

Nitric Oxide Inhibits IgE-Dependent Cytokine Production and Fos and Jun Activation in Mast Cells¹

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NO is a cell-derived radical reported to inhibit mast cell degranulation and subsequent allergic inflammation, although whether its action is nonspecific or occurs via specific molecular mechanisms remains unknown. To examine this question, we set out to determine whether NO inhibits mast cell cytokine production, and, if so, whether it also alters FcεRI-dependent signal transduction. As hypothesized, the radical inhibited IgE/Ag-induced IL-4, IL-6, and TNF production. Although NO did not influence phosphorylated JNK, p38 MAPK, or p44/42 MAPK, it did inhibit phosphorylation of phospholipase Cγ1 and the AP-1 transcription factor protein c-Jun, but not NF-κB or CREB. NO further completely abrogated IgE/Ag-induced DNA-binding activity of the nuclear AP-1 proteins Fos and Jun. These results show that NO is capable of inhibiting FcεRI-dependent mast cell cytokine production at the level of gene regulation, and suggest too that NO may contribute to resolution of allergic inflammation. *The Journal of Immunology*, 2004, 173: 6914–6920.

Nitric oxide is a cell-derived radical with diverse roles as a messenger in physiological, pathological, and immunological processes (1, 2). In immunity, NO is generated largely by cells of the innate response, namely macrophages, granulocytes, and epithelial cells (3–7). Upon activation by inflammatory cytokines and/or bacterial LPS, these cells express NO synthase-2 (NOS-2),³ the inducible form of the NOS family of enzymes that catalyze NO production from L-arginine and molecular oxygen (8). Once expressed, NOS-2 is continuously active and generates NO at levels that are sufficiently high and sustained to influence immune cell function (1, 2). NO so produced exerts regulatory effects on several cell types, including mast cells and T cells (9–16). For example, macrophage-derived NO inhibits Ag-induced mast cell degranulation (9–11), T lymphocyte proliferation (12–14), and T cell responses during cognate Ag recognition (15, 16).

NO is known to suppress mast cell activation and subsequent features of inflammation (17, 18). For example, cell- or chemical-derived NO inhibits calcium ionophore- and IgE/Ag-induced release of granule-associated mediators from rodent peritoneal mast cells (9, 10, 19), adhesion of mast cells to fibronectin (20, 21), as well as mast cell-mediated changes in gut epithelial permeability (22), adherence of leukocytes to the vascular endothelium (23),

and microvascular leakage (24). Furthermore, the suppressive effect of LPS on mast cell-dependent passive cutaneous anaphylaxis, pulmonary inflammation, airway eosinophilia, mucus production, and airway hyperactivity is mediated by NO (25).

To date, there is little information available with regard to the mechanism of action of NO on mast cells, other than that guanylate cyclase and peroxynitrite are not involved (10, 21) and that the cysteine protease calpain is a target in NO inhibition of mast cell adhesion (21). Particularly, the means by which NO is able to affect mast cell-dependent inflammation and the specific molecular mechanisms involved are incompletely understood. To explore this question, we first determined whether NO can alter IgE/Ag-induced mast cell cytokine production. We then examined FcεRI-dependent signal transduction to identify specific NO-regulated events. As will be shown, NO inhibits Ag-driven mast cell cytokine expression, indeed considerably more effectively than it inhibits cell degranulation. This effect is associated with selective suppression of Ag-induced DNA-binding activity of the AP-1 transcription factor proteins Fos and Jun. We conclude that NO is highly effective as an inhibitor of Ag-driven cytokine responses, and that Fos and Jun are key NO-regulated components of Ag-induced mast cell cytokine production. NO suppression of mast cell cytokine production may be an important component of the processes that lead to resolution of allergic inflammation.

Materials and Methods

Cell culture and activation

RBL-2H3 mast cells were grown to confluence in RPMI 1640 medium (Invitrogen Life Technologies, Paisley, U.K.) supplemented with 10% FBS. Mouse bone marrow-derived cells (BMMC) were grown from femoral marrow cells of BALB/c mice and cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 mM HEPES, 1.0 mM sodium pyruvate, nonessential amino acids (all from BioSource International, Camarillo, CA), 0.0035% 2-ME, and 300 ng/ml mouse rIL-3 (PeproTech, Rocky Hill, NJ). BMMC were used after 4–6 wk of culture. For mediator release assays, RBL-2H3 cells or BMMC were seeded at 25,000 per well (100 μl) of a 96-well flat-bottom cell culture plate and cultured overnight at 37°C with 50 ng/ml mouse monoclonal IgE anti-DNP (Sigma-Aldrich, St. Louis, MO). The NO chemical donors S-nitrosoglutathione (GSNO), S-nitroso-N-acetylpenicillamine (SNAP), 2,2'-(hydroxynitrosohydrazino)bis-ethanamine (NOC-18), or N-(2-aminoethyl)-N-(2-hydroxy-2-nitrosohydrazino)-1,2-ethylenediamine

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Received for publication July 27, 2004. Accepted for publication August 29, 2004.

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¹ This work was supported by The Wellcome Trust and National Institutes of Health intramural funds.

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³ Abbreviations used in this paper: NOS, NO synthase; β-hex, β-hexosaminidase; BMMC, bone marrow-derived mast cell; GSNO, S-nitrosoglutathione; IKK, IκB kinase; NOC-18, 2,2'-(hydroxynitrosohydrazino)bis-ethanamine; P, phospho; PLC, phospholipase C; SNAP, S-nitroso-N-acetylpenicillamine; SPER-NO, N-(2-aminoethyl)-N-(2-hydroxy-2-nitrosohydrazino)-1,2-ethylenediamine.

(SPER-NO) (all from Calbiochem, San Diego, CA) were added to the cultures at appropriate times and concentrations, and the cells were subsequently challenged at 37°C with 10 ng/ml DNP₃₀-human serum albumin Ag (Sigma-Aldrich) or control medium as negative control. NO donors were routinely used at 125–500 μ M because this was the dose-dependent range. It should be noted that these are concentrations of NO donors and not of the liberated NO, which is considerably ($\sim$ 100-fold) lower. None of the NO donors reduced mast cell uptake of [³H]serotonin, total cell content of the granule marker β -hexosaminidase (β -hex), viable cell number by trypan blue staining, or levels of the housekeeping genes L32 and GAPDH.

Degranulation and cytokine release

Mast cell degranulation was measured as release of incorporated [³H]serotonin (9) or endogenous β -hex (26) 30 min after cell challenge and calculated as percentage of cell total after subtracting background release (<3%). Cytokine content of cell culture supernatants obtained 20 h after Ag challenge was measured by ELISA (OPTEIA rat IL-4, IL-6, and TNF sets, BD Biosciences, San Diego, CA; Cytoscreen mouse IL-4, IL-6, and TNF kits, BioSource International), according to the suppliers' instructions. Within the assay sensitivities, rat IL-4, but not IL-6 and TNF, and mouse IL-6 and TNF, but not IL-4, were detected.

Cytokine mRNA

Cytokine mRNA was measured by RNase protection assay (RiboQuant rCK-1 kit, BD Biosciences; incorporating probes for IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, TNF, lymphotoxin, IFN- γ , GAPDH, and L32), according to the supplier's instructions. Briefly, at appropriate times after Ag challenge, RBL-2H3 cells (2.0×10^6) were solubilized in TRI reagent (1.0 ml; Sigma-Aldrich), and RNA (10–20 μ g) was extracted and hybridized to ³²P-radiolabeled antisense RNA probes at 56°C overnight. The samples were digested with RNase A and T1 (BD Biosciences) to remove nonhybridized ssRNA, after which the protected fragments were purified by phenol/chloroform extraction and ethanol precipitation and loaded onto a 6% polyacrylamide urea gel, run at 38 mA in 0.5 $\times$ Tris-borate EDTA buffer alongside the undigested probes as size markers. Gels were exposed overnight to x-ray film, and densitometric analysis was conducted using the Image program (National Institutes of Health).

Western blots

BMMC were cultured (2×10^6 in 1.0 ml) in 24-well culture plates, sensitized with IgE anti-DNP overnight, and then incubated with or without GSNO (125–500 μ M) for 4 h. The cells were then centrifuged, resuspended, and challenged with Ag in a final volume of 200 μ l. At 1 and 30 min after challenge, the cells were lysed by addition of 200 μ l of boiling lysis buffer (2 $\times$ NuPage LDS sample buffer, Invitrogen Life Technologies; supplemented with 100 mM DTT, protease inhibitor mixture, and 2-ME, as described) (27). The cell extracts were boiled for 3 min and electrophoresed on 4–12% SDS-polyacrylamide gels. The separated proteins were electrophoretically transferred to nitrocellulose membranes, and the blots were probed with the following rabbit polyclonal Abs: anti-phospho (P)-phospholipase C γ 1 (PLC γ 1) (Tyr⁷⁸³) (BioSource International), anti-P-p38 MAPK (Thr¹⁸⁰, Tyr¹⁸²), anti-P-p42/44 MAPK (Thr²⁰², Tyr²⁰⁴), anti-P-I κ B kinase (IKK α , Ser¹⁸⁰; IKK β , Ser¹⁸¹), anti-P-JNK (JNK, Thr¹⁸³,

Tyr¹⁸⁵), anti-P-c-Jun (Ser⁷³), anti-P-NF- κ B p65 (Ser⁵³⁶), or anti-P-CREB (Ser¹³³) (all from Cell Signaling Technology, Beverly, MA), followed by HRP-linked donkey anti-rabbit Ig (Amersham Biosciences, Piscataway, NJ). To monitor protein loading, blots were stripped and reprobed with rabbit anti-Lyn (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were developed in chemiluminescent reagent (Western Lightening; PerkinElmer Life Science, Boston, MA) and exposed to x-ray film.

DNA-binding activity of nuclear transcription factor proteins

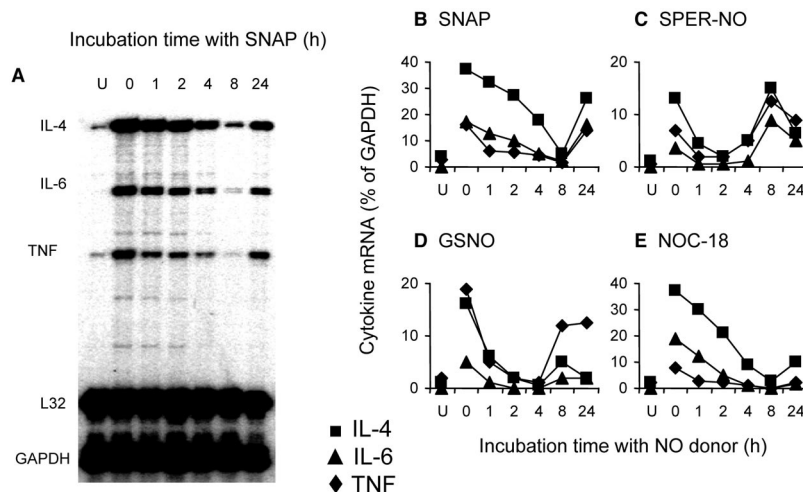
Nuclear protein was extracted from IgE-sensitized BMMC 30 min after Ag challenge using the nuclear extract kit supplied by Active Motif (Carlsbad, CA), according to the supplier's instructions. The nuclear extracts (10 μ g of protein/aliquot) were assayed by ELISA (TransAM kits; Active Motif) for DNA-binding activity of phospho-c-Jun, c-Fos, FosB, Fra-1, Fra-2, JunB, JunD, NF-ATc, and NF- κ B p50. The transcription factor proteins, bound to immobilized oligonucleotides corresponding to appropriate consensus gene response elements, were detected with specific Abs, followed by HRP-conjugated anti-Ig, according to the supplier's instructions. DNA-binding activity was expressed as a ratio to the positive control cell extract as supplied.

Results

NO regulation of RBL-2H3 cell cytokine mRNA expression

To determine whether NO regulates mast cell cytokine mRNA, IgE-sensitized RBL-2H3 cells were incubated with a panel of NO donors for various time periods before challenge with Ag and RNA was extracted 2 h later. Four compounds of diverse chemical structure and $t_{1/2}$ were used as a source of NO: two S-nitrosothiols (SNAP and GSNO) and two 1-substituted diazen-1-ium-1,2-diols (NONOates: NOC-18 and SPER-NO). SNAP produced a time-dependent inhibition of Ag-induced IL-4, IL-6, and TNF mRNA (Fig. 1A). Of the panel of mRNA species probed by RNase protection assay, these three cytokines were consistently the most strongly induced by Ag (Fig. 1A). Furthermore, each of these three types of mRNA showed parallel patterns of inhibition by each of the four NO donors (Fig. 1, B–E). However, each NO donor produced a characteristic time-dependent reduction of Ag-stimulated cytokine mRNA expression. SNAP gave maximal inhibition at 8 h (Fig. 1, A and B), SPER-NO at 2 h (Fig. 1C), GSNO at 4 h (Fig. 1D), and NOC-18 at 8 h (Fig. 1E). With shorter acting NO donors, cytokine responses recovered with time. For example, IL-4, IL-6, and TNF responses recovered partially 24 h after exposure to SNAP (Fig. 1, A and B), fully 8 h after exposure to SPER-NO (Fig. 1C), and partially 8 h after exposure to GSNO (Fig. 1D). None of the NO donors over 24 h led to any reduction in mRNA levels for the housekeeping genes GAPDH or L32, showing that this was not a general effect on mRNA production.

FIGURE 1. NO inhibits Ag-induced mast cell cytokine mRNA. IgE-sensitized RBL-2H3 cells (1.5×10^6 in 1.0 ml) were incubated with or without NO donor (250 μ M) for various time periods, as indicated, before challenge with Ag. RNA was extracted 2 h later and analyzed by RNase protection assay. A, Representative RNase protection assay showing the effect of SNAP on Ag-induced IL-4, IL-6, and TNF mRNA. B–E, Densitometric analyses of IL-4, IL-6, and TNF mRNA for experiments with SNAP, SPER-NO, GSNO, and NOC-18, respectively. U, Represents unstimulated cells; 0, represents Ag-challenged cells that had not been exposed to NO. Results are representative of two to three experiments with each NO donor.



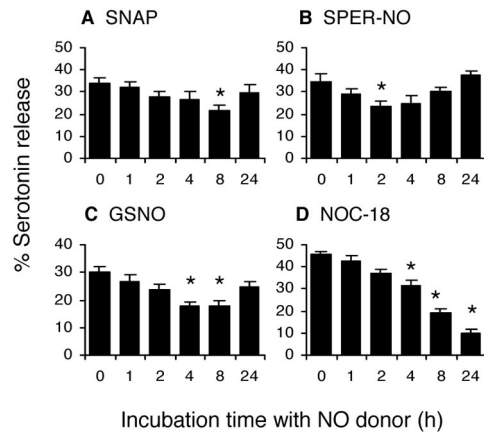


FIGURE 2. Effect of NO on Ag-induced serotonin release. IgE-sensitized RBL-2H3 cells (25,000/well) were incubated with or without NO donor (250 μ M) for various time periods, as indicated, before challenge with Ag, and degranulation was measured as release of serotonin. Results are for SNAP (A), SPER-NO (B), GSNO (C), and NOC-18 (D). 0, Represents Ag-challenged cells that had not been exposed to NO. Data are means $\pm$ SEM for three to five separate experiments. *, $p < 0.05$, by ANOVA, followed by two-tailed unpaired Student's t test with Bonferroni correction for comparisons with cells cultured without NO.

NO regulation of RBL-2H3 cell degranulation and cytokine release

Each of the panel of NO donors, as expected, inhibited IgE/Ag-induced degranulation of RBL-2H3 cells. The kinetics were found to be similar to those seen for inhibition of cytokine mRNA. However, the magnitude of the effect was less (Fig. 2) with inhibition not exceeding 40%, except in the case of NOC-18 at 24 h (Fig. 2D). GSNO and NOC-18 were then added to the cells over a range of concentrations for 4 h, and the release of β -hex and IL-4 protein was determined. GSNO and NOC-18 both significantly inhibited Ag-induced β -hex release (by 25 and 16%, respectively, at 500

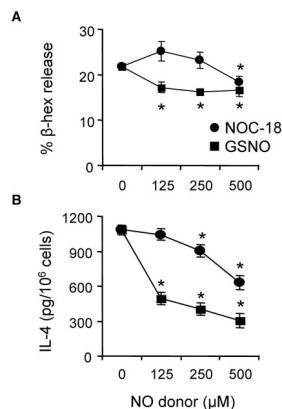


FIGURE 3. NO suppression of Ag-induced β -hex and IL-4 release. IgE-sensitized RBL-2H3 cells (25,000/well) were incubated with or without NO donor (125–500 μ M) for 4 h before challenge with Ag. β -hex release (A) was measured at 30 min, and IL-4 release (B) at 20 h post-Ag challenge. 0, Represents Ag-challenged cells that had not been exposed to NO. Data are means $\pm$ SD for quadruplicate wells from one experiment representative of four. *, $p < 0.05$, by ANOVA, followed by two-tailed unpaired Student's t test with Bonferroni correction for comparisons with cells cultured without NO.

μ M) (Fig. 3A), but inhibited IL-4 release more strongly (by 42 and 72%, respectively, at the same concentration) (Fig. 3B).

NO effects on BMMC degranulation and cytokine release

Because RBL-2H3 cells are of basophilic tumor origin, we considered it desirable to investigate whether NO inhibits cytokine production in a second cell type. We selected BMMC, as these are of more classic mast cell phenotype, are primary cultured, and nonmutant. These cells showed patterns of susceptibility to NO similar to those shown by RBL-2H3 cells. Addition of NOC-18 or GSNO to BMMC for 4 or 20 h led to 20% or less inhibition of Ag-induced degranulation (Fig. 4, A and B). In contrast, both NO donors inhibited cytokine release strongly. At 4 h, GSNO proved more effective than NOC-18 at inhibiting release of IL-6 (80 vs <10% inhibition at 500 μ M) (Fig. 4C), while at 20 h NOC-18 was more effective than GSNO (87 vs 65% at 500 μ M) (Fig. 4D). Likewise, at 4 h, GSNO inhibited TNF release by 95%, while NOC-18 was ineffective (Fig. 4E), and at 20 h, NOC-18 was more effective than GSNO (90 vs 80% inhibition at 500 μ M) (Fig. 4F). Thus, for BMMC, as for RBL-2H3 cells, NOC-18 requires longer

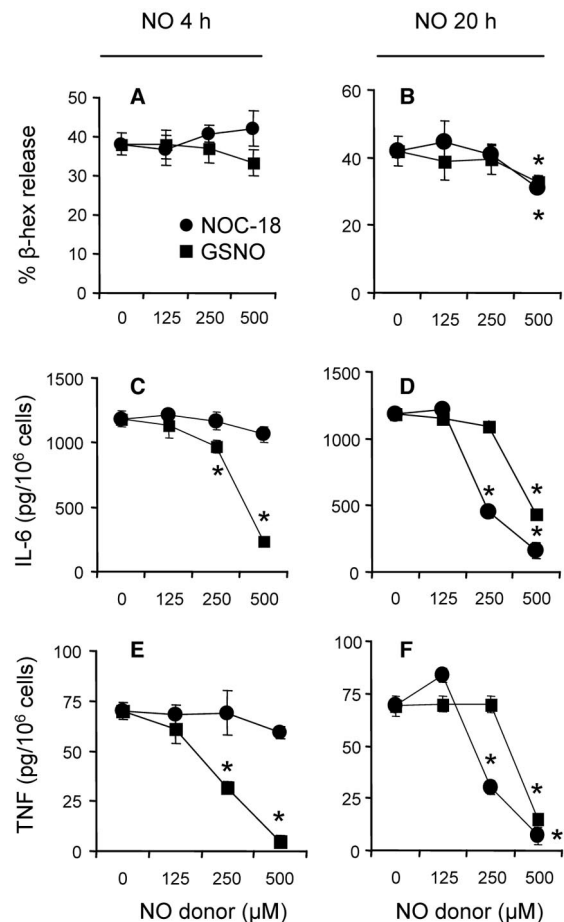
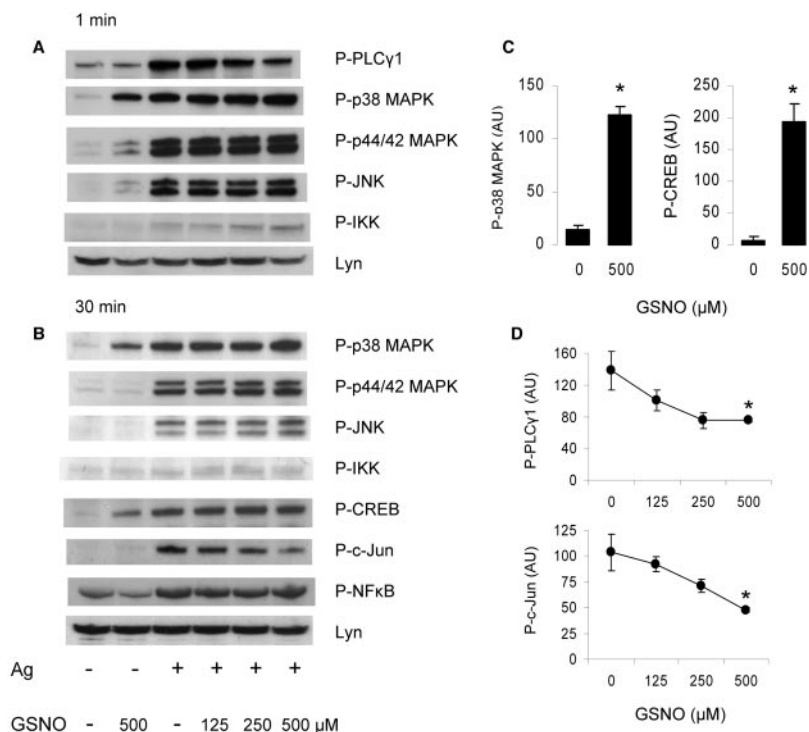


FIGURE 4. Regulation of BMMC β -hex and cytokine release by NO. IgE-sensitized BMMC (25,000/well) were incubated with or without NO donor (125–500 μ M) for 4 h (A, C, and E) or 20 h (B, D, and F) before challenge with Ag. β -hex release (A and B) was measured at 30 min, and IL-6 release (C and D) and TNF release (E and F) at 20 h post-Ag challenge. 0, Represents Ag-challenged cells that had not been exposed to NO. Unstimulated cells released <3% β -hex, <25 pg of IL-6/10⁶ cells, and <5 pg of TNF/10⁶ cells. Data are means $\pm$ SD for quadruplicate wells from one experiment representative of four. *, $p < 0.05$, by ANOVA, followed by two-tailed unpaired Student's t test with Bonferroni correction for comparisons with cells cultured without NO.

FIGURE 5. NO effects on protein phosphorylation. A–D, IgE-sensitized BMMC (10^6 in 1.0 ml) were incubated with or without GSNO (125–500 μ M) for 4 h before challenge with Ag, and proteins were extracted at 1 or 30 min post-Ag for Western blot analysis. A and B, Lane 1, Shows unstimulated cells without NO pre-exposure; lane 2, shows cells exposed to GSNO (500 μ M), but not challenged with Ag; lane 3, shows cells challenged with Ag without exposure to NO; lanes 4–6, show cells exposed to 125, 250, or 500 μ M GSNO, respectively, before Ag challenge. C, Densitometric analyses of P-p38 MAPK and P-CREB extracted after 4-h culture with or without 500 μ M GSNO. D, Densitometric analyses of P-PLC γ 1 and P-c-Jun extracted 1 or 30 min, respectively, after Ag challenge, following preincubation with or without GSNO for 4 h. C and D, Results are means $\pm$ SEM for three experiments; AU = arbitrary units. *, $p < 0.05$, by ANOVA, followed by two-tailed unpaired Student's t test with Bonferroni correction for comparisons with cells cultured without NO.



incubation times than GSNO to exert maximal inhibition. These differences between NO donors reflect their chemical $t_{1/2}$ (see Discussion).

Effects of NO on mast cell signaling events

After characterizing coincident NO effects on mediator release and cytokine production by RBL-2H3 cells and BMMC, and showing that these were comparable for both cell types, we selected BMMC for detailed signaling studies. GSNO was added to BMMC for 4 h, and the cells were then challenged with Ag or control medium. We examined levels of phosphorylated proteins at previously optimized time points and according to the time sequence of protein activation: PLC γ 1 at 1 min; p38 MAPK, p44/42 MAPK, and JNK at both 1 and 30 min; and transcription factor proteins at 30 min. Challenge of the cells with Ag for 1 min led to elevated levels of P-PLC γ 1, P-p38 MAPK, P-p44/42 MAPK, and P-JNK at both 1 and 30 min, because these kinases slightly raised levels of P-IKK, but did not influence levels of total Lyn (housekeeping protein) compared with unchallenged cells (compare lanes 3 and 1, Fig. 5A). Cells that had been preincubated with GSNO for 4 h without Ag challenge showed elevated levels of P-p38 MAPK, but not other phosphorylated proteins (compare lanes 2 and 1, Fig. 5A). Preincubation of the cells with GSNO led to a concentration-dependent partial reduction in levels of Ag-induced P-PLC γ 1, but not P-p38 MAPK, P-p44/42 MAPK, P-JNK, or P-IKK (Fig. 5A, lanes 3–6). The NO inhibition of PLC γ 1 phosphorylation was significant over repeated experiments (Fig. 5D).

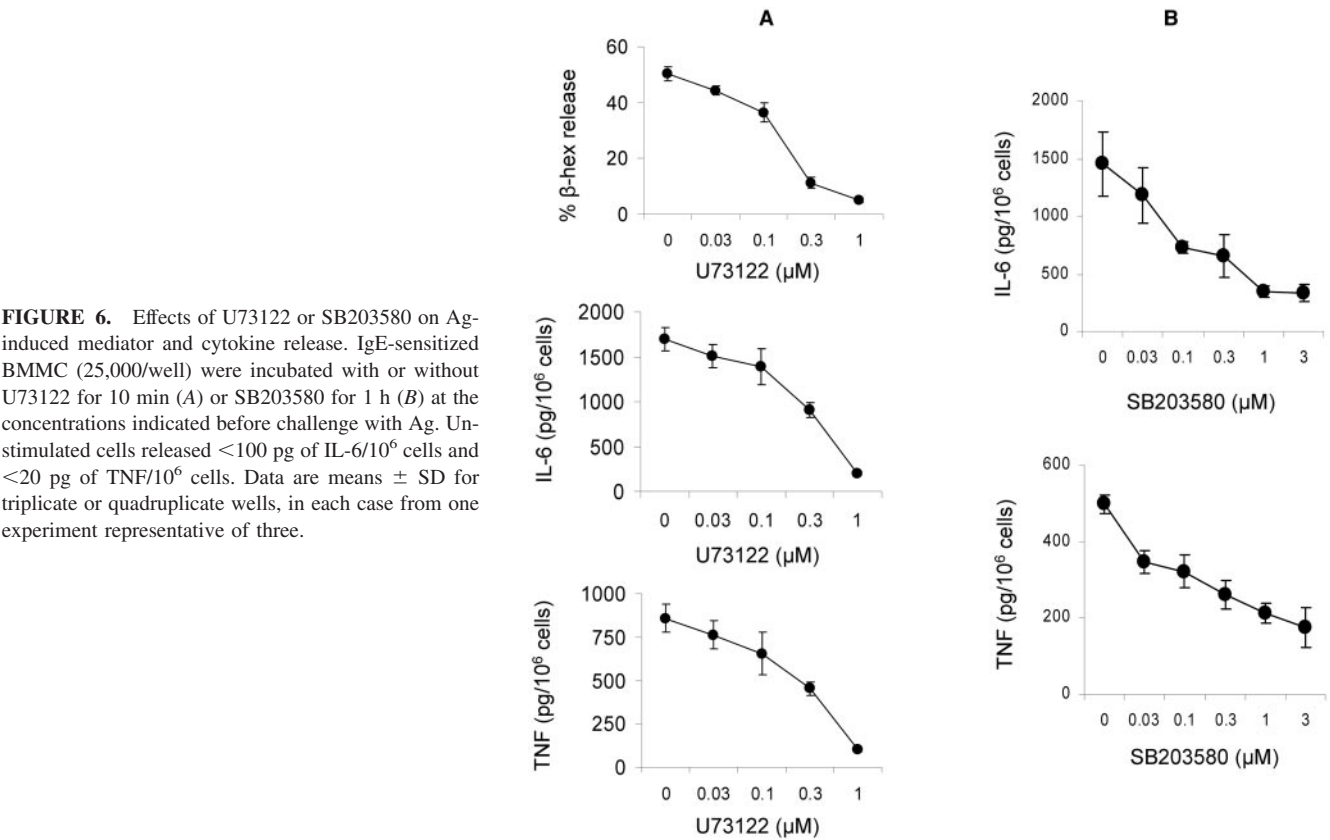
Challenge of cells with Ag for 30 min led to raised levels of P-p38 MAPK, P-p44/42 MAPK, P-JNK, P-CREB, P-c-Jun, and P-NF- κ B (lanes 3 and 1, Fig. 5B). Incubation with GSNO (without Ag challenge) led to elevated P-p38 MAPK and P-CREB (lanes 2 and 1, Fig. 5B), and this was statistically significant for both proteins over repeated experiments (Fig. 5C). GSNO produced a concentration-dependent reduction of Ag-induced phosphorylation of c-Jun, but none of the other proteins (lanes 3–6, Fig. 5B), and again this was significant over repeated experiments (Fig. 5D).

PLC γ and p38 MAPK inhibitors on degranulation and cytokine release

Because NO inhibited Ag-induced phosphorylation of PLC γ 1, we examined whether this effect might account for the differential inhibitory effect of NO on cytokine production vs degranulation. This was not the case, as the PLC γ inhibitor U73122 was equally potent in inhibiting Ag-induced release of either β -hex, IL-6, or TNF (Fig. 6A). Because NO elevated P-p38 MAPK, we examined whether this might account for inhibition of cytokine production. If this were so, then inhibition of p38 MAPK activity would be expected to enhance Ag-induced cytokine production. However, the p38 inhibitor SB203580 inhibited rather than enhanced Ag-induced release of IL-6 and TNF (Fig. 6B). Thus, PLC γ is required equally for mast cell degranulation and cytokine production, and p38 MAPK activity is facilitatory rather than inhibitory for cytokine production.

NO regulation of transcription factor activity

As NO inhibited cytokine production and levels of phosphorylated c-Jun, we next examined its effects on Fc ϵ RI-dependent activation of transcription factors associated with these pathways. Ag challenge of BMMC led to induction of DNA-binding activity of P-c-Jun, JunB, JunD, FosB, c-Fos, and NF-AT (Fig. 7), but not Fra-1, Fra-2, or NF- κ B p50 (data not shown). GSNO alone, for 4 h, did not induce activity of any of these transcription factor proteins (Fig. 7, and data not shown). However, culture of cells with GSNO for 4 h reversed fully the Ag-induced DNA-binding activity of P-c-Jun, JunB, JunD, FosB, and c-Fos (Fig. 7). Under the same conditions, Ag activation of NF-AT activity was partially inhibited, by $\sim$ 33% (Fig. 7). To examine whether NO inhibits directly the binding of transcription factor proteins to their respective oligonucleotide response elements, a pooled sample of nuclear proteins, extracted from Ag-activated cells, was incubated with either GSNO or NOC-18 for 1, 2, or 4 h, and the samples were assayed for DNA-binding activity. Over a series of experiments, no effect of NO on binding of NF-AT, P-c-Jun, JunB, JunD, FosB, and



c-Fos to DNA was observed. Thus, we were unable to obtain evidence that NO directly interacts with transcription factors.

Discussion

In this study, we demonstrate that NO inhibits IgE/Ag-induced mast cell cytokine expression. This effect is characterized by distinctive features. First, regulation by NO requires exposure of the cells before cell activation, and this varies in turn according to *t*_{1/2} of the NO source. Second, NO preferentially inhibits cytokine production compared with degranulation. Third, NO selectively targets certain cell signaling events while leaving others unscathed. Most notably, NO inhibits Ag-induced activation of nuclear Fos and Jun proteins, components of the AP-1 transcription factor involved in signaling pathways leading to cytokine expression.

To study the time requirements of cell exposure to NO, before proceeding to signaling studies, we used a panel of NO donors that differ in chemical characteristics and *t*_{1/2}: SPER-NO and NOC-18,

both nucleophile adducts of NO with *t*_{1/2} of 39 min and 20 h, respectively (28, 29), and GSNO and SNAP, *S*-nitrosothiols that generate nitrite at a steady rate over 30–60 min (30, 31). SPER-NO was the most rapidly acting, producing maximal inhibition of Ag-induced cytokine mRNA expression after only 2 h, while GSNO, SNAP, and NOC-18 produced maximal inhibition of cytokine mRNA at 4, 8, and 8–24 h, respectively. Both RBL-2H3 cells and BMMC recovered cytokine responsiveness after exposure to the more rapidly acting NO donors. Our finding that optimal mast cell inhibition requires pre-exposure to NO is consistent with sustained NO production by NOS-2 in vivo, and suggests that NO may be most effective in suppressing mast cell-dependent inflammation upon secondary exposure to allergen. As reported in this work for mast cells, human PBMC likewise require prior exposure to NO for inhibition of proliferation and cytokine production (30).

We observed that NO was considerably more effective at reducing Ag-induced mast cell cytokine expression than degranulation

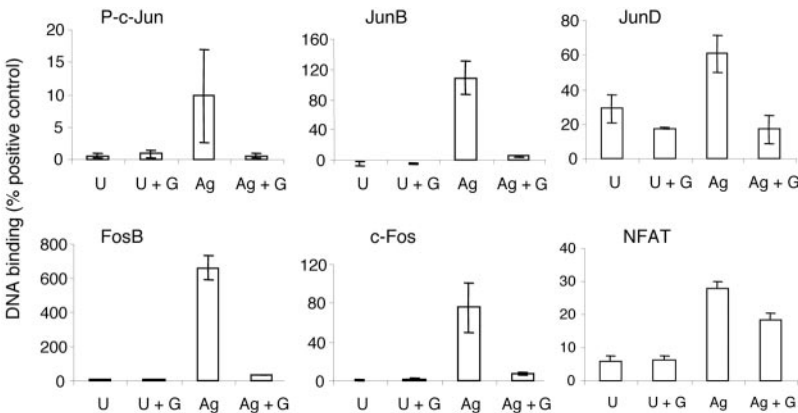


FIGURE 7. NO inhibition of Ag-induced transcription factor activation. BMMC (10⁷ cells per extract) were cultured with or without GSNO (500 μM) for 4 h before challenge with or without Ag. Nuclear proteins were extracted 30 min later and 10 μg assayed for binding activity to immobilized oligonucleotides representative of appropriate gene response elements. U = cells unexposed to GSNO or Ag; G = cells cultured with GSNO (500 μM) alone for 4 h; Ag = cells challenged with Ag for 30 min; Ag + G = cells cultured with GSNO for 4 h and then challenged with Ag for 30 min. Results are means ± SEM for three separate experiments.

in both RBL-2H3 cells and BMMC. This relative, but not absolute selectivity of NO for mast cell cytokine expression suggested that NO might exert actions at more than one point in mast cell signaling pathways, i.e., at events that are shared by both the degranulation and cytokine pathways, as well as events further downstream that are exclusive to the cytokine response. Our signaling studies confirm that this is the case. We found that GSNO partially inhibited Ag-induced phosphorylation of PLC γ 1, the enzyme that generates inositol triphosphate and diacylglycerol in pathways leading to degranulation and cytokine expression in mast cells (32–34). The degree of inhibition of PLC γ 1 by NO was comparable to the degree of inhibition of degranulation, indicating that inhibition of this enzyme can account for this NO effect. However, the PLC γ inhibitor U73122 inhibited Ag-induced BMMC degranulation and cytokine release equally, establishing that inhibition of the enzyme cannot account for selective inhibition by NO of cytokine expression. The results indicate again that NO must target additional downstream events that are unique to pathways leading to cytokine expression.

Further potential targets for NO might be p38 MAPK, p44/42 MAPK, JNK, and IKK, kinases involved in mast cell cytokine production (35–38), and their associated transcription factors CREB, c-Jun, and NF- κ B. NO elevated phosphorylation of p38 MAPK and its downstream transcription factor CREB, but not other kinases and transcription factor proteins. This raised the possibility that induction of the p38 MAPK/CREB pathway might relate to inhibition of cytokine production. However, the p38 inhibitor SB203580 inhibited Ag-induced release of TNF and IL-6, indicating that p38 augments rather than curbs cytokine production in these cells. Thus, activation of p38 does not explain the cytokine inhibitory action of NO.

With regard to Ag-driven downstream (post-PLC γ) events, NO did not influence Fc ϵ RI-induced phosphorylation of p38 MAPK, p44/42 MAPK, JNK, IKK, CREB, and NF- κ B. However, NO did significantly inhibit Ag-induced phosphorylation of c-Jun. In additional experiments, the NO donor completely blocked Ag-induced induction of DNA-binding activity of the nuclear proteins c-Jun, JunB, JunD, FosB, and c-Fos, which as homo- or heterodimers make up the AP-1 transcription factor (39). In parallel, Ag-induced DNA binding of NF-AT to its DNA response element was only partially inhibited by NO. We observed no Ag-induced DNA-binding activity of the AP-1 proteins Fra-1 or Fra-2, or of the NF- κ B p50 protein that is activated in human lung mast cells (40). Alongside our finding that IKK and NF- κ B were only weakly phosphorylated in BMMC, we believe that AP-1 and NF-AT may be more important transcription factors than NF- κ B in these cells.

In conclusion, our results show that NO inhibits Ag-induced mast cell cytokine expression and to a lesser extent degranulation. Inhibition of degranulation is accounted for by partial inhibition of phosphorylation of PLC- γ 1, whereas, in relation to cytokine expression, NO acts post-MAPK/JNK to completely abrogate Ag-induced activation of Fos and Jun. This study represents the first step in defining at the molecular level the actions of NO in gene regulation in mast cells, and suggests that NO inhibition of mast cell cytokine production might be an important component in the resolution or self-limitation of allergic inflammation.

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