RESEARCH COMMUNICATION

A redox-based mechanism for induction of interleukin-1 production by nitric oxide in a human colonic epithelial cell line (HT29-Cl.16E)

Geneviève VALLETTE*, Anne JARRY, Jean-Eric BRANKA and Christian L. LABOISSE

Groupe de Recherche 'Fonctions Sécrétoires des Epithéliums Digestifs', INSERM CJF 94-04, Faculté de Médecine, 1 Rue Gaston Veil, 44035 Nantes, France

We evaluated the effects of two NO donors, sodium nitroprusside (SNP) and 3-morpholino-sydnominine (SIN-1), characterized by alternative redox states, i.e. nitrosonium ion (NO^+) and nitric oxide ($\text{NO}^\bullet$) respectively, on intracellular interleukin-1 (IL-1) production, by a human colonic epithelial cell line (HT29-Cl.16E). SNP was able to induce intracellular IL-1 α production up to 10 h incubation, in a dose-dependent manner. Several experiments provide evidence that the NO^+ redox form, and not the free radical $\text{NO}^\bullet$, is implicated in the IL-1 α production: (i) SIN-1, devoid of any NO^+ character, led to a very weak IL-1

production as compared with SNP; (ii) the reductive action of a thiol such as cysteine on NO^+ led to a dose-dependent increase in $\text{NO}^\bullet$ concentration, measured as $\text{NO}_2^-/\text{NO}_3^-$ accumulation, and to a large decrease in IL-1 production. Dibutyryl cGMP had no effect on IL-1 production, this finding supporting the concept that a cGMP-independent pathway is involved in the intracellular signalling of NO^+ . Together these results point out that NO , depending on its redox form, is able to modulate IL-1 production in cultured colonic epithelial cells.

INTRODUCTION

Several lines of investigation have recently suggested novel functions for intestinal epithelial cells (IEC). Under certain conditions these cells have phenotypic characteristics of antigen-presenting cells, including presentation of peptides and proteins to immune cells [1], and secretion of pro-inflammatory cytokines such as interleukin (IL)-8 in response to bacterial antigens or lipopolysaccharide [2]. Among the pro-inflammatory cytokines that play a major role in triggering an inflammatory/immune response, IL-1 has been reported to be secreted by several epithelial cell types [3–7]. However, whether this cytokine is produced by IEC is currently under investigation. While the issue of the constitutive production of IL-1 by IEC remains highly debated, there is growing evidence that IL-1 production is induced in these cells under inflammatory conditions. The evidence includes increased IL-1 mRNA expression by enterocytes in an experimental model of rat colitis [8], and increased immunoreactive IL-1 in IEC isolated from patients with inflammatory bowel disease [7].

Based on these findings, interest is centring now on the way in which IL-1 production is induced in IEC during inflammation. In this work, we have focused on the effects of NO on IL-1 synthesis because several studies point to NO as an intercellular mediator produced by activated inflammatory/immune cells [9], which exhibits pleiotropic effects, including a regulatory role in the production and/or release of IL-8, IL-6 and tumour necrosis factor α by several cell types, including endothelial cells [10] and macrophages [11,12].

The ideal approach to evaluating the effects of NO on IL-1 synthesis would involve treating isolated colonic crypt cells maintained in culture with NO donors. However, there are some major drawbacks currently encountered concerning primary cultures of human normal colonocytes that are linked to (1) the poor viability of these cells, and (2) the possible contamination of colonocyte cultures by *lamina propria* inflammatory/immune cells.

Based on this rationale, we devised a reductionist experimental approach based on the evaluation of the effects of several NO donors in a differentiated human colonic epithelial cell line (HT29-Cl.16E), which expresses *in vitro* specialized functions that are close to those of human colonocytes *in vivo* [13–16]. Altogether, our results demonstrate that, depending on its redox state, NO is able to induce IL-1 production in the colonic epithelial cell line HT29-Cl.16E.

MATERIALS AND METHODS

Reagents

Sodium nitroprusside (SNP), 3-morpholino-sydnominine (SIN-1), cysteine, dibutyryl guanosine 3',5'-cyclic monophosphate (dbcGMP) and *N*^G-methyl-L-arginine (NMMA) were obtained from Sigma.

Cell culture

HT29-Cl.16E cells were maintained as monolayers of polarized cells by plating the cells on filters mounted in chamber cultures (Millicell culture plate insert, 0.45 μm porosity, 1.2×10^6 cells per chamber; Millipore), which delineate an apical and a serosal bathing medium. These cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco), supplemented with 10 % heat-inactivated fetal-calf serum (Gibco), for 8–10 days before the experiment. At this time point, the integrity of the confluent polarized monolayers was determined by measuring trans-epithelial membrane resistance with a volt-ohmmeter (Millicell ERS; Millipore). The NO donors were then added to both apical and basolateral media at the concentrations mentioned; the supernatant was removed at various time points and stored at -20°C . Cell lysates were obtained by submitting the chamber-grown cells to three cycles of freezing and thawing in 2 ml of Ham F12/DMEM supplemented with anti-proteases (leupeptin

Abbreviations used: dbcGMP, dibutyryl guanosine 3',5'-cyclic monophosphate; DMEM, Dulbecco's modified Eagle's medium; IEC, intestinal epithelial cells; IL-1, interleukin-1; LDH, lactate dehydrogenase; NMMA, *N*^G-methyl-L-arginine; SIN-1, 3-morpholino-sydnominine; SNP, sodium nitroprusside.

* To whom correspondence should be addressed.

and aprotinin, 2 $\mu\text{g}/\text{ml}$ each; Sigma), and centrifuging. The supernatant was separated into aliquots and frozen at -20°C until use.

Cell viability

Cell viability was assessed by measuring lactate dehydrogenase (LDH) activity in both the supernatant and cell lysate, using a standardized kinetic determination (LDH Enzyline Kit; Bio-mérieux). Cell lysis was expressed as the percentage of LDH released, which equalled the activity of extracellular LDH divided by the activity of (extracellular + intracellular) LDH.

IL-1 α and IL-1 β ELISA

The production of intracellular IL-1 α or IL-1 β was measured in cell lysates by a sensitive and specific ELISA (R&D Systems). Results were expressed as $\text{pg}/10^6$ cells.

Measurement of NO $_2^-$ /NO $_3^-$ levels

Extracellular nitrite and nitrate levels were measured as nitrite after enzymic conversion of nitrate into nitrite by nitrate reductase, using a spectrophotometric assay (Cayman Chemicals) based on the Griess reagent [17]. The absorbance at 540 nm was measured using an Argus 400 microplate reader (Packard Instrument).

Statistical analysis

Data are expressed as means $\pm$ S.E.M. of several experiments, with at least three monolayers per experiment. The significance of differences was assessed by a Student's *t* test.

RESULTS

We evaluated the effects of compounds characterized by alternative redox states, i.e. nitrosonium ion (NO $^+$) and nitric oxide (NO $^\bullet$), on intracellular IL-1 α and/or IL-1 β production by HT29-Cl.16E cells. To this end, SNP, a nitroso compound with strong NO $^+$ character, and SIN-1, an agent generating NO $^\bullet$, were added to chamber-grown HT29-Cl.16E cells.

As shown in Figure 1, the addition of 1 mM SNP to chamber-

Figure 1 Kinetics of intracellular IL-1 α accumulation in HT29-Cl.16E cells stimulated by SNP

Chamber-grown HT29-Cl.16E monolayers were incubated in the presence of 1 mM SNP. Results are expressed as means $\pm$ S.E.M. of nine monolayers from three separate experiments. Data points without error bars indicate S.E.M. values less than the symbol size.

Table 1 Effect of transient exposure to SNP on IL-1 α synthesis in HT29-Cl.16E cells

HT29-Cl.16E monolayers were exposed to 1 mM SNP for various periods of time and then returned to standard medium prior to IL-1 α measurement at 10 h. Results are means $\pm$ S.E.M. of three monolayers.

Treatment	Incubation time	IL-1 α (pg/10 ⁶ cells)
Control		2.2 $\pm$ 0.03
SNP (1 mM)	15 min	2.8 $\pm$ 0.15
	30 min	2.6 $\pm$ 0.1
	1 h	2.8 $\pm$ 0.1
	2 h	4.3 $\pm$ 0.15*
	5 h	8.4 $\pm$ 0.2*
	10 h	14.5 $\pm$ 1.5*

* $P \leq 0.002$ versus control.

Figure 2 Dose-dependent effect of NO donors (SNP and SIN-1) on intracellular IL-1 α production by HT29-Cl.16E cells

Monolayers were treated for 10 h with the indicated concentrations of SNP (●) or SIN-1 (○). Results are expressed relative to the baseline value of IL-1 α production (1.1 $\text{pg}/10^6$ cells). Results are means $\pm$ S.E.M. of six monolayers from two separate experiments for SNP and SIN-1. Data points without error bars indicate S.E.M. values less than the symbol size.

grown HT29-Cl.16E cells elicited an increase in IL-1 α production, with an onset at 4 h and a maximum at 8–10 h of incubation. This IL-1 α production was restricted to cell lysates and was never found in the supernatants. In these experimental conditions, SNP had no effect on IL-1 β production.

To examine whether a continuous exposure to SNP was necessary to induce IL-1 α , HT29-Cl.16E cells were incubated for various periods of time in the presence of SNP, and then returned to standard medium. IL-1 measurement was performed at 10 h. As shown in Table 1, 2 h was the minimum incubation time required to induce a significant increase in IL-1 at 10 h, while a continuous exposure to SNP was necessary to obtain the maximal stimulatory effect.

IL-1 α production induced by SNP was dose dependent up to 1 mM SNP (Figure 2). At these concentrations, SNP did not alter transepithelial monolayer resistance and did not induce cell cytotoxicity, as assessed by the measurement of LDH activity in the supernatant. However, beyond 1 mM SNP, the production of IL-1 dramatically fell, this effect being concomitant with a drop in the resistance of monolayers [ΔR of cell monolayers at 5 mM SNP was $15 \pm 1.5 \Omega \cdot \text{cm}^2$, significantly lower than for control monolayers ($\Delta R = 220 \pm 4 \Omega \cdot \text{cm}^2$) ($P < 0.001$, $n = 3$)] without cell death as measured by LDH assay.

Table 2 $\text{NO}_2^-/\text{NO}_3^-$ accumulation in incubation media of HT29-Cl.16E cells treated with SNP or SIN-1

Chamber-grown HT29-Cl.16E monolayers were treated with 1 mM SNP or 1 mM SIN-1 for 10 h. $\text{NO}_2^-/\text{NO}_3^-$ accumulation was measured in the supernatants with the Griess reagent as described in the Materials and methods section. Results are means $\pm$ S.E.M. of three to six monolayers.

Treatment	$\text{NO}_2^-/\text{NO}_3^-$ (μM)
Control	2.4 ± 0.3
SNP	15 ± 0.2
SIN-1	234 ± 7

Table 3 Effect of cysteine on SNP-induced IL-1 α production and $\text{NO}_2^-/\text{NO}_3^-$ accumulation

Chamber-grown HT29-Cl.16E monolayers were treated with 1 mM SNP with or without various concentrations of cysteine for 10 h. Results are means $\pm$ S.E.M. of three to six monolayers.

Cysteine (mM)	IL-1 α (% of maximal response)	$\text{NO}_2^-/\text{NO}_3^-$ (μM)
0	100	11 ± 0.7
0.05	54 ± 6	14 ± 0.8
0.2	54 ± 2	23 ± 0.7
0.5	17 ± 1	48 ± 2

Finally, to rule out the possibility that SNP-induced IL-1 α production was due to an indirect effect through the stimulation of intracellular NO synthase, we incubated the cells in the presence of the NO synthase inhibitor NMMA (500 μM). In these conditions, NMMA did not affect SNP-induced IL-1 α production (results not shown).

In contrast with the findings with SNP, SIN-1 was found to be a very weak inducer of IL-1 accumulation even at concentrations up to 5 mM (Figure 2). When measuring the accumulation of NO as its end products $\text{NO}_2^-/\text{NO}_3^-$, we observed that SIN-1 released 16 times as much $\text{NO}_2^-/\text{NO}_3^-$ as SNP did (Table 2). Moreover, SIN-1 altered neither the transepithelial monolayer resistance nor cell viability (results not shown). Together these findings strongly suggest that the NO^+ character of SNP is responsible for IL-1 α production.

To prove more directly that manipulation of the NO redox state influences its effect on IL-1 production, we measured both IL-1 production and $\text{NO}_2^-/\text{NO}_3^-$ accumulation after treatment with SNP under reducing conditions with a thiol compound such as cysteine, which, by a reductive activation, gives NO^+ ($\text{NO}^+ \xrightarrow{e^-} \text{NO}^*$) [16]. The addition of cysteine to the incubation medium led to a dose-dependent (1) decrease in SNP-induced IL-1 production (Table 3) and (2) increase in $\text{NO}_2^-/\text{NO}_3^-$ accumulation (Table 3). To test whether cysteine was able to alter the functional integrity of the polarized monolayer and/or cell viability, we measured both the transepithelial resistance and LDH release. None of these parameters was affected by the presence of cysteine (results not shown).

Interestingly, as the reduction of nitroprusside generates both NO and cyanide, it is clear from the results of the experiments shown in Table 3 that cyanide is not involved in the induction of IL-1 by SNP (negative control).

Finally, these foregoing experiments predict that the NO-cGMP pathway is not involved in SNP-induced IL-1 production. In fact, the addition of dbcGMP to the culture

medium did not induce any significant IL-1 production over a 10 h period (IL-1 level at 1 mM dbcGMP was 1.3 ± 0.08 pg/10⁶ cells; control level was 1.7 ± 0.08 pg/10⁶ cells; $n = 3$).

DISCUSSION

Recent investigations of NO biochemistry show that this molecule exists as several inter-related redox forms: free radical nitric oxide NO^* , nitrosonium cation NO^+ and nitroxyl anion NO^- , with distinctive properties and biological functions [18–21]. For example, it has been demonstrated that, depending on the redox form delivered in neuronal tissue, NO could exert a cytotoxic or cytoprotective action [18]. Finally, given the variety of (1) the redox forms of NO and (2) the potential intracellular targets, several intracellular pathways could account for NO biological effects.

Our results demonstrate that, depending on its redox forms, NO is able to induce the production of the pro-inflammatory cytokine IL-1 α in a human colonic epithelial cell line (HT29-Cl.16E). Indeed, SNP, a nitroso compound with a strong NO^+ character, induced a time-related intracellular IL-1 α production in a dose-dependent manner up to a 1 mM concentration. A higher concentration of SNP (5 mM) led to a drop in IL-1 production and a concomitant loss of transepithelial monolayer resistance. These two effects can be traced back to the metabolic stress induced by a very high dose of SNP, an effect recently described by Salzman et al. [22], which includes a dramatic loss in intracellular ATP and increased permeability of the tight junctions.

It is to be noted that the accumulation of IL-1 α in the presence of SNP was exclusively intracellular. As IL-1 is a protein without a signal peptide that accumulates in the cytosol, it is generally assumed that its release into the extracellular medium depends on cell death [23], a process that did not occur in our experimental conditions.

Different experimental approaches show that SNP-induced IL-1 α production was due to the NO^+ redox form. (1) SIN-1, another NO donor, which at the same concentration of SNP released 16 times as much NO as SNP did, did not induce significant IL-1 α production. (2) The inhibition of the SNP-induced IL-1 production in the presence of cysteine was due to the reductive activation of NO^+ by thiol. This is confirmed by an increase in the concentration of end products of NO ($\text{NO}_2^-/\text{NO}_3^-$) in the extracellular medium. (3) Finally, the absence of an effect of dbcGMP, a membrane-permeant and non-hydrolysable form of cGMP, on IL-1 α production shows that the classical NO/guanylate cyclase/cGMP pathway is not involved.

Finally, it was also important to rule out the possibility that IL-1 induction could be due to (1) cyanide release by SNP and (2) the formation of peroxynitrite. First, the release of CN^- , resulting from the reduction of SNP by thiol [18,19,24], was ineffective in inducing IL-1. Secondly, the fact that SIN-1, a product that generates both NO and O_2^- , resulting in the formation of peroxynitrite, was a very poor inducer of IL-1 rules out the possible involvement of peroxynitrite in IL-1 induction.

Our findings could have an important biological implication in so far as NO^+ is available to epithelial cells. First, a variety of nitroso compounds formed in physiological conditions can be viewed as NO^+ carriers. Examples of such compounds are thionitrites and nitrosamines [19,21]. In addition, many of the metal nitrosyl complexes that form in the cytokine-treated cells are now considered as NO^+ donors [19,25]. These data argue that NO^+ donors are being produced during inflammatory conditions.

Finally, the mechanism by which NO^+ results in IL-1 production in HT29-Cl.16E cells remains to be elucidated. A

potential pathway is that NO⁺, through protein nitrosylation, could activate transcription factor(s), and thus initiate IL-1 production. Further work is needed to explore this point.

We gratefully thank Mrs. M. Robard for skilful technical assistance. This work was supported in part by the Association pour la Recherche sur le Cancer (A.R.C.) and by the Conseil Régional des Pays de la Loire.

REFERENCES

- 1 Panja, A. and Mayer, L. (1994) in *Handbook of Mucosal Immunology* (Ogra, P. L., Strober, W., Mestecky, J., Mcghee, J. R., Lamm, M. E. and Bienenstock, J., eds.), pp. 177–183, Academic Press, New York
- 2 Eckmann, L., Kagnoff, M. F. and Fierer, J. (1993) *Infect. Immun.* **61**, 4569–4574
- 3 Mattoli, S. F., Colotta, G., Fincato, M., Mezetti, M., Mantovani, A., Patalano, F. and Fasoli, A. (1991) *J. Cell Physiol.* **149**, 260–266
- 4 Jaffe, G. J., Van Le, L., Valea, F., Haskill, S., Roberts, W., Arend, W. P., Stuart, A. and Peters, W. (1992) *Exp. Eye Res.* **55**, 325–335
- 5 Stadnyk, A. W. (1994) *FASEB J.* **8**, 1041–1047
- 6 Corradi, A., Franzi, A. T. and Rubbartelli, A. (1995) *Exp. Cell Res.* **217**, 355–362
- 7 Casini-Raggi, V., Kam, L., Chong, Y. J. T., Fiocchi, C., Pizarro, T. T. and Cominelli, F. (1995) *J. Immunol.* **154**, 2434–2440
- 8 Radema, S. A., Van Deventer, S. J. H. and Cerami, A. (1991) *Gastroenterology* **100**, 1180–1186
- 9 Schmidt, H. H. W. and Walter, U. (1994) *Cell* **78**, 919–925
- 10 Villarete, L. H. and Remick, D. G. (1995) *Biochem. Biophys. Res. Commun.* **211**, 671–676
- 11 Marcinkiewicz, J., Grabowska, A. and Chain, B. (1995) *Eur. J. Immunol.* **25**, 947–951
- 12 Deakin, A. M., Payne, A. M., Whittle, B. J. R. and Moncada, S. (1995) *Cytokine* **7**, 408–416
- 13 Augeron, C. and Labois, C. L. (1984) *Cancer Res.* **44**, 3961–3969
- 14 Augeron, C., Voisin, T., Maoret, J. J., Berthon, B., Laburthe M. and Labois, C. L. (1992) *Am. J. Physiol.* **262**, G470–G476
- 15 Jarry, A., Merlin, D., Velcich, A., Hopfer, U., Augenlicht, L. H. and Labois, C. (1994) *Eur. J. Pharmacol. Mol. Pharmacol. Sect.* **267**, 95–103
- 16 Jarry, A., Vallette, G., Branka, J.-E. and Labois, C. (1996) *Gut*, in the press
- 17 Green L., Wagner, D., Glogowski, J., Skipper, P. L., Wishnok, J. S. and Tannenbaum, S. R. (1982) *Anal. Biochem.* **126**, 131–138
- 18 Lipton, S. A., Chol, Y. B., Pan, Z. H., Sucher, N. S., Loscalzo, J., Singel, D. J. and Stamler, J. S. (1993) *Nature (London)* **364**, 626–634
- 19 Stamler, J. R., Singel, D. J. and Loscalzo, J. (1992) *Science* **258**, 1898–1902
- 20 Zhang, J., Dawson, V. L., Dawson, T. M. and Snyder, S. H. (1994) *Science* **263**, 687–689
- 21 Arnelle, D. R. and Stamler, J. S. (1995) *Arch. Biochem. Biophys.* **318**, 279–285
- 22 Salzman, A. L., Menconi, M. J., Unno, N., Ezzell, R. M., Casey, J. M., Gonzalez, P. K. and Fink, M. P. (1995) *Am. J. Physiol.* **268**, G361–G373
- 23 Hogquist, K. A., Nett, M. A., Unanue, E. R. and Chaplin, D. D. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 8485–8489
- 24 Bates, J. N., Baker, N. T., Guerra, R. and Harrison, D. G. (1991) *Biochem. Pharmacol.* **42**, S162–S165
- 25 Corbett, J. A., Lancaster, J. R., Sweetland, J. M. A. and Mc Daniel, M. C. (1991) *J. Biol. Chem.* **266**, 21351–21354