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Role of nitric oxide on *in vitro* human eosinophil migration

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Abstract

Eosinophils purified from the rat peritoneal cavity have been found to contain nitric oxide synthase (NOS) functionally coupled to a cyclic GMP transduction pathway that is involved in *in vitro* eosinophil migration, but no studies on cell locomotion have been done with purified human eosinophils. Therefore, this study was carried out to investigate the effects of N^{ω} -nitro-L-arginine methyl ester (L-NAME; a non-selective NOS inhibitor), 1-(2-trifluoromethylphenyl) imidazole (TRIM; a type I/type II NOS inhibitor), 2-amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine (AMT; a selective type II NOS inhibitor), and 1*H*-[1,2,4]-oxidiazolo[4,3-*a*] quinoxalin-1-one (ODQ; a soluble guanylate cyclase inhibitor) on human eosinophil migration induced by *N*-formyl-methionyl-leucyl-phenylalanine (fMLP). Human eosinophils were purified from peripheral blood of healthy volunteers using a Percoll gradient followed by an immunomagnetic cell separator. Chemotaxis was evaluated using a 48-well microchemotaxis chamber. The fMLP (1.0×10^{-7} M)-induced eosinophil migration was reduced significantly by l-NAME (0.1 and 1.0 mM), whereas the inactive enantiomer N^{ω}-nitro-D-arginine methyl ester (D-NAME) had no effect. The inhibition by l-NAME was restored by sodium nitroprusside (0.25 mM). The NOS inhibitors AMT and TRIM (0.05 to 0.25 mM each) also markedly attenuated fMLP-induced chemotaxis. Additionally, ODQ (0.01 to 0.5 mM) concentration-dependently inhibited fMLP-induced migration, and the inhibition was restored by 2.0 mM dibutyryl cyclic GMP. In conclusion, this study demonstrates that human eosinophils present a nitric oxide-cyclic GMP pathway that is involved in the *in vitro* locomotion of this cell type. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Leucocyte migration; Soluble guanylate cyclase; Nitric oxide synthase inhibitors; L-NAME

1. Introduction

Eosinophils play an important role in host defense mechanisms in parasitic infestation and pathogenesis of allergic, immunological, and malignant disorders [1,2]. A number of both preformed (major basic protein, eosinophil cationic protein, eosinophil peroxidase, and protein X) and *de novo* synthesized (eicosanoids, platelet-activating factor, oxygen metabolites, cytokines, and neuropeptides) proinflammatory substances are produced and released by eosinophils [3]. Recently, immunohistochemical studies revealed that rat [4] and human [5] eosinophils express type II (inducible) and type III (endothelial) NOSs, and activated rat peritoneal eosinophils are able to release NO that is responsible for their microbicidal activity against *Leishmania major* [6]. Furthermore, studies carried out in rats demonstrated that L-NAME [7], TRIM (a type I/type II NOS inhibitor) [8], and/or AMT (a selective type II NOS inhibitor) [9] attenuate *in vitro* and *in vivo* eosinophil chemotaxis, strongly indicating an important role for NO in eosinophil locomotion [4,10,11]. Since no evaluation of the role of the NO-cGMP pathway in human eosinophil locomotion was done, the present study was undertaken to examine the effects of non-selective (L-NAME) and selective (TRIM and AMT) NOS inhibitors, as well as of the soluble guanylate cyclase inhibitor ODQ, on *in vitro* chemotaxis of human purified eosinophils stimulated with fMLP.

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Abbreviations: AMT, 2-amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; cGMP, db-cGMP, dibutyryl cyclic GMP; D-NAME, N^{ω}-nitro-D-arginine methyl ester; L-NAME, N^{ω}-nitro-L-arginine methyl ester; L-NIL, L-N^{6}-(1-iminoethyl) lysine; NO, nitric oxide; NOS, nitric oxide synthase; ODQ, 1*H*-[1,2,4]-oxidiazolo[4,3-*a*] quinoxalin-1-one; and TRIM, 1-(2-trifluoromethylphenyl) imidazole.

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2. Materials and methods

2.1. Materials

The VarioMACS system and microbeads were purchased from Miltenyi Biotec Inc. fMLP, L-NAME, D-NAME, db-cGMP, sodium nitroprusside, ODQ, AMT, and Percoll were purchased from the Sigma Chemical Co. Polycarbonate filters (5 μm) were obtained from Nucleopore. Diff-Quik and TRIM were obtained from the Baxter Healthcare Corp. and Tocris Cookson, respectively.

2.2. Eosinophil isolation

Blood was collected from healthy volunteers (male and female, aged 18–50 years) who were not on medication. Informed consent and approval from the local ethical committee were obtained before the study.

Human eosinophils were isolated from peripheral blood using a method adapted from that of Hansel *et al.* [12]. Briefly, 60 mL of blood collected in 3.13% (w/v) sodium citrate from a healthy subject was diluted 1:1 with PBS and 30 mL of diluted blood overlaid onto a 15-mL Percoll gradient (1.130 $\pm$ 0.005 g/mL, pH 7.4, 340 mosmol/kg of H_2O). Gradients were centrifuged at 700 *g* for 20 min at 4° (Hermle model Z360k centrifuge), and the cell pellet was collected. Red cells contained in the granulocyte pellet were lysed with lysing buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA pH 7.4). Washed granulocytes were incubated with anti-CD16 immunomagnetic microbeads (Miltenyi Biotec) before passing on a steel-matrix column in a magnetic field (Miltenyi Biotec) and the CD16-negative eosinophils (92–99% purity) were then resuspended in Eagle's minimum essential medium (MEM; pH 7.2). Contaminating cells were mononuclear cells.

2.3 Chemotaxis assay

Eosinophils were resuspended at a concentration of 5×10^6 cells/mL in MEM/ovalbumin, and migration assays were performed using a 48-well microchemotaxis chamber [13]. The bottom wells of the chamber were filled with the chemoattractant agent fMLP (2.5×10^{-8} to 2.5×10^{-7} M) in 27 μL MEM, whereas the upper wells were filled with eosinophils (50 μL) that had been treated with drugs or MEM. The bottom and upper cells were separated by a polycarbonate filter of 5 μm (Nucleopore). The chamber was then incubated for 1 hr at 37° in a 5% CO_2 atmosphere. At the end of the incubation period, the filter was removed, washed, fixed in methanol for 2 min, stained with Diff-Quik, and mounted on a glass slide. Each incubation was carried out in triplicate, and migration was determined by counting eosinophils that had migrated completely through the filter in five random high-power fields (HPF; 1000×) per well.

2.4. In vitro treatment of eosinophils

Before performing the chemotaxis assay, eosinophil suspensions were treated with L-NAME (0.1 and 1.0 mM), D-NAME (0.1 and 1.0 mM), ODQ (0.01 to 0.5 mM), AMT (0.05 to 0.25 mM), TRIM (0.05 to 0.25 mM), or their corresponding vehicle (5 μL/mL) for 30 min, at 37° in a 5% CO_2 atmosphere. In some experiments, sodium nitroprusside (0.25 mM) and db-cGMP (2.0 mM) were co-incubated with L-NAME (1.0 mM) or ODQ (0.25 mM), respectively.

2.5. Statistical analysis

Data are expressed as the means $\pm$ SEM of at least three separate experiments, each carried out in triplicate. Data were analysed by ANOVA for multiple comparisons followed by Tukey's test. A value of $P < 0.05$ was taken as significant.

3. Results

3.1. Effects of different NOS inhibitors on fMLP-stimulated eosinophil chemotaxis

The chemotactic agent fMLP caused a concentration-dependent human eosinophil chemotaxis (8.4 $\pm$ 1.2, 9.8 $\pm$ 2.0, 13.5 $\pm$ 0.5, and 12.4 $\pm$ 0.5 cells/HPF for 2.5×10^{-8}, 5.0×10^{-8}, 1.0×10^{-7}, and 2.5×10^{-7} M, respectively) compared with random migration (3.8 $\pm$ 1.0 cells/HPF; N = 4). For further studies, a concentration of 1.0×10^{-7} M fMLP was used routinely.

Figure 1 shows that incubation of human eosinophils (30 min, 37°) with the non-selective NOS inhibitor L-NAME (0.1 and 1.0 mM; N = 4) significantly inhibited fMLP-induced chemotaxis, whereas the inactive enantiomer D-NAME (same concentrations) had no effect. The inhibition of fMLP-induced chemotaxis by L-NAME (1.0 mM; N = 3) was restored by co-incubating the eosinophil suspension with 0.25 mM sodium nitroprusside (Fig. 2). Sodium nitroprusside alone did not change the fMLP-induced eosinophil migration significantly (Fig. 2).

The type I/type II NOS inhibitor TRIM (0.05 to 0.25 mM) and the selective type II NOS inhibitor AMT (0.05 to 0.25 mM) also concentration-dependently reduced fMLP-induced eosinophil migration (Fig. 3; N = 3). At the same concentrations used, no statistical differences were found between AMT and TRIM.

Random migration (non-stimulated cells) was not affected significantly by L-NAME (2.2 $\pm$ 0.6, 2.2 $\pm$ 0.5, and 2.0 $\pm$ 0.7 for control and L-NAME at 0.1 and 1.0 mM, respectively; N = 4). Similarly, AMT and TRIM (up to 1.0 mM each) had no effect on random migration (N = 3; not shown).

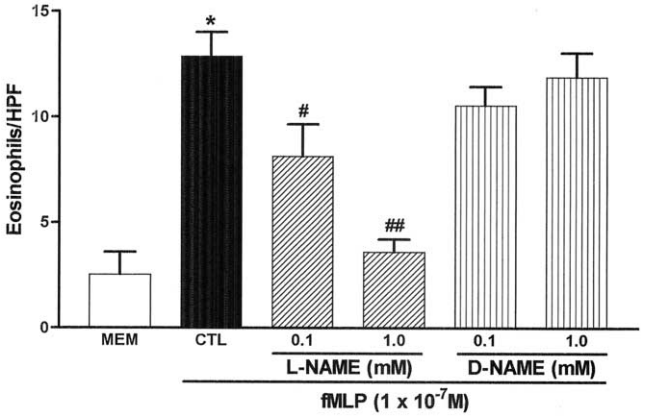

Fig. 1. Inhibition by L-NAME of fMLP (1.0×10^{-7} M)-induced human eosinophil migration. The human eosinophil suspension (5×10^6 cells/mL) was incubated previously (37°, 30 min) with either L-NAME (0.1 and 1.0 mM; right-hatched columns) or the inactive enantiomer D-NAME (0.1 and 1.0 mM; striped columns). Control migration (CTL) is represented by the solid column, whereas random chemotaxis (MEM; in the absence of fMLP) is represented by the open column. Each experiment was carried out in triplicate. Eosinophil migration is expressed as the mean number of migrated cells per high-power field (HPF). Results are means ± SEM. Key: (*) $P < 0.001$ compared with MEM; and (#) $P < 0.01$ and (##) $P < 0.001$ compared with CTL.

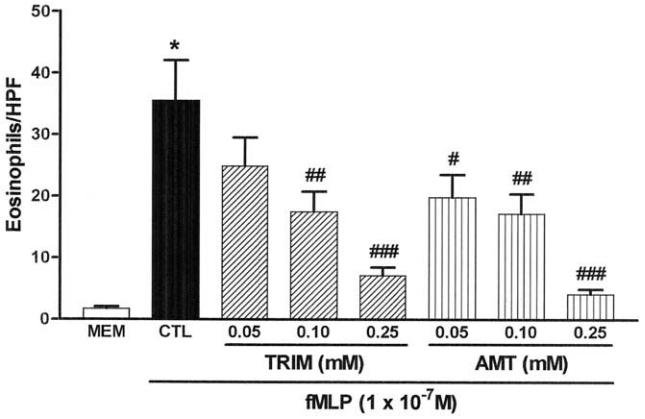

Fig. 3. Inhibition by TRIM and AMT of fMLP (1.0×10^{-7} M)-induced human eosinophil migration. The human eosinophil suspension (5×10^6 cells/mL) was preincubated (37°, 30 min) with either TRIM (0.05 to 0.25 mM; right-hatched columns) or AMT (0.05 to 0.25 mM; striped columns). Control migration (CTL) is represented by the solid column, whereas random chemotaxis (MEM; in the absence of fMLP) is represented by the open column. Each experiment was carried out in triplicate. Eosinophil migration is expressed as the mean number of migrated cells per high-power field (HPF). Results are means ± SEM. Key: (*) $P < 0.001$ compared with MEM; and (#) $P < 0.05$, (##) $P < 0.01$, and (###) $P < 0.001$ compared with CTL.

3.2. Effect of the soluble guanylate cyclase inhibitor ODQ on fMLP-stimulated eosinophil chemotaxis

Figure 4 shows that incubation of eosinophils with ODQ (0.01 to 0.5 mM) concentration-dependently inhibited fMLP-induced eosinophil chemotaxis as compared with the vehicle (5 μL/mL of DMSO; N = 3). The inhibition of fMLP-induced eosinophil chemotaxis by ODQ (0.25 mM) was restored by co-incubating the eosinophil suspension with db-cGMP (2.0 mM, N = 4; Fig. 5). The random migration was not affected significantly by ODQ (N = 3; not shown).

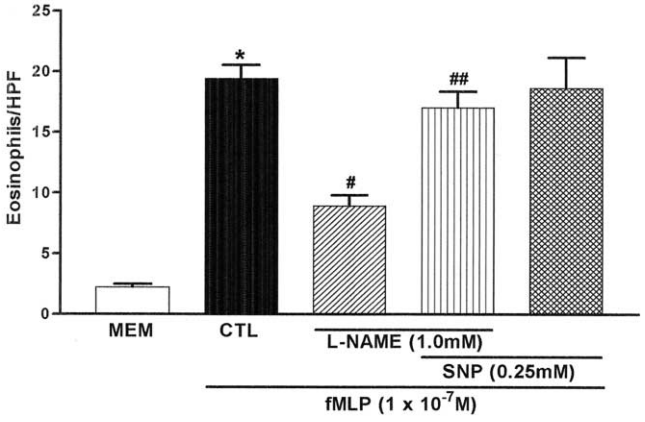

Fig. 2. Reversal by sodium nitroprusside (SNP, 0.25 mM) of L-NAME inhibition of fMLP (1.0×10^{-7} M)-induced human eosinophil migration. The human eosinophil suspension (5×10^6 cells/mL) was preincubated (37°, 30 min) with L-NAME (1.0 mM) in the absence (right-hatched column) or the presence of SNP (0.25 mM; striped column). The response to SNP alone is shown by the cross-hatched column. Control migration (CTL) is represented by the solid column, whereas random chemotaxis (MEM; in the absence of fMLP) is represented by the open column. Each experiment was carried out in triplicate. Eosinophil migration is expressed as the mean number of migrated cells per high-power field (HPF). Results are means ± SEM. Key: (*) $P < 0.001$ compared with MEM; (#) $P < 0.001$ compared with CTL; and (##) $P < 0.001$ compared with L-NAME in the absence of SNP.

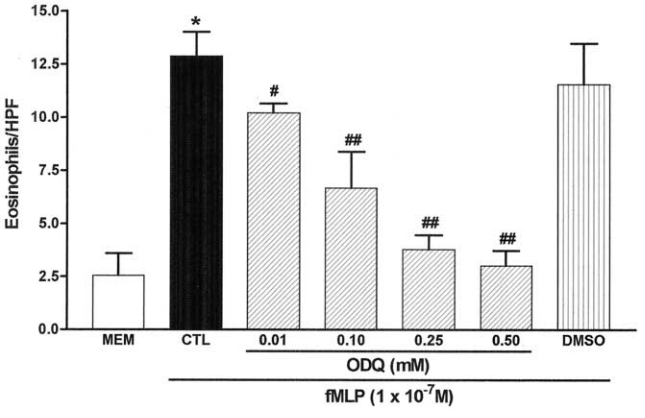

Fig. 4. Inhibition by ODQ of fMLP (1.0×10^{-7} M)-induced human eosinophil migration. The human eosinophil suspension (5×10^6 cells/mL) was preincubated (37°, 30 min) with ODQ (0.01 to 0.5 mM; right-hatched columns) or its vehicle (5 μL/mL of DMSO; striped column). Control migration (CTL) is represented by the solid column, whereas random chemotaxis (MEM; in the absence of fMLP) is represented by the open column. Each experiment was carried out in triplicate. Eosinophil migration is expressed as the mean number of migrated cells per high-power field (HPF). Results are means ± SEM. Key: (*) $P < 0.001$ compared with MEM; and (#) $P < 0.05$ and (##) $P < 0.001$ compared with CTL.

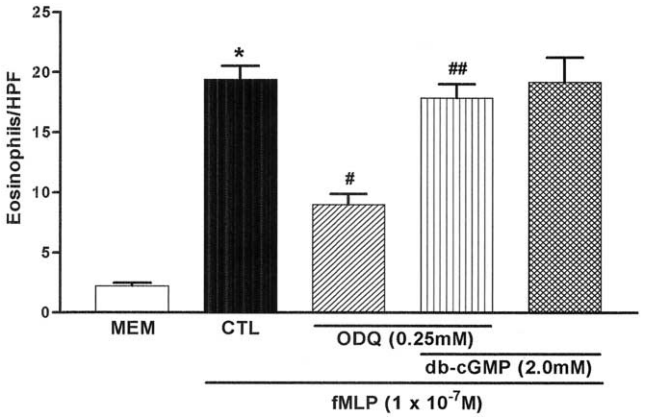

Fig. 5. Effect of db-cGMP on the inhibition by ODQ of fMLP (1.0×10^{-7} M)-induced human eosinophil migration. The human eosinophil suspension (5×10^6 cells/mL) was preincubated (37°, 30 min) with ODQ (0.25 mM) in the absence (right-hatched column) or the presence of 2.0 mM db-cGMP (striped column). The response to db-cGMP alone is shown by the cross-hatched column. Control migration (CTL) is represented by the solid column, whereas random chemotaxis (MEM; in the absence of fMLP) is represented by the open column. Each experiment was carried out in triplicate. Eosinophil migration is expressed as the mean number of migrated cells per high-power field (HPF). Results are means ± SEM. Key: (*) $P < 0.001$ compared with MEM; (#) $P < 0.001$ compared with CTL; and (##) $P < 0.001$ compared with ODQ alone.

4. Discussion

Our results demonstrate for the first time the existence of a functional NO-cGMP pathway in human purified eosinophils that modulate the *in vitro* locomotion of this cell type. This conclusion is based on our findings that L-NAME, AMT, and TRIM markedly attenuated fMLP-induced eosinophil migration, an effect completely reversed by the NO-donor compound sodium nitroprusside. Additionally, the soluble guanylate cyclase inhibitor ODQ reduced eosinophil chemotaxis, and db-cGMP reversed this effect, thus indicating that the transduction mechanism involves intracellular cGMP accumulation. Since these inhibitors exert a similar inhibitory effect on the chemotactic responses of eosinophils purified from the rat peritoneal cavity [4], we suggest that NO has a crucial role for eosinophil locomotion, irrespective of the animal species studied.

The selective migration of eosinophils from the circulation into tissues involves a stepwise interaction between eosinophils and endothelial cells, including reversible binding and subsequent rolling, firm adhesion, and transmigration of adherent eosinophils through adhesion molecules on endothelial cells and counter-ligands on eosinophils [14,15]. The migration of eosinophils into tissues is initiated by local chemoattractant molecules and numerous chemotactic substances from tissue and/or resident cells acting on eosinophils [3]. The role of NO on *in vivo* leucocyte adhesion and migration has been shown to be controversial. While some authors report that *in vivo* NO blockade leads to an increase in the leucocyte rolling and adhesion to vascular endothelium in cat and rat intravital microscopy preparations [16–18], others have reported that it markedly attenuates the eosinophil accumulation in the guinea pig cutaneous microcirculation [19], rat pleural cavity [10], and mouse and rat bronchoalveolar lavage fluid [11,20]. This suggests that, depending on the type and phase of inflammation and the vascular or cellular response studied, NO may present pro-inflammatory or anti-inflammatory properties. Although reduction of infiltrating leucocytes into inflammatory sites by NOS inhibitors may be due to mechanisms dependent upon [19] and independent [21] of the local reduction of blood flow as well as on the potential involvement of $CD4^+$ lymphocytes [22,23], our findings that they attenuate the eosinophil migration in isolated cells indicate that this phenomenon reflects a direct effect on the eosinophil itself.

Human eosinophils contain an inducible NOS of high identity with macrophage/monocyte inducible NOS, as detected by immunocytochemistry, immunoblotting, and RT–PCR [5]. Accordingly, our study showed that, in addition to L-NAME, the migration of stimulated eosinophils was largely attenuated by the inducible NOS inhibitors AMT and TRIM, suggesting that *in vitro* eosinophil locomotion is driven by NO through the activity of a type II NOS. Interestingly, the inducible NOS inhibitor L-NIL affected neither eosinophilic lung infiltration nor eosinophil release from bone marrow in allergic mice, and also failed to increase the levels of inducible NOS protein (or mRNA) in the lungs and nitrite in BAL fluid [20]. Since rodent eosinophils also present constitutive type III NOS [4], one may speculate that this isoform also plays a role in *in vivo* eosinophil locomotion.

NO activates soluble guanylate cyclase, thereby increasing intracellular levels of cGMP. Cyclic nucleotides are known to modulate leucocyte activation processes, but their role in cell locomotion is controversial. In isolated neutrophils and mononuclear cells, an increase of cGMP can either stimulate [24–29] or inhibit [30–32] *in vitro* chemotaxis. Similar to rats [4], our present study showed that the soluble guanylate cyclase inhibitor ODQ [33] concentration-dependently reduced fMLP-induced eosinophil chemotaxis, an effect reversed by the addition of db-cGMP, indicating that this second messenger has an essential role in eosinophil migration. The findings that the NO-donor compounds azide and hydroxylamine inhibit programmed cell death of human isolated eosinophils and that this effect is mimicked by permeable cGMP analogues [34] reinforce our proposal.

In conclusion, eosinophil chemotaxis induced by fMLP was attenuated markedly by inhibitors of NOS (L-NAME, AMT, and TRIM) and soluble guanylate cyclase (ODQ), and this reduction was reversed significantly by NO-donor and/or db-cGMP, suggesting that NO through increased cGMP is implicated in human eosinophil chemotaxis.

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