Stimulation of mucin exocytosis from human epithelial cells by nitric oxide: evidence for a cGMP-dependent and a cGMP-independent pathway

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The aim of this work was to investigate the role of nitric oxide (NO) on the macromolecular exocytotic function of human epithelial cells. We tested the effects of two NO-generating drugs, i.e. 1-hexanamine 6-(2-hydroxy-1-methyl-2-nitrosohydrazine)-*N*-methyl (MAHMA NONOate) and sodium nitroprusside (SNP), on mucin exocytosis from the human colonic epithelial HT29-Cl.16E cell line. Our results show that MAHMA NONOate and SNP elicited a rapid mucin exocytotic response through a cGMP-dependent and a cGMP-independent pathway respectively. Indeed, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxaline-1-one (ODQ), a newly available specific inhibitor of soluble guanylate cyclase, inhibited both cGMP accumulation and subsequent mucin exocytosis evoked by MAHMA NONOate. By contrast, SNP

did not alter intracellular cGMP levels, and SNP-mediated mucin exocytosis was not inhibited by ODQ. As expected from two NO donors acting through distinct pathways, the combined action of MAHMA NONOate and SNP led to an additive effect on mucin exocytosis. SNP was likely to act through S-nitrosylation of a cellular target, because cysteine, a reductive thiol that provides decoy targets for SNP through the formation of nitrosocysteine, abolished the early stimulatory effect of SNP on mucin exocytosis. Finally, the fact that in the presence of cysteine SNP was able to trigger a late, ODQ-inhibitable, mucin exocytotic response demonstrates the ability of NO to shift its intracellular signalling pathway depending on the changes of the redox state of the milieu.

INTRODUCTION

The intestinal epithelial barrier consists of a continuous monolayer of polarized, highly specialized cells capable of vectorially secreting water, ions and macromolecules (mucins) [1]. The mucin exocytotic function of the intestinal mucous cells, the so-called goblet cells, is regulated by neurocrine, endocrine, paracrine and immune pathways acting on specific receptors on epithelial cells [2]. Several lines of investigation tend to show that various neuro-immune mediators produced in the environment of the epithelial barrier during inflammation can stimulate the mucin exocytotic function of goblet cells [3–5]. The fact that NO is produced in the environment of the epithelial barrier under normal conditions and in inflammatory states, provides a rationale for examining the effects of this mediator on the mucin exocytotic function of goblet cells. Whereas recent experiments have suggested that NO is able to stimulate the secretion of mucins from the rat gastrointestinal tract [6] there is no evidence to date of whether this mediator can affect directly the secretory function of human mucous cells. In this work we took advantage of a differentiated human mucus-secreting epithelial cell line, the so-called HT29-Cl.16E cell line [7], to directly examine the effects of NO donors on human goblet cells. Our results show that (1) NO triggers an exocytotic response from HT29-Cl.16E cells, and (2) NO-stimulated exocytosis is mediated by two signalling pathways, each related to a distinct redox form of NO. In addition, our results illustrate a singular property of NO that resides in its ability to shift its intracellular signalling pathway depending on a change of the redox state of the milieu, a property that is relevant to the biology of epithelial cells during inflammation.

MATERIALS AND METHODS

Reagents

D-[6-³H]Glucosamine (specific radioactivity 20 to 40 Ci/mmol) was from Amersham (Paris, France), sodium nitroprusside (SNP), cysteine and BSA were from Sigma Chemicals (Cogen, Paris, France), superoxide dismutase (SOD) and catalase from Boehringer Mannheim (Meylan, France), 1-hexanamine 6-(2-hydroxy-1-methyl-2-nitrosohydrazine)-*N*-methyl (MAHMA NONOate) was from Cayman Chemical Company (Spi Bio, Gif sur Yvette, France), and 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxaline-1-one (ODQ) was purchased from Tocris Cookson (Bristol, U.K.).

Cell culture

The HT29-Cl.16E cell line, a well characterized, polarized, human colonic mucus-secreting cell line [7], was routinely grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with heat-inactivated 10% (v/v) fetal-calf serum (FCS; Gibco). For the experiments, HT29-Cl.16E cells were maintained as monolayers of polarized cells by plating the cells on filters mounted in chamber cultures (culture plate insert, Costar; 0.4 μm porosity, 1.2 × 10⁶ cells per chamber). The monolayers were maintained in DMEM/FCS for 8–10 days before the stimulation of mucin exocytosis. At this time point, the integrity of the confluent polarized monolayers was checked by measuring transepithelial membrane resistance with a volt-ohmmeter (Millicell ERS, Millipore).

Abbreviations used: MAHMA NONOate, 1-hexanamine 6-(2-hydroxy-1-methyl-2-nitrosohydrazine)-*N*-methyl; SNP, sodium nitroprusside; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxaline-1-one; SOD, superoxide dismutase; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; F12, Ham's F12 medium; IBMX, 3-isobutyl-1-methylxanthine; sGC, soluble guanylate cyclase; ROS, reactive oxygen species; IL-1, interleukin-1; VIP, vasoactive intestinal peptide; LY 83583, 6-anilinoquinoline-5,8-dione.

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Stimulation of mucin exocytosis by NO donors and measurement of secretory mucins

Secretory mucins were quantified using a previously described electrophoretic assay [8]. Briefly, confluent monolayers of HT29-Cl.16E cells were labelled with 8 $\mu\text{Ci/ml}$ D-[6- ^3H]glucosamine in DMEM plus 10% FCS over 24 h. The labelled filters were washed three times with serum-free medium consisting of a 1:1 mixture of DMEM and Ham's F12 medium (F12; Gibco) plus 0.01% (w/v) BSA (BSA/F12/DMEM). The cells were then exposed at 37 °C to NO donors, added at the concentrations mentioned to both apical and basolateral media, in BSA/F12/DMEM. At the indicated time points, the medium was removed and the monolayer rinsed with additional BSA/F12/DMEM to remove adherent mucins. The mucin-containing medium was dialysed at 4 °C for 36 to 48 h against several changes of deionized water and subsequently freeze-dried. The secretory glycoproteins were separated by SDS/PAGE on 3% gels. The band at the stacking/running gel interface, which contains the mucins, was cut out, placed into a borosilicate vial, and incubated overnight at 40 °C in the presence of 0.5 ml of Soluene 350. Radioactivity was determined by liquid-scintillation counting. When tested, the compound ODQ was added to both apical and basolateral media at the concentrations mentioned, 30 min before the effector agent. SOD (250 units/ml) and catalase (100 units/ml) were added to both apical and basolateral media when SNP was used.

Measurement of cGMP concentration

Filter-grown HT29-Cl.16E cells were incubated for 30 min with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; 1 mM) before the addition of SNP (1 mM) or MAHMA NONOate (1 μM) or vehicle alone (control). When used, the specific inhibitor of soluble guanylate cyclase (sGC), ODQ (5 μM) [9], was added along with IBMX. At the indicated time point, cell lysates were obtained by submitting cells to three cycles of freezing and thawing. All samples were subsequently boiled at 90 °C for 5 min and then centrifuged at 12000 g for 15 min. The supernatants were assayed for cGMP by using a commercially available cGMP enzyme immunoassay kit (Cayman Chemical Company).

Statistics

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

RESULTS AND DISCUSSION

To assess the role of NO on the exocytotic function of epithelial cells, we used two NO-generating drugs, i.e. MAHMA NONOate and SNP. These agents were tested on the human colonic cell line HT29-Cl.16E, a well characterized model system for studying the regulated exocytosis of mucins [4,5,7,8,10,11].

Effects of MAHMA NONOate and SNP on mucin exocytosis

Time-course experiments showed that the addition of MAHMA NONOate (1 μM) or SNP (1 mM) to the incubation medium of HT29-Cl.16E cells elicited an increased exocytotic response (Figure 1). The stimulatory action of MAHMA NONOate was detectable as early as 5 min after incubation, was maximal after 15 min (Figure 1) and was observed in the dose range of 10^{-8} M to 10^{-6} M (results not shown). SNP (1 mM) induced an increase in [^3H]mucin exocytosis peaking at 5 min, followed by a decline

Figure 1 Effects of MAHMA NONOate and SNP on mucin exocytosis from HT29-Cl.16E cells

HT29-Cl.16E monolayers were incubated in the presence of 1 μM MAHMA NONOate (---○---) or 1 mM SNP with (—●—) or without (—○—) SOD (250 units/ml)/catalase (100 units/ml). The total release of mucins in the incubation medium over time periods of 0–5, 0–15 and 0–30 min was quantified as described in the Materials and methods section. Results are expressed relative to the baseline value of [^3H]mucin exocytosis. Results are means $\pm$ S.E.M. of six monolayers from two separate experiments for MAHMA NONOate, and 14 monolayers from four separate experiments for SNP. Inset: spontaneous dissociation of MAHMA NONOate (1 μM) into NO $^+$ at pH 7.4 and 37 °C in the incubation medium (UV absorbance at 250 nm).

of the level of secretory [^3H]mucins at 15 min, and by a return to the baseline level at time point 30 min (Figure 1). As shown in Figure 1, the decline phase of secretory [^3H]mucins was prevented by simultaneous addition of SOD (250 units/ml) and catalase (100 units/ml) to the incubation medium for the duration of the experiment. As SOD and catalase play a pivotal role in scavenging reactive oxygen species (O_2^- and H_2O_2), it is likely that the apparent loss of secretory [^3H]mucins was accounted for by an oxidative attack. Our data suggest that SNP evoked an oxidative attack of mucins through the production of reactive oxygen species (ROS), a finding in line with the recent demonstration of Rao and Cederbaum [12] that ROS are produced during the redox cycling of nitroprusside in biological systems. However, the production of ROS elicited by SNP did not play a role in the stimulation of mucin exocytosis, as SOD/catalase did not alter the magnitude of the SNP-induced exocytotic response from HT29-Cl.16E cells. Finally, as ferricyanide (tested in the dose range 10^{-3} – 10^{-6} M) was unable to alter the rate of mucin secretion from HT29-Cl.16E cells (results not shown), we ruled out the possibility that the ferricyanide moiety of SNP was responsible for its stimulatory effect.

Our results are consistent with the concept derived from studies on rat gastric mucosa that NO stimulates the exocytotic function of mucous cells [6]. In addition, we provide an unambiguous demonstration of a direct effect of NO on human mucous cells. The potential importance of NO-mediated mucin secretion can be evaluated by comparing the efficacy of this agent with that of other secretagogues, e.g. ATP, which stimulates P2 receptors on HT29-Cl.16E cells, carbachol, a stable analogue of

Figure 2 Effects of MAHMA NONOate and SNP on cGMP accumulation in HT29-Cl.16E cells

HT29-Cl.16E monolayers were preincubated for 30 min at 37 °C in the presence of 1 mM IBMX before treatment by 1 μ M MAHMA NONOate (○) or 1 mM SNP (●). At the indicated time points intracellular cGMP was extracted and measured by enzyme immunoassay. Results are expressed relative to the baseline value of cGMP accumulation. Results are means $\pm$ S.E.M. of nine monolayers from three separate experiments for MAHMA NONOate, and six monolayers from two separate experiments for SNP.

acetylcholine acting on muscarinic receptors, interleukin-1 (IL-1), a cytokine acting through specific receptors on epithelial cells (IL-1R1), and vasoactive intestinal peptide (VIP) or forskolin, acting through the cAMP pathway. From our published data [4,5,8] together with our present results we can infer the following order of efficacy of several secretagogues including NO in promoting mucin release: ATP > carbachol > VIP = IL-1 = NO.

Effects of MAHMA NONOate and SNP on cGMP accumulation in HT29-Cl.16E cells

As several lines of investigations have largely shown that the enzyme sGC serves as a receptor for NO⁺ [13–17] and mediates its intracellular effects, we measured the time course of cGMP accumulation in HT29-Cl.16E cells under MAHMA NONOate and SNP stimulation. As shown in Figure 2, MAHMA NONOate (10⁻⁶ M) induced a very rapid intracellular accumulation of cGMP, significant as early as 1 min incubation, whereas SNP did not induce any modification of intracellular cGMP levels. Together our results strongly suggest that MAHMA NONOate and SNP stimulate mucin exocytosis through two independent signalling pathways. This conclusion is further strengthened by our finding that the combined action of both agents added to the incubation medium resulted in an additive effect on [³H]mucin exocytosis [at time point 15 min: SNP/SOD/catalase = 87($\pm$ 6.5)% over baseline, MAHMA NONOate = 93($\pm$ 11)% over baseline and SNP/SOD/catalase + MAHMA NONOate = 212($\pm$ 15)% over baseline, n = 6, P < 0.001 versus SNP/SOD/catalase or MAHMA NONOate alone].

To test whether MAHMA NONOate-induced cGMP accumulation was causally related to mucin exocytosis (stimulus–secretion coupling), we examined the effect of ODQ, a newly available specific inhibitor of sGC, on both cGMP accumulation and mucin exocytosis. ODQ was chosen to examine the stimulus–secretion coupling because it does not have the inconveniences of

Table 1 Effect of ODQ on mucin exocytosis in MAHMA NONOate- and SNP-treated HT29-Cl.16E cells

HT29-Cl.16E monolayers were preincubated for 30 min at 37 °C in the presence of various concentrations of ODQ (0.5 μ M, 5 μ M or 10 μ M) before treatment with 1 μ M MAHMA NONOate or 1 mM SNP. Mucin exocytosis was measured at the time point 15 min as described in the Materials and methods section. Results are expressed as the percentage of maximal stimulatory effect of MAHMA NONOate or SNP on [³H]-mucin exocytosis. Results are means $\pm$ S.E.M. of six monolayers from two separate experiments for MAHMA NONOate, and three monolayers from one experiment for SNP. (* P < 0.05, ** P < 0.01 versus MAHMA NONOate; ns, not significant; nd, not determined.)

	[ODQ] (μ M) ...	0.5	5	10
MAHMA NONOate		70 $\pm$ 1 ns	53 $\pm$ 5**	54 $\pm$ 18*
SNP		87 $\pm$ 6 ns	89 $\pm$ 18 ns	nd

other currently employed inhibitors of sGC such as Methylene Blue and 6-anilino-5,8-quinolinedione (LY 83583), which can exert a number of undesirable effects through their known ability to generate superoxide ions [18,19]. Moreover, LY 83583 was originally reported to enhance sGC activity, but paradoxically to lower cGMP levels non-specifically and by an unknown mechanism [20]. As shown in Table 1, ODQ inhibited MAHMA NONOate-induced mucin exocytosis in a dose-dependent manner. The maximal inhibitory effect [47($\pm$ 5)% inhibition] was obtained at 5 μ M ODQ. This effect was preceded by an inhibition of intracellular cGMP accumulation [inhibitory effect of 5 μ M ODQ: 60($\pm$ 10)% of maximal effect of MAHMA NONOate, P < 0.01, n = 6 monolayers]. Together these results provide direct evidence that the stimulatory effect of NO on the exocytotic function of epithelial cells is mediated through the sGC/cGMP pathway. This conclusion is further strengthened by our demonstration that 1 mM dibutyl cGMP induced a maximal stimulation of mucin exocytosis at time point 15 min [115($\pm$ 22)% over baseline, n = 6, P < 0.001].

As expected, ODQ did not inhibit SNP-induced mucin exocytosis (Table 1), a finding which strengthens the view that the sGC/cGMP pathway is not involved in the exocytotic response to SNP. Our observation that SNP, added to an essentially thiol-free incubation medium, failed to activate sGC is consistent with spectroscopic data showing that the addition of SNP to purified sGC does not yield Fe(II)sGC(NO), with characteristic Soret absorption at 398 nm [21]. Moreover, our result is in line with the finding by Ignarro et al. [22] that in the absence of thiols, SNP failed to activate sGC. Due to the strong NO⁺ character of SNP, rendering it very reactive with thiol groups in general, the signalling pathway of SNP is best rationalized in terms of S-nitrosation [23]. Furthermore, several lines of investigation suggest that S-nitrosation modulates membrane receptors, membrane ion channels, GTP-binding proteins, protein kinases, phosphotyrosine protein phosphatases, or cytoskeletal proteins [24]. Further work is needed to decipher the S-nitrosation-based mechanisms underlying the effects of SNP on the exocytotic machinery.

Conversion of NO⁺ into NO[•] leads to a shift of the signalling pathway

As the most obvious signal transduction mechanism for NO⁺ is S-nitrosation of proteins [23,25], we reasoned that the addition of a thiol such as cysteine in excess to the incubation medium would create decoy targets for NO⁺ through the formation of S-nitrosocysteine. Indeed, 1.2 mM cysteine abolished the SNP-

Table 2 Effect of cysteine on mucin exocytosis stimulated by NO⁺ or NO⁻

HT29-Cl.16E monolayers were incubated in the presence of 1 μ M MAHMA NONOate or 1 mM SNP in the absence or presence of 1.2 mM cysteine. Mucin exocytosis was measured after 15 min incubation, as described in the Materials and methods section. Results are expressed as the percentage over the baseline value of [³H]mucin exocytosis. Results are means $\pm$ S.E.M. of seven monolayers from two separate experiments for SNP, and four monolayers from one experiment for MAHMA NONOate (****P* < 0.001 versus SNP alone; ns, not significant).

Treatment ...	None	1.2 mM Cysteine
SNP	117 $\pm$ 7	0 $\pm$ 13***
MAHMA NONOate	102 $\pm$ 9	104 $\pm$ 9 ns

Figure 3 Delayed stimulatory effect of SNP plus cysteine on mucin exocytosis: effect of ODQ

HT29-Cl.16E monolayers were incubated in the presence of 1 mM SNP + 1.2 mM cysteine with (●) or without (○) 5 μ M ODQ. When used, ODQ was added to the incubation medium 30 min before the SNP + cysteine treatment. Mucin exocytosis was measured at the indicated time points as described in the Materials and methods section. Results are expressed relative to the baseline value of [³H]mucin exocytosis measured in the presence of 1.2 mM cysteine. Results are means $\pm$ S.E.M. of six monolayers from two separate experiments.

induced mucin exocytosis measured at time point 15 min, whereas it did not affect the response to MAHMA NONOate (Table 2).

As shown in Figure 3, an extended incubation of HT29-Cl.16E cells with SNP plus cysteine resulted in a late stimulatory effect on mucin exocytosis, this effect being significant at time point 30 min. Interestingly, this late stimulatory effect was completely blocked by ODQ. The most likely explanation is that NO⁺ was released in a time-dependent manner from *S*-nitrosocysteine, thus leading to mucin exocytosis through the activation of sGC.

Our demonstration that, in the presence of cysteine, SNP is able to activate mucin exocytosis through a cGMP-dependent pathway is perfectly in line with previous findings by Ignarro et al. [22], who showed that activation of arterial GC by SNP was markedly enhanced by the addition of thiols, e.g. cysteine, to the reaction mixture. Our finding is best rationalized by a recent study showing that several *S*-nitrosothiols, including *S*-nitroso-

cysteine are able to release NO⁺ via homolytic cleavage under physiological conditions [26]. The estimated half-life of 60 min for NO⁺ release from *S*-nitrosocysteine, as evaluated by an oxymyoglobin-NO⁺ capture assay [26], explains why a lag phase preceded the onset of a significant increase in mucin secretion upon addition of SNP plus cysteine to the incubation medium.

The biological relevance of our findings relates to the known presence of *S*-nitrosothiols in the environment of epithelial cells, e.g. plasma, red blood cells, human airway lining fluids, platelets and neutrophils [27,28]. Under physiological conditions these *S*-nitrosothiols can act as NO⁺, NO⁺ and NO⁻ donors, which can co-operate in modulating the exocytotic function of epithelial cells. Furthermore, we demonstrate that the redox versatility of NO allows for a shift in its cellular signalling pathway mediating epithelial secretory function, depending on changes in the ambient redox milieu. As a consequence, NO is likely to maintain its up-regulatory role in mucin exocytosis, whatever the redox environment changes initiated by inflammatory conditions.

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