the expression of markers of terminal differentiation in human keratinocytes. From a physiological perspective, the ability of peroxynitrite, a known genotoxic and potentially carcinogenic agent, to direct proliferating keratinocytes towards terminal differentiation may be crucial to protect the genomic stability of this barrier epithelium.

INTRODUCTION

Inflammatory stress is associated with enhanced production of NO and NO-related species, including peroxynitrite ($ONOO^-$) resulting from the combination of NO and $O_2^{-\bullet}$. While numerous studies have delineated antiproliferative, cytotoxic or pro-apoptotic effects of NO and NO-related species on several cell types [1–3], very few studies have addressed the effects of these compounds on the biology of human barrier epithelia (keratinocytes, gastro-intestinal cells). Interestingly, several lines of investigation have shown that NO, up to high concentrations, has no direct cytotoxic effect on several epithelial cell types, including intestinal cells [4,5] and retinal pigmental cells [6]. In addition, several epithelial cell lines were found to be remarkably resistant to the cytotoxic action of peroxynitrite [4,7]. These findings, together with the recent demonstration that NO can trigger growth arrest and differentiation of several non-epithelial cell types, including leukaemia and pheochromocytoma cells [8–12], prompted us to examine the effects of NO and $ONOO^-$ on growth, viability and differentiation of an *in vitro* model of barrier epithelial cells.

Skin keratinocytes provide an appropriate model for this kind of study. In fact these cells have the unique advantage of being an experimentally amenable barrier, as they can be maintained as proliferating populations of undifferentiated basal-like cells, which can undergo growth arrest and differentiation when triggered by the appropriate signals.

Here we demonstrate that both exogenous NO and $ONOO^-$ induced a dose-related growth arrest in human keratinocytes without cytotoxic effect. In addition, $ONOO^-$ is able to induce the expression of keratinocyte differentiation markers. These experiments unveil a new biological role for peroxynitrite. From a physiological perspective, the ability of peroxynitrite, a known genotoxic and potentially carcinogenic agent [13], to direct proliferating cells towards terminal differentiation may be crucial to protect the genomic stability of the barrier epithelium.

EXPERIMENTAL

Cell culture and treatment of keratinocytes

Human normal keratinocytes were obtained from foreskins and expanded using a low Ca^{2+} serum-free medium (KSFM medium, Life Technologies) supplemented with 0.5 ng/ml epidermal growth factor (Life Technologies), 25 μg/ml bovine pituitary extract (Life Technologies), 200 units/ml penicillin, 200 μg/ml streptomycin (Boehringer Mannheim) and 0.25 μg/ml fungizone (Bristol-Myers Squibb), according to the technique of Boyce and Ham [14]. After two passages, the cells were seeded into 6-well multi-well plates and grown in keratinocyte growth medium without hydrocortisone (KGM medium, Promocell). Seventy-two hours after seeding, when the cells were in a proliferating state, the following agents were added to the culture medium: 3-morpholinosydnonimine (SIN-1) (1-propanamine, 3-(2-hydroxy-2-nitroso-1-propyl hydrazino) (PAPA NONOate) or peroxynitrite (Cayman Chemical Company, Spibio). In all control experiments, the vehicle alone was added to the incubation medium at the same final concentration as in the treated cells. For experiments designed to scavenge $O_2^{-\bullet}$, superoxide dismutase (SOD, 50-units/ml; Boehringer) and catalase (100 units/ml; Boehringer) were added to the culture medium carboxy-1H-imidazol-1-yloxy,2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-3-oxide. (CPTIO, Cayman Chemical Company, Spibio) was used as an NO scavenger.

Cell growth and cell viability assays

For [^{3}H]thymidine incorporation experiments, the agent to

Abbreviations used: NOS, nitric oxide synthase; $ONOO^-$, peroxynitrite; SIN-1,3-morpholino-sydnonimine; PAPA NONOate, 1-propanamine, 3-(2-hydroxy-2-nitroso-1-propyl hydrazino); SOD, superoxide dismutase; CPTIO, carboxy-1H-imidazol-1-yloxy,2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-3-oxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide; LDH, lactate dehydrogenase; PARP, poly(ADP-ribose) polymerase.

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be tested was added concomitantly with [^{3}H]thymidine and incubation was then for 10 h in 6-well multi-well chambers. Incorporated radioactivity was measured as previously described [15]. For cell counts, cells were treated for 24 h with the agent to be tested, and counted.

Cellular metabolic activity was assessed by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT) to formazan using a commercially available kit (Boehringer-Mannheim). Lactate dehydrogenase (LDH) release was measured using an Enzyline LDH kit (Biomérieux).

Immunofluorescence

For immunofluorescence studies, keratinocytes were plated and cultured on microscope slides with attached multi-well chambers (Sonic Seal Well, Polylabo). After a 24 h incubation with NO donors or peroxynitrite, the slides were fixed in cold acetone. PBS-washed slides were treated with either anti-keratins 5–6 (1:10 dilution; Clone B5-16B4, Boehringer-Mannheim), anti-keratin 10 (1:10 dilution, Clone D6-K10; Dako), or anti-filaggrin (1:30 dilution; Biomedical Technologies Inc.) antibodies, for 30 min at room temperature. After washing with PBS, the slides were treated with FITC-conjugated goat-anti-mouse Fab′$_2$ antibody (1:15 dilution; Caltag, San Franscisco CA, U.S.A.) for 30 min. The slides were washed again, mounted in aqueous medium and examined with a fluorescence microscope. Negative controls included the FITC-conjugated second antibody alone and an antibody of the same isotype.

RESULTS AND DISCUSSION

In a first series of experiments, we studied the effects of SIN-1 on the proliferation of human keratinocytes in culture. In fact, SIN-1 has the unique property of generating $ONOO^-$ by releasing $O_2^{-\bullet}$ and $NO^\bullet$, essentially in a simultaneous manner [16,17]. Therefore, when used in combination with appropriate scavengers, this compound provides a tool to study the differential effects of NO, superoxide and peroxynitrite.

SIN-1 produced a dose-dependent inhibitory effect on [^{3}H]thymidine incorporation in exponentially growing cultures of human keratinocytes with a maximal inhibitory effect at 1 mM SIN-1 (Figure 1) and an IC_{50} of 0.1 mM. In order to decipher the effect of each product released by SIN-1, i.e. NO and $O_2^{-\bullet}$, we added to the medium containing SIN-1 (1 mM), SOD, a scavenger of $O_2^{-\bullet}$ and catalase which eliminated H_2O_2 formed as a result of SOD activity. Under these conditions SIN-1 can be considered as an $NO^\bullet$ donor. As shown in Table 1, addition of SOD/catalase resulted in a dramatic reduction in the

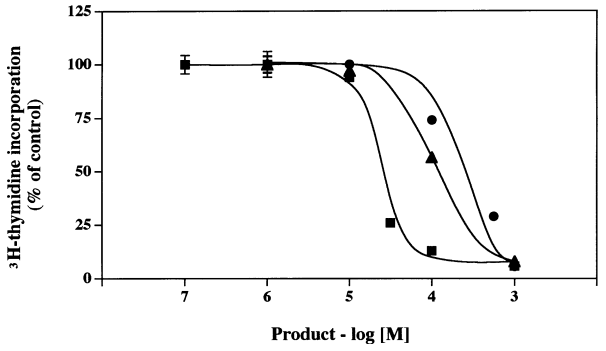

Figure 1 Inhibitory effect of NO and peroxynitrite on [^{3}H]thymidline incorporation by human keratinocytes in culture

Human keratinocytes were incubated with increasing concentrations of SIN-1 (▲), PAPA NONOate (●) and peroxynitrite (■) in the presence of [^{3}H]thymidine (2 μCi/ml) for 10 h. Each point represents the mean ± S.E.M. of 6–9 determinations of [^{3}H]thymidine incorporation from 2–3 separate experiments. Data points without error bars indicate S.E.M. values less than the symbol size.

Table 1 Partial prevention of SIN-1-mediated growth arrest by SOD/catalase

[^{3}H]Thymidine incorporation was determined after 10 h with [^{3}H]thymidine (2 μCi/ml) in the presence of 1 mM SIN-1 or PAPA NONOate alone or with SOD (50 units/ml) and catalase (100 units/ml). Results are expressed as a percentage of the control value. Each point represents the mean ± S.E.M. of 6–9 wells from two experiments. *Significantly different from SIN-1 without SOD/catalase ($P < 0.001$); †significantly different from control with SOD/catalase ($P < 0.001$).

Treatment	[^{3}H]Thymidine incorporation (% of control)	
	Without SOD/catalase	With SOD/catalase
None	100 ± 3	101 ± 3
SIN-1	15 ± 3	75 ± 1*†
PAPA NONOate	34 ± 3	30 ± 1

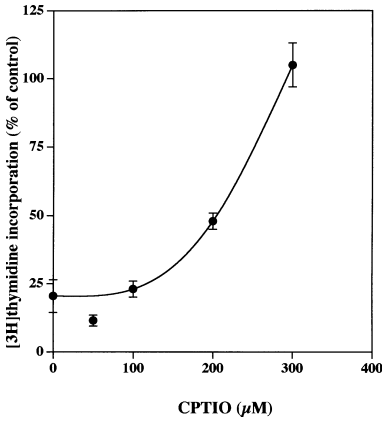

Figure 2 CPTIO dose-dependently prevents SIN-1-mediated growth arrest of human keratinocytes

Human keratinocytes were incubated with increasing concentrations of CPTIO in the presence of 1 mM SIN-1. [^{3}H]Thymidine incorporation was determined as described in the Experimental section. Results are expressed as a percentage of the control (CPTIO alone). Each point represents the mean ± S.E.M. of three determinations.

Table 2 Differential effects of NO and peroxynitrite on keratinocyte adhesion

Human keratinocytes were incubated for 24 h with 1 mM PAPA NONOate or 1 mM SIN-1. Then both adherent cells and cells in suspension were counted. Results are expressed as a percentage of total cells (adherent cells + cells in suspension). Values represent the means ± S.E.M. of six determinations from two separate experiments.

Treatment	Adherent cells (%)	Cells in suspension (%)
None	99.0 ± 0.4	1.0 ± 0.4
SIN-1	80 ± 6	20 ± 6
PAPA NONOate	95 ± 1	5 ± 1

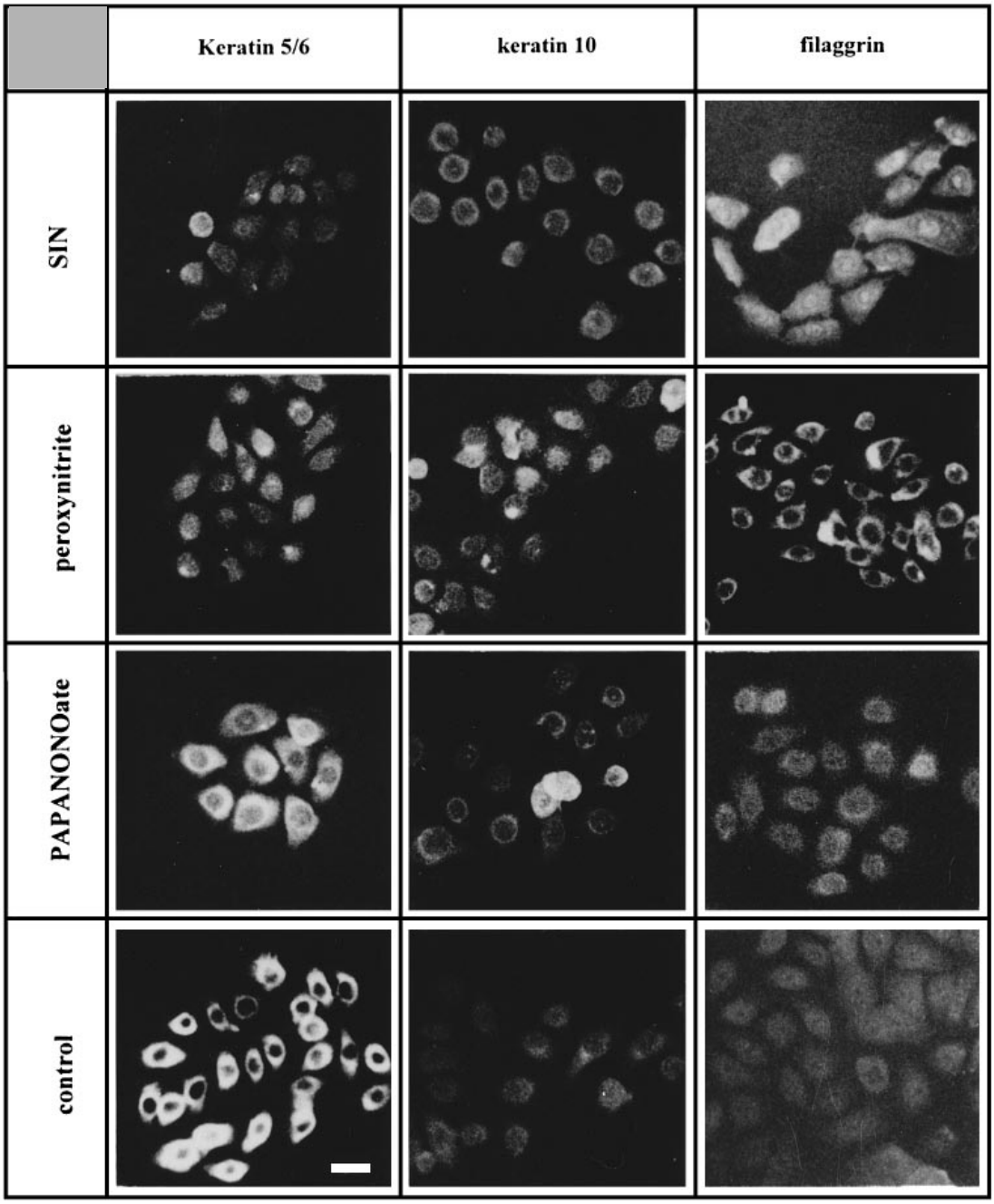

Figure 3 Effects of NO and peroxynitrite or keratinocyte differentiation

Keratinocytes were seeded at low density on microscope slides with attached multi-well chambers and treated during the exponential phase of growth with various agents at the indicated concentrations for 24 h. Immunofluorescence staining of cultured cells was performed with antibodies specific for proliferation-associated or differentiation-associated markers, as described in the Experimental section. The scale bar represents 50 μm.

inhibitory effect of 1 mM SIN-1 on [^{3}H]thymidine incorporation. In the presence of SOD/catalase SIN-1 still had a weak but significant inhibitory effect on [^{3}H]thymidine incorporation, predicting that NO could alter the growth rate of keratinocytes. To test this hypothesis we determined the effect of PAPA NONOate, a pure NO donor, on [^{3}H]thymidine incorporation. PAPA NONOate dose-dependently inhibited the [^{3}H]thymidine incorporation of human normal keratinocytes with an IC_{50} of 0.45 mM and a maximal effect at 1 mM (Figure 1). To rule out the possibility that the inhibitory effect of PAPA NONOate resulted from the recombination of NO with endogenous $O_2^{-\cdot}$ leading to $ONOO^-$, we showed that the addition of SOD/catalase to PAPA NONOate-treated cells did not alter the growth inhibitory effect of this NO donor (Table 1). Interestingly, PAPA NONOate, employed at a concentration (200 μM) that released the same amount of NO_2^-/NO_3^- into the medium using the Griess assay [18] as did 1 mM SIN-1 + SOD/catalase, had a similar inhibitory effect on [^{3}H]thymidine incorporation. To examine

Table 3 Quantitative evaluation of the effects of NO and peroxynitrite on keratinocyte differentiation

Keratinocytes were cultured as described in Figure 3. Quantitative evaluation of expression of proliferation-associated markers (keratins 5–6) and differentiation-associated markers (keratin 10 and filaggrin) took into account: (1) the percentage of cells scoring positive with the indicated antibody (mean ± S.E.M. of cell counts performed on four successive fields per slide at a ×40 magnification; two slides for each experimental condition; two independent experiments); and (2) the intensity of fluorescence evaluated as ± (weak fluorescence), + (moderate fluorescence) or ++ (strong fluorescence).

	Keratins 5–6	Keratin 10	Filaggrin
Control	85 ± 2.5 (++)	0	0
PAPA NONOate (1 mM)	80 ± 7.5 (+)	80 ± 7 (±)	0
Peroxynitrite (0.1 mM)	63 ± 9.5 (±)	58 ± 11 (++)	71 ± 7 (++)
SIN-1 (1 mM)	0	77 ± 2 (+)	81 ± 9 (++)

whether $O_2^{-\bullet}$ can affect the growth of human keratinocytes, CPTIO, a compound designed to trap NO [19], was added to the incubation medium in the presence of SIN-1. As shown in Figure 2, CPTIO dose-dependently relieved the inhibitory effect of 1 mM SIN-1. The inhibitory effect of 1 mM SIN-1 was completely abolished by 300 μM CPTIO, a finding showing that $O_2^{-\bullet}$ released by 1 mM SIN-1 does not affect the growth rate of keratinocytes.

Finally we examined the effects of directly adding peroxynitrite to the culture medium. Peroxynitrite dose-dependently inhibited [^{3}H]thymidine incorporation by human keratinocytes with an IC_{50} of 0.045 mM and had a maximal effect at 0.1 mM (Figure 1). Together, these experiments clearly show that both peroxynitrite and NO exert a growth inhibitory effect on human keratinocytes.

Cell counts performed after a 24 h incubation with 1 mM PAPA NONOate or SIN-1 confirmed that the cells underwent growth arrest (results not shown). SIN-1 induced the detachment of a fraction of the cultured cells (Table 2). Moreover, the non-adherent cells were responsible for 25 ± 10% of the transformation of MTT into formazan measured in the entire cell population (adherent + non-adherent cells), showing that SIN-1-induced cellular detachment did not impair cell viability. The absence of LDH release (results not shown) upon treatment with both PAPA NONOate and SIN-1 confirmed that these agents did not exert any acute cytotoxic effect. Finally, apoptotic cell death was assessed using two assays, i.e. nuclear staining with Hoechst 33258 and DNA laddering after a 24 h incubation. The results ruled out the possibility that SIN-1 or PAPA NONOate induced an apoptotic process in this culture (results not shown).

Thus, in the absence of any cytotoxicity or apoptotic effect, the emerging picture is that of a cytostatic effect of NO and of the NO-related compound peroxynitrite.

As arrest of cell division is a prerequisite for cells to enter differentiation, we examined whether NO and/or peroxynitrite were able to induce differentiation of keratinocytes. To this end, exponentially-growing human keratinocytes were maintained for 24 h after addition of SIN-1, PAPA NONOate or peroxynitrite, and the cells were tested by immunocytochemistry for the expression of a family of proliferation- or differentiation-specific proteins, i.e. keratins 5–6, characteristic of proliferating undifferentiated basal cell-like keratinocytes, keratin 10, expressed by spinous cells, and filaggrin which is considered as a late differentiation marker, expressed in the granular layers [20]. Results are shown in Figure 3 and Table 3. As expected from proliferating keratinocytes, exponentially growing control cultures expressed solely keratins 5–6, whereas keratin 10 and filaggrin remained undetectable by immunocytochemistry. The response of keratinocytes to PAPA NONOate consisted in an uninterrupted expression of the marker keratin 5–6, while a very weak expression of keratin 10 was observed. Filaggrin, a late differentiation marker, remained unexpressed.

The cellular response to SIN-1 resulted in coordinate changes consisting in a strong inhibition of keratin 5–6 expression coupled with the emergence of early (keratin 10) and late (filaggrin) markers of differentiation. Treatment with 0.1 mM peroxynitrite resulted in essentially the same pattern of differentiation marker expression (albeit with a less marked inhibitory effect on keratin 5–6 expression).

Together these results suggest a model for NO and $ONOO^-$ action on keratinocytes, in which two actions can be outlined. First, NO is able to switch growing keratinocytes to the cytostatic phase without inducing late differentiation markers. A second kind of action is generated by peroxynitrite which, in addition to inhibiting cell growth, is able to trigger the expression of markers of terminal differentiation. Our findings raise the issue of the underlying mechanisms. From this perspective, it is worth considering a pathway involving DNA single-strand breakage by peroxynitrite and consequent activation of poly(ADP-ribose) polymerase (PARP) [21]. In fact this hypothesis is supported by the recent demonstration that PARP-deleted mice are susceptible to the development of epidermal hyperplasia in response to environmental stress [22]. Further studies are needed to explore this issue.

The potential significance of our results can be considered in relation to the finding that NO and peroxynitrite are produced within the skin in response to various kinds of stress. Peroxynitrite is produced by activated human leukocytes in the vicinity of keratinocytes during inflammatory stress [23]. In addition, keratinocytes themselves are able to produce both NO and $ONOO^-$ through the activation of NOS and xanthine oxidase upon stimulation by UV light [24]. Commitment to differentiation by $ONOO^-$ could provide a mechanism by which proliferating keratinocytes prevent the deleterious consequences of a genotoxic attack that otherwise could eventually lead to a malignant transformation.

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