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Nitric oxide has a role in regulating VLA-4-integrin expression on the human neutrophil cell surface

Nicola Conran[a,*], Alessandra Gambero[b], Heloisa H.A. Ferreira[b], Edson Antunes[a], Gilberto de Nucci[a]

[a]*Department of Pharmacology, FCM, UNICAMP, Campinas, Brazil*
[b]*Clinical Pharmacology Unit, USF, Bragança Paulista, Brazil*

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Abstract

Recent research demonstrates that the β1 integrins may be involved in neutrophil migration. Here, we investigate the role of nitric oxide in the expression and function of the very late antigen-4 (VLA-4) and Mac-1 integrins on human neutrophils. Human blood neutrophils were treated with N^{ω}-nitro-L-arginine methyl ester (L-NAME) and their adhesion to fibronectin (FN) and serum observed. Adhesion of neutrophils to FN and serum increased significantly following incubation with 0.1 mM L-NAME by 65.5 and 44.6%, respectively. Increased adhesions to FN and serum were abolished by a VLA-4-specific monoclonal antibody, HP2/1, and a Mac-1-specific monoclonal antibody, ICRF 44, respectively. The microfilament- and microtubule-depolymerizing agents, dihydrochalasin B and nocodazole, were also able to reverse L-NAME-induced adhesion to both FN and serum. L-NAME induced a discrete increase in the expression of CD49d (VLA-4, 25.3 ± 4.8%), but not CD11b, on the neutrophil cell surface, as detected by flow cytometry. Results indicate that NO has a role in regulating VLA-4 and Mac-1 function on the human neutrophil cell surface and that this modulation in integrin function is accompanied by cytoskeletal rearrangements and changes in the ability of the neutrophil to adhere to the extracellular matrix.

Keywords: Adhesion molecules; Cytoskeleton; Mac-1; Neutrophil; Nitric oxide synthase inhibitors; VLA-4

1. Introduction

Leukocyte emigration from the blood vessel into surrounding tissue is a major feature of inflammatory diseases. This transmigration process involves a number of steps involving a sequence of leukocyte adhesion molecule interactions with the endothelial cell and extracellular matrix (ECM) ligands. Tethering and rolling of the leukocyte on the endothelial cell layer is thought to be mediated by a number of neutrophil ligands, including L-selectin and P-selectin glycoprotein ligand-1 with their corresponding endothelial cell ligands [1,2]. This step is followed by 'firm adhesion' of the leukocyte to the endothelial cell layer mediated largely by the β_2 integrins (Mac-1, LFA-1) on the neutrophil cell surface [3], non-neutrophilic leukocytes can also use the VLA-4 integrin ($\alpha_4\beta_1$) in this and possibly the rolling stage [4]. Final transendothelial migration is facilitated principally by Mac-1 and VLA-4 in non-neutrophilic leukocytes [5].

Whilst the β_2 integrins are accepted to be the major integrin mediators of neutrophil adhesion to endothelial cells during migration, a β_2-independent mechanism may also be involved in the infiltration of neutrophils into lungs, joints, and peritoneum [6–8]. Chemotactically stimulated human neutrophils express VLA-4 when allowed to transmigrate across endothelial layers or when treated with dihydrocytochalasin B (DHCB) together with *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), PAF, or IL-8 [9]. Furthermore, these DHCB-treated neutrophils are able to

* Corresponding author. Present address: Av. Jesuino Marcondes Machado, 415, 13092-320, Campinas, SP, Brazil. Tel.: +55-19-3788-8734; fax: +55-19-3252-1516.
E-mail address: conran@lexxa.com.br (N. Conran).

Abbreviations: cGMP, cyclic guanosine 3′,5′-monophosphate; DHCB, dihydrocytochalasin B; ECM, extracellular matrix; FITC, fluorescein isothiocyanate; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; FN, fibronectin; HUVEC, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule; LFA-1, leukocyte functional antigen-1; L-NAME, N^{ω}-nitro-L-arginine methyl ester; mAb, monoclonal antibody; MEM, Eagle's minimum essential medium; MPO, myeloperoxidase; NO, nitric oxide; NOS, nitric oxide synthase; ODQ, *H*-[1,2,4] oxadiazolo quinoxalin-1-one; PAF, platelet activating factor; VCAM-1, vascular cell adhesion molecule 1; VLA-4, very late antigen-4.

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adhere to endothelium *via* an alternative VLA-4-dependent pathway under flow conditions [10]. VLA-4 can be mobilized by human peripheral blood neutrophils and utilized in conjunction with VLA-6 and Mac-1 to mediate neutrophil migration through connective tissue [11]. A special role for the VLA-9 integrin ($\alpha_9\beta_1$), in conjunction with other β_1 integrins, has also been postulated by Shang *et al*. [12] in mediating neutrophil migration in the extravascular space.

Human neutrophil *in vitro* chemotaxis can be inhibited by inhibitors of nitric oxide (NO) synthesis [13] and evidence suggests that NO may effect neutrophil chemotaxis *via* a cyclic guanosine 3′,5′-monophosphate (cGMP)-dependent pathway [14–16]. To date there has been very little research into the role of NO in human neutrophil adhesion molecule interactions. NO donors inhibit both TNF-α-induced human neutrophil adhesion to endothelial cells [17] and E-selectin expression in neutrophils and their adhesion to lipopolysaccaride-stimulated vascular endothelial cells [18]. The nitric oxide synthase (NOS) inhibitor, N^G-monomethyl-L-arginine (L-NMMA), increased the expression of CD11/CD18 on cat neutrophils [19]. Furthermore, administration of NO, or NO-donating species, inhibits neutrophil adhesion in animal models of ischemia/reperfusion, lung injury, and peritonitis [20].

The effect of blocking neutrophil NO synthesis upon cell surface integrin expression and cell adhesion interactions with ECM components and endothelial cell ligands was investigated herein.

2. Materials and methods

2.1. Materials

Blood was collected from healthy volunteers. Mouse IgG1 monoclonal antibodies (mAbs) specific for the α_4 and α_M integrin subunits (clones HP2/1 and ICRF 44, respectively) and the FITC-conjugated goat anti-mouse IgG secondary antibody were purchased from Serotec USA. The IgG1 isotype control mAb was obtained from Rockland. All other products were bought from Sigma Co, unless otherwise stated.

2.2. Isolation of human neutrophils from peripheral blood

Neutrophils were separated from peripheral blood collected in 3.13% (w/v) sodium citrate (10:1), whole blood was laid over two layers of Ficoll-Paque of densities of 1.077 and 1.019 g/L, respectively. After separation of monocytes and granulocytes by centrifugation at 700 *g* (Hermle model Z 360k centrifuge) for 28 min, the granulocyte layer was washed once in Eagle's minimum essential medium (MEM), pH 7.2, before performing a hypotonic lysis to lyse contaminating red cells. Cells were washed once again in MEM before resuspending finally in MEM/0.1% ovalbumin. The total cell number was calculated (cells were diluted 1:20 and counted in an Improved Neubauer Haemocytometer) and cells were cytospinned onto slides and a cell differentiation count performed. Cells were stored at 4° until use.

2.3. Neutrophil adhesion assays

Cells were treated with L-NAME, D-NAME, DHCB, or nocodazole with or without the presence of the antibodies listed above before performance of adhesion assays. Cells suspended in MEM/0.1% ovalbumin were incubated with the drug and/or antibody of choice for 10 min at 37°, 5% CO_2. Treated cells were then used immediately in the assay.

Wells of 96-well plates were prepared by coating individual wells with 60 μL of either 20 μg/mL FN or 10% (v/v) serum (in PBS) overnight at 4°. Wells were then washed twice with PBS before blocking non-coated sites with 0.1% (w/v) BSA for 60 min at 37°. Wells were washed twice again with PBS before allowing plates to dry.

Neutrophils (50 μL of 2×10^6 cells/mL in MEM/ovalbumin) were seeded onto the coated wells of a 96-well plate and cells were allowed to adhere to FN and serum for 15 min (optimum times for cell adhesion demonstrated by time course experiments, results not shown) at 37°, 5% CO_2.

Following incubation, non-adhered cells were washed thrice with PBS. MEM (50 μL) was added to each well and varying concentrations of the original neutrophil cell suspension were added to empty wells to form a standard curve. Plates were then stored frozen overnight before measuring the MPO content of adherent cells and calculating percentage neutrophil adhesion [21]. Briefly, plates were defrosted on ice before extracting MPO from adhered cells in each well by adding 0.5% (w/v) hexadecyltrimethylammonium bromide in 50 mM potassium phosphate buffer, pH 6.0. Twenty microliters of each well sample to be measured were mixed with 200 μL of *o*-dianisidine solution (0.167 mg/mL *o*-dianisidine dihydrochloride, 0.0005% hydrogen peroxide in 50 mM phosphate buffer, pH 6.0) immediately prior to reading change of absorbance at 460 nm over 5 min using a microplate reader (Multiscan MS, Labsystems). Adherence was calculated by comparing absorbance changes of unknowns to those of the standard curve.

2.4. Flow cytometry

Expression of adhesion molecules on the surface of neutrophils was detected using flow cytometry.

Isolated neutrophils (5×10^6 cells/mL) were incubated with the drug of choice (30 min, L-NAME/D-NAME; 5 min, fMLP; optimum incubation times were determined by time course experiments) before incubating 50 μL of the cell suspension with a saturating concentration of adhesion molecule mAb (ICRF 44, anti-α_M; HP2/1, anti-α_4), or a suitable isotype control for 30 min, 4°. After centrifugation of cells (300 *g*, 10 min) and removal of supernatant, cells were incubated with FITC-conjugated secondary antibody

(30 min, 4° in dark). The cells were then fixed in 0.5 mL 1% paraformaldehyde (10 min, 4 °) before washing twice with buffer (PBS/0.1% BSA). Cells (50,000) were analyzed at 488 nm on a Becton-Dickinson FACS Calibur. SSC/FSC (side scatter/forward scatter) dot plots were used to gate the neutrophil population ensuring that only the cell population of interest was analyzed. The fluorescence intensity of each cell was compared to that of isotype controls.

2.5. Statistical analysis

Results are expressed as means ± SE, with the number of different experiments given in Section 3 and figure legends. Unless otherwise stated, statistical significance between groups was determined using the Tukey test for analysis of variance. Where appropriate, Student's *t*-test (unpaired) was used to compare specific groups. Significance was established at $P < 0.05$.

3. Results

3.1. Effect of L-NAME upon human neutrophil adhesion to human FN and serum

Pre-incubation of neutrophils (10 min, 37°, 5% CO_2) with L-NAME (0.01–1.0 mM) significantly increased the adhesion of cells to both FN (20 μg/mL) and serum (10% v/v)-coated plates (15 min, 37°, 5% CO_2) (see Fig. 1). D-NAME had no significant effect upon the adhesion of neutrophils to either FN or serum. Basal adhesion to FN and serum was 26.6 ± 2.0% and 32.7 ± 1.4%, respectively, and following treatment with 0.1 mM D-NAME, adhesion was 28.3 ± 2.9% (N = 4) and 29.7 ± 2.7% (N = 4), respectively.

3.2. Effect of VLA-4- and Mac-1-blocking mAbs upon basal neutrophil adhesion to FN and serum

Incubation of neutrophils (10 min, 37°, 5% CO_2) with the anti-VLA-4 mAb, HP2/1 (at a saturating concentration of 10 μg/mL), did not significantly reduce basal neutrophil adhesion to FN or serum. In contrast, pre-incubation of cells with the Mac-1-specific mAb, ICRF 44 (at a saturating concentration of 10 μg/mL), significantly decreased the basal adhesion of neutrophils to FN and to serum. Similar results were obtained when cells were pre-incubated with both ICRF 44 and HP2/1 together, whilst a non-specific control mAb did not significantly affect neutrophil adhesion to FN or serum (see Fig. 2).

3.3. Effect of VLA-4- and Mac-1-blocking mAbs upon L-NAME-induced neutrophil adhesion to FN and serum

The increased adhesion of neutrophils to FN (15 min, 37°, 5% CO_2) seen after treatment of cells with L-NAME (0.1 mM) (10 min, 37°, 5% CO_2) was reversed when cells were co-incubated with HP2/1 (10 μg/mL) (Fig. 3a). Neither the Mac-1-specific mAb, ICRF 44, nor a non-specific control mAb (10 μg/mL) had any significant effect upon the adhesion of L-NAME-treated cells to FN.

Pre-incubation of neutrophils with ICRF 44 (10 μg/mL) as well as L-NAME abolished the increase in adhesion to serum seen when cells were pre-incubated with L-NAME alone (see Fig. 3b). The HP2/1 mAb also significantly reversed the L-NAME-induced increase in adhesion to serum, but did not totally abolish it, whilst a non-specific mAb had no significant effect upon the increased adhesion.

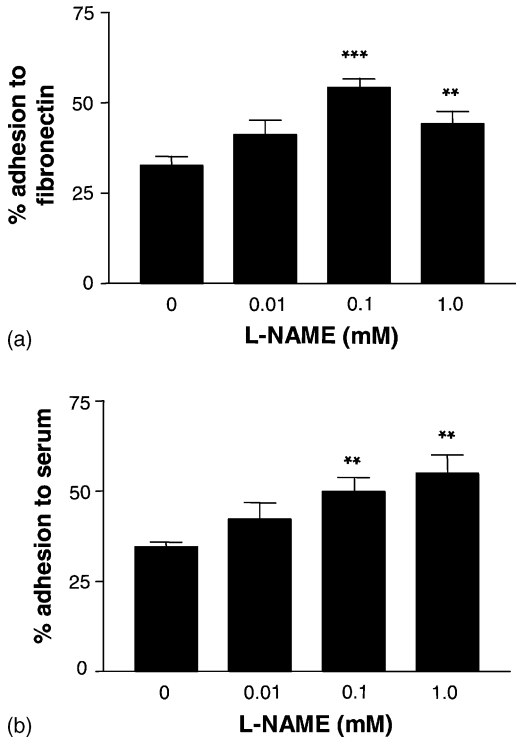

Fig. 1. Effect of L-NAME (0.01–1.0 mM) upon neutrophil adhesion to (a) 20 μg/mL FN and (b) 10% (v/v) human serum. Neutrophils (>95% pure) were incubated with L-NAME (10 min, 37°, 5% CO_2) before allowing cells to adhere to FN/serum-coated plates (15 min, 37°, 5% CO_2). Results are expressed as adhered cell percentages of total cell number ± SE of five independent experiments with three replicates in each. $^{**}P < 0.01$, $^{***}P < 0.001$, compared to control cell basal adhesion.

3.4. Effect of the soluble guanylate cyclase inhibitor, H-[1,2,4] oxadiazolo quinoxalin-1-one (ODQ), upon neutrophil adhesion to fibronectin and serum

Incubation of neutrophils with ODQ had no significant effect upon cell adhesion to FN or serum. Control cell adhesion to FN was 31.8 ± 1.9% and following incubation with ODQ (25 min, 37°, 5% CO_2) was 27.6 ± 4.1% (0.01 mM ODQ), 30.1 ± 5.1% (0.1 mM ODQ), 27.0± 6.3% (0.25 mM ODQ) (N = 6). Untreated cell adhesion to serum was 36.9 ± 4.2% and following incubation with ODQ was 33.2 ± 5.0% (0.01 mM ODQ), 26.3 ± 5.2%

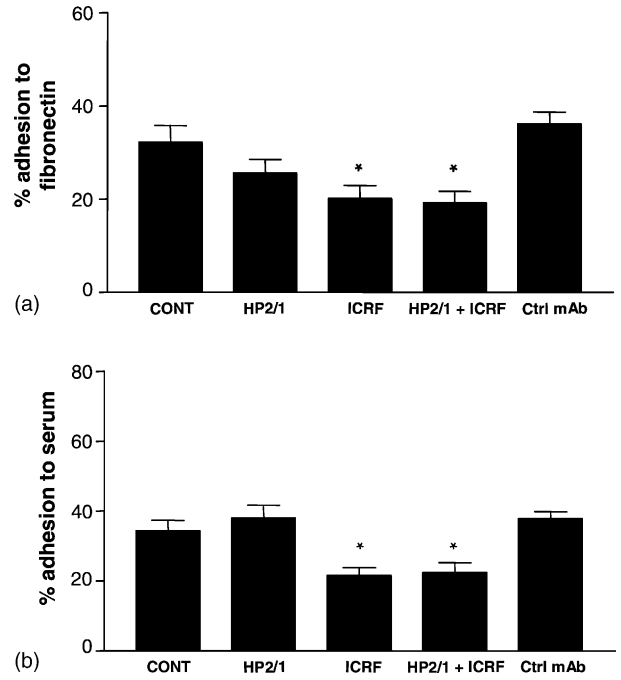

Fig. 2. Effects of the anti-VLA-4 (HP2/1, 10 μg/mL), anti-Mac-1 (ICRF 44, 10 μg/mL) mAbs, and a non-specific control mAb (Ctrl mAb, 10 μg/mL) upon basal human neutrophil adhesion to (a) 20 μg/mL FN and (b) 10% (v/v) human serum. Neutrophils (>95% pure) were incubated with mAbs (10 min, 37°, 5% CO_2) before allowing cells to adhere to FN/serum-coated plates (15 min, 37°, 5% CO_2). Results are expressed as adhered cell percentages of total cell number ± SE of five independent experiments with three replicates in each. $^{*}P < 0.05$ compared to cells control cell basal adhesion.

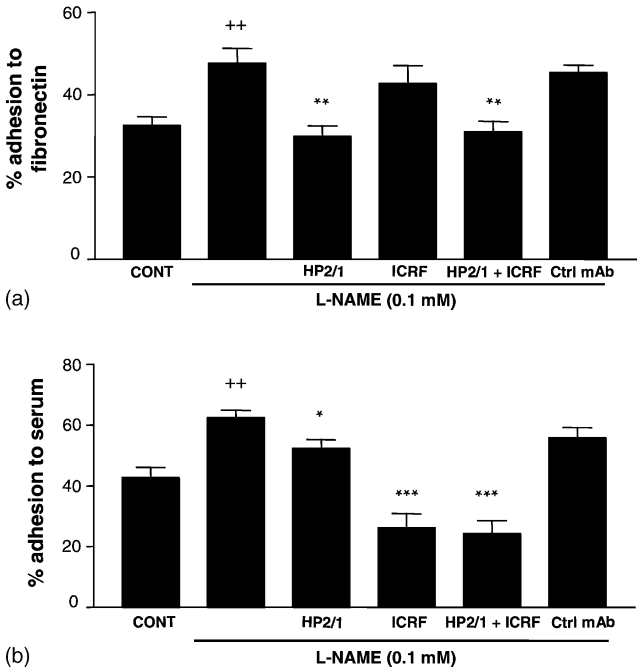

Fig. 3. Effects of the anti-VLA-4 (HP2/1, 10 μg/mL), anti-Mac-1 (ICRF 44, 10 μg/mL) mAbs, and a non-specific control mAb (Ctrl mAb, 10 μg/mL) upon human neutrophil adhesion after treatment with L-NAME (0.1 mM) to (a) 20 μg/mL FN and (b) 10% (v/v) human serum. Neutrophils (>95% pure) were incubated with mAbs (±L-NAME) (10 min, 37°, 5% CO_2) before allowing cells to adhere to FN/serum-coated plates (15 min, 37°, 5% CO_2). Results are expressed as adhered cell percentages of total cell number ± SE of four independent experiments with three replicates in each. $^{++}P < 0.01$, compared to untreated cells. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared to cells treated with L-NAME alone (0.1 mM).

(0.1 mM ODQ), 26.7 ± 7.2% (0.25 mM ODQ) (N = 8). The vehicle in which ODQ was solubilized (DMSO) had no effect upon neutrophil adhesion to either FN or serum (28.7 ± 6.7% and 36.8 ± 7.1%, respectively, N = 6/8).

3.5. Effect of L-NAME upon the expression of Mac-1 and VLA-4 integrins on the human neutrophil cell surface

The Mac-1 integrin ($\alpha_M\beta_2$) was detected on the surface of 90.9 ± 1.7% of human neutrophils using flow cytometry. Treatment of neutrophils with 0.05 μM fMLP significantly increased the mean expression of the adhesion molecule on each cell, whilst L-NAME (0.1–2.5 mM) had no effect upon the mean expression of Mac-1 on each cell (see Table 1).

Expression of VLA-4 on neutrophils was almost undetectable, but a significant increase in the expression of the integrin was seen to occur after treatment of cells with L-NAME (1.0, 2.0 mM) (see Table 1). Treatment of cells with D-NAME (1.0 mM) had no significant effect upon VLA-4 expression.

3.6. Effect of microfilament- and microtubule-inhibiting agents upon L-NAME-induced adhesion to FN and serum

The microfilament-depolymerizing agent, DHCB (0.1–2.5 μg/mL), did not affect basal adhesion of neutrophils to either FN or serum (basal adhesion to FN: 18.1 ± 2.3%; 0.1 μg/mL DHCB, 17.2 ± 3.7%; 0.5 μg/mL DHCB, 20.4 ± 4.2%; N = 3) (basal adhesion to serum: 20.1 ± 2.4%; 0.1 μg/mL DHCB, 22.2 ± 2.6%; 0.5 μg/mL DHCB, 22.8 ± 1.8%; N = 3). Pre-incubation of neutrophils with both L-NAME (0.1 mM) and DHCB (0.5 μg/mL) (10 min, 37°, 5% CO_2) abolished the L-NAME-induced adhesion to both FN and serum (Fig. 4).

Table 1
Effect of L-NAME/D-NAME/fMLP upon α_4 and α_M expressions on human neutrophils (50,000) as determined by flow cytometry

Neutrophil treatment	α_M expression (Mac-1 expression) mean fluorescence intensity	α_4 expression (VLA-4 expression) mean fluorescence intensity
Untreated neutrophils	52.59 ± 1.32	9.50 ± 0.38
fMLP (0.05 μM)	66.99 ± 2.87**	10.90 ± 0.43
D-NAME (1.0 mM)	51.52 ± 2.10	9.98 ± 1.02
L-NAME (0.1 mM)	50.33 ± 3.27	11.34 ± 0.71
L-NAME (1.0 mM)	52.66 ± 2.31	11.90 ± 0.46*
L-NAME (2.0 mM)	n/m	12.16 ± 0.64*

Data are expressed as mean fluorescence units per cell ± SE of greater than five independent experiments with duplicates in each. n/m, not measured.

$^{*}P < 0.05$; $^{**}P < 0.01$.

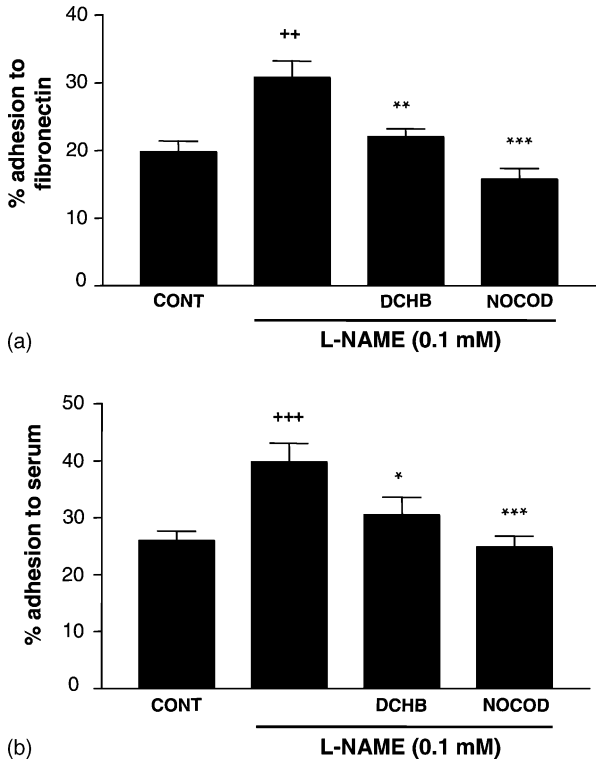

Fig. 4. Effects of the actin filament-depolymerizing agent, dihydrocytochalasin B (DHCB, 0.5 μg/mL), and the microtubule disrupting agent, nocodazole (NOCOD, 10 μM), upon human neutrophil adhesion following treatment with L-NAME (0.1 mM) to (a) 20 μg/mL FN and (b) 10% (v/v) human serum. Neutrophils (>95% pure) were pre-incubated with DHCB or nocodazole (±L-NAME) (10 min, 37°, 5% CO_2) before allowing cells to adhere to FN/serum-coated plates (15 min, 37°, 5% CO_2). Results are expressed as adhered cell percentages of total cell number ± SE of four independent experiments with three replicates in each. $^{++}P < 0.01$, $^{+++}P < 0.001$, compared to control cells. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared to cells treated with L-NAME alone (0.1 mM).

Pre-incubation (10 min, 37°, 5% CO_2) of neutrophils with the microtubule-depolymerizing agent, nocodazole, decreased basal adhesion to FN and serum only at the higher concentration of 25 μM. Basal adhesion to FN was 23.3 ± 2.1%, following treatment with nocodazole adhesion was 22.0 ± 1.7% (1 μM nocodazole), 19.7 ± 2.8% (10 μM), and 12.9 ± 1.6% (25 μM, $P = 0.004$, N = 4). Basal adhesion to serum was 33.4 ± 4.4%, following treatment with nocodazole, adhesion was 29.7 ± 4.6% (1 μM), 32.3 ± 4.4% (10 μM), and 22.9 ± 4.4% (25 μM, N = 4).

Pre-incubation of cells with nocodazole at the lower concentration of 10 μM abolished the L-NAME (0.1 mM)-induced adhesion to both FN and serum (see Fig. 4).

4. Discussion

Previous research has demonstrated that a β_2-independent mechanism may also be involved in the neutrophil migration process [9,11], theories suggest that the β_1 integrin, VLA-4 (hitherto thought not to be expressed by human neutrophils) and possibly VLA-9 [12] may be the principle adhesion molecules involved in this mechanism. Evidence also shows that NO plays a central role in the regulation of leukocyte migration. Leukocytes of eNOS knockout mice have an increased basal adhesion to post-capillary venules compared to wild-type mice [22,23] and L-NAME has also been demonstrated to affect leukocyte migration both *in vitro* [13,24] and *in vivo* [25]. In addition, NO inhibits vascular cell adhesion molecule 1 (VCAM-1) expression on human mesothelial and endothelial cells in a cGMP-independent manner, preventing the focal adhesion and accumulation of leukocytes to vessel walls [26,27]. Here, we present evidence to suggest that NO may have a regulatory role in the expression and function of VLA-4 on human neutrophils in addition to its role in modulating the function of Mac-1, a β_2 integrin.

Purified human neutrophils were seen to adhere, to some extent, to both FN and human serum without any stimulation. This basal adhesion to serum and FN varied considerably from individual to individual and was partially inhibited by the ICRF 44 mAb, demonstrating that the Mac-1 integrin plays a role in the basal adhesion of neutrophils to both FN and serum. Treatment of human neutrophils with L-NAME significantly increased cell adhesion to FN and serum in a concentration-dependent manner, demonstrating a role for NO in regulating adhesion molecule function on the human neutrophil. The mAbs specific for the VLA-4 and Mac-1 α-subunits were able to block the L-NAME-induced adhesion of neutrophils to FN and serum in different manners. The VLA-4 mAb, HP2/1, entirely reversed the increased neutrophil adhesion to FN following treatment with L-NAME, whilst the Mac-1-specific mAb, ICRF 44, completely reversed the L-NAME-induced adhesion to human serum with HP2/1 able to partially reverse L-NAME-induced adhesion to serum. These findings reflect the different affinities of the VLA-4 and Mac-1 integrins for different ligands. VLA-4 binds predominantly to FN and VCAM-1 with high affinity, whilst Mac-1 has a higher affinity for a range of ligands [28], including fibrinogen and intercellular adhesion molecule (ICAM-1), which may be encountered in the human serum utilized in our study.

Flow cytometry experiments detected an increased α_4-subunit expression on neutrophils following L-NAME treatment. This increase, although significant, was slight and could be argued as being biologically insignificant. A similar low increase in α_4-subunit expression, however, was reported on human neutrophils following stimulation with C5a [11] (mean fluorescence units increased from 8.7 for the control to 14.1 following stimulation, using anti-α_4-subunit mAbs) and the authors suggested that this increase in VLA-4 expression contributed to neutrophil migration through connective tissue fibroblast barriers. The extremely low expression of α_4, described in the current study, was somewhat surprising, since increased adhesion to FN following L-NAME treatment was reversed by an anti-VLA-4 mAb. Furthermore, the actin-depolymerizing agent, DHCB, and the microtubule disrupter, nocodazole, were able to abolish

this increased adhesion, supporting the theory that L-NAME up-regulated VLA-4 *via* mobilization of $\alpha_4\beta_1$ from neutrophil granules and cytoskeletal rearrangement. Whilst L-NAME-induced expression of VLA-4 is extremely low level, it seems that those VLA-4 integrins expressed are capable of supporting firm adhesion and possess a very high affinity for their ligands. It may be that L-NAME induces low affinity neutrophil adhesion, which in turn, up-regulates VLA-4 expression and function *via* outside-in signaling. Ligand binding by integrins can generate outside-in signaling which is instrumental in various cell processes, for example, binding of LFA-1 to ICAM-1 decreases VLA-4 avidity on T cells [29]. In addition, phosphorylation events mediated by Src kinase p56 (Ick) have been shown to be a key regulator of VLA-4 affinity for FN and VCAM-1 on T cells and NO is known to stimulate tyrosine phosphorylation of various kinases [30,31]. Thus, in addition to the increased expression of VLA-4 on neutrophils following inhibition of NO production, the increase in VLA-4 adhesion observed may also be mediated by a significant increase in the affinity of the expressed VLA-4 molecules for their ligand.

The Mac-1 ($\alpha_M\beta_2$) adhesion molecule is already expressed abundantly on the surface of over 90% of neutrophils, inhibition of NO synthesis in human neutrophils did not produce any detectable change in this expression. The chemotactic peptide, fMLP, was used as a positive control to demonstrate that Mac-1 expression on neutrophils can be up-regulated by certain stimuli. Increased adhesion of neutrophils to serum following incubation of neutrophils with L-NAME, though, was seen to be mediated by an increase in Mac-1 activity. Thus, inhibition of NO synthesis in neutrophils stimulated an increase in Mac-1 function either by increasing the ligand affinity of Mac-1 or by increasing the avidity of Mac-1. The I-domain of α_M is known to have two different conformations that may be involved in regulating the affinity conformation of the Mac-1 molecule [32]. There is also some evidence to suggest that an enhancement in Mac-1 avidity may be responsible for its increased function. Neutrophil contact with HUVECs results in an accumulation of Mac-1 at the contact area [33] and phosphorylation of the β_2 integrin activates the integrin molecule, possibly by inducing an enhancement in integrin mobility in the plane of the membrane and by a reorganization of the cytoskeleton [34]. Neutrophil F-actin and myosin, but not microtubules, functionally regulate IL-8-induced transepithelial migration [35] and the cytoskeleton has been shown to take part in β_2 integrin avidity [36] and to actively regulate the binding conformation of CD11b/CD18 as well as its mobilization in the membrane [37]. Our experiments demonstrating that DHCB and nocodazole, at low concentrations, inhibit L-NAME-induced Mac-1 adhesion, but not basal adhesion, suggest that a rearrangement of the cytoskeleton, involving actin filaments and microtubules, is indeed involved in the adhesion observed and that NO may have a role in modulating integrin avidity as well as expression and affinity. Indeed, NO has been shown to disassemble actin filaments in chondrocyte lineage cells [38] and the presence of eNOS has been observed localized at endothelial cell-to-cell contact sites, suggesting an association of eNOS with the actin cytoskeleton [39].

Whether human neutrophils express NOS under resting conditions, and which isoforms, is unclear. Cytokines are able to induce inducible NOS (iNOS) production in buffy coat preparation neutrophils, although no increase in iNOS expression was demonstrated following cytokine stimulation of purified neutrophils in suspension [40]. Interestingly, when purified neutrophils are allowed to adhere (for 2 hr) to glass surfaces uncoated or coated with fetal calf serum, plasma, FN, or laminin, iNOS production increases significantly [40]. In contrast, constituitively expressed neuronal NOS (nNOS) has been demonstrated in all cells of neutrophil preparations using immunocytochemistry [41] and human neutrophils release NO under basal conditions [42,43].

Whether the mechanism by which L-NAME increases adhesion in neutrophils functions by inhibiting the iNOS expressed upon basal neutrophil adhesion to coated surfaces or by inhibiting constituitively expressed NOS remains to be clarified, but its relatively rapid effect in the experimental system described herein (approximately 30 min) suggests that L-NAME may act upon constituitively expressed NOS, rather than iNOS. Further experiments employing selective inhibitors of specific NOS isoforms, such as the iNOS inhibitor, 1400W (*N*-3-aminomethyl-benzyl-acetamide-dihydrochloride) and the nNOS-selective inhibitor, L-VNIO (*N*-5-(1-imino-3-butenyl)-L-ornithine), may shed light on this question [44].

Although L-NAME has some putative effects in certain situations (L-NAME induces mast cell degranulation *in vivo* as a consequence of cationic charges [45]), we would expect to see at least some effect of D-NAME, the inactive enantiomer of L-NAME upon neutrophil adhesion molecule function. However, D-NAME had no effect upon neutrophil adhesion to FN or serum, supporting the theory that L-NAME induces neutrophil adhesion molecule function *via* an NO-dependent mechanism. The effects of L-NAME upon leukocyte adhesion are generally reversed by co-incubation with a donor of NO. L-NAME-induced human eosinophil adhesion to FN and serum is abolished by co-incubation with sodium nitroprusside[1] and L-arginine, but not D-arginine, is able to inhibit L-NAME-induced leukocyte adhesion to vascular endothelium under shear stress [19], further supporting the theory that L-NAME-induced leukocyte adhesion is NO dependent.

NO is thought to mediate fMLP-stimulated leukocyte chemotaxis *via* a cGMP-dependent pathway [13,16], however, we were unable to demonstrate any effect of ODQ, a soluble guanylate cyclase inhibitor, upon neutrophil adhesion to FN or serum. Results contrasted with those obtained in eosinophils where ODQ mimicked the effect of L-NAME

[1] Conran *et al.*, unpublished observations.

upon cell adhesion to FN [46]. Whilst these results cannot be taken as conclusive evidence that L-NAME does not exert its effect upon neutrophil adhesion *via* a cGMP-dependent mechanism, it is possible that ODQ does not inhibit neutrophil guanylate cyclase. Indeed, ODQ has no inhibitory effect upon soluble guanylyl cyclase in rat ventricular cardiomyocytes [47] due to scavenging by myoglobin and there is evidence to suggest that not all NO-dependent effects are mediated by the cGMP pathway. Treatment of HUVECs with NO resulted in a change in adhesion of neutrophils mediated by an increase in CD18 (β_2) and ICAM-1 function that was not mediated by changes in cGMP levels [48]. Protein kinase C may activate the β_2 integrin subunit [49] and NO induces changes in neutrophil adhesion to human endothelial monolayers *via* a protein kinase G mechanism [50]. NO and its metabolites are known to nitrate a number of proteins other than guanylate cyclase, including tyrosine residues [51], and it may be that NOs or NO-derived species have a more direct effect upon the cytoskeleton/integrin complex, indeed eNOS has been found localized at intercellular junctions in endothelial cells [39]. Alternatively, evidence exists to suggest that when NOS is inhibited, changes in superoxide production may occur. An inverse correlation between NO and superoxide levels has been observed, for example, during human neutrophil–endothelial cell interactions [52]. In addition, superoxide and other oxygen free radicals have been shown to trigger neutrophil activation and adhesion to vascular endothelium *via* up-regulation of CD11b and CD18 expressions [53] and it may be that the changes in neutrophil adhesion mechanisms, following inhibition of NOS effects, may involve changes in superoxide production.

In conclusion, we postulate that endogenously produced NO has a role in inhibiting the adhesion of human neutrophils to ECM components by inhibiting the expression of the VLA-4 integrin on the cell surface and decreasing the functional activity of the Mac-1 integrin already expressed on the neutrophil cell surface. These changes involve cytoskeletal rearrangements and are possibly cGMP independent.

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