Nitric Oxide and Prostacyclin

Divergence of Inhibitory Effects on Monocyte Chemotaxis and Adhesion to Endothelium In Vitro

Philip M.W. Bath, David G. Hassall, Ann-Marie Gladwin, Richard M.J. Palmer, and John F. Martin

Monocyte--endothelial interactions are of fundamental importance in determining the movement of monocytes from the blood stream into the vessel wall. This study reports that two endothelium-derived factors, nitric oxide and prostacyclin, alter in vitro monocyte behavior. Nitric oxide (>10^-6 M) inhibited monocyte adhesion to porcine aortic endothelial cell monolayers, whereas prostacyclin (10^-9 to 10^-5 M) had no effect. Both nitric oxide and prostacyclin inhibited monocyte chemotaxis stimulated by N-formyl-methionyl-leucyl-phenylalanine and induced dose-dependent increases in intracellular cyclic guanosine monophosphate and cyclic adenosine monophosphate concentrations, respectively. The cell surface expression of the CD11b/CD18 adhesion receptor, a glycoprotein complex known to mediate monocyte intercellular adhesion, was not altered by either nitric oxide or by prostacyclin. Thus, endothelium-derived nitric oxide and prostacyclin may have a physiological role in modulating monocyte--vascular wall interactions. Alterations in this system may contribute to the increased monocyte emigration from the blood stream into the vessel wall observed in atherogenesis.

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Nitric Oxide and Monocytes

**Mononuclear Cell Preparation**

Human mononuclear cells (MNCs) were prepared as described previously; this involved density gradient separation on Ficoll-Paque (Pharmacia, Milton Keynes, U.K.) of freshly drawn blood anticoagulated with EDTA (2.7%). MNCs were used at a concentration of \(5 \times 10^6\) cells/ml and their viability assessed with Trypan Blue. Histological staining of MNCs with modified Giemsa demonstrated that 33% of the cells were monocytes, 66% were lymphocytes, and less than 1% were neutrophils.

**Porcine Aortic Endothelial Cell Culture**

Culture of porcine aortic endothelial cells (PAECs) was performed according to the method of Gryglewski et al. Aortas were cleaned of their adventitial fat and were washed to remove blood and cell debris, and the lumen was incubated for 30 minutes with collagenase in PBS. The aortas were rewarshed and the loosened cells placed in minimal essential medium (containing 1 mM glutamate, 100 \(\mu\)g/ml streptomycin, 100 IU penicillin, and 15% fetal calf serum) and plated into 25-cm\(^2\) flasks. The cells were cultured at 37°C and reached confluence within 3–4 days, after which time they were trypsinized and then plated onto 96-well plates (Falcon, Becton Dickinson, Oxford, U.K.). The cells were used within 72 hours and only at passage 1. Before use, each monolayer was examined by microscopy to ensure that only endothelial cells, identifiable by their characteristic cobblestone appearance, were present. Endothelial morphology was confirmed by electron microscopy and a positive factor VIII–related antigen test.

**Nitric Oxide and Monocytes**

In experiments involving NO, MNCs were preincubated with SOD (15 units/ml) and M&B 22948 (1 \(\mu\)M) for 10 minutes at room temperature. NO was removed from its vial in a gas-tight syringe, injected into the MNC preparation, and mixed by syringe aspiration and reinjection. In experiments involving MNC stimulation, fMLP (5 \(\times 10^{-8}\) M) was added immediately before the addition of NO and/or PGI\(_2\). The potential synergy between NO and PGI\(_2\) with respect to inhibition of monocyte adhesion to PAECs was investigated in the presence of M&B 22948 (1 \(\mu\)M), HL 725 (1 \(\mu\)M), or IBMX (1 mM); PGI\(_2\) (10\(^{-9}\) M) was added to the MNCs immediately before NO (10\(^{-7}\) M) was added.

**Monocyte Adhesion**

MNCs were incubated with PAEC monolayers for 30 minutes in humidified 5% CO\(_2\) in air at 37°C, after which time the nonadherent cells were removed by washing twice with PBS. The adherent cells were lysed in 50 \(\mu\)l 0.5% hexadecyltrimethylammonium bromide. Dianisidine dihydrochloride (0.2 mg/ml) containing hydrogen peroxide (0.4 mM) containing perchorlic acid (final concentration, 0.5 M). After neutralization with 1.08 M tripotassium phosphate, cAMP and cGMP concentrations were measured by radioimmunoassay (Amersham, Aylesbury, U.K.). The results are expressed as femtomoles per micromgram protein; protein concentration was determined using a modified Lowry procedure.

**CD11b Adhesion Receptor Expression**

NO (in the presence of SOD and M&B 22948) or PGI\(_2\) was added to the MNCs, which were then incubated for 20 minutes at 37°C in humidified air. The reaction was terminated with sodium azide (final concentration, 3 \(\times 10^{-2}\) M) and the cells labeled for surface expression of the adhesion protein CD11b/CD18 with a mouse anti-human CD11b antibody (Monosan, Sanbio, Market Harborough, U.K.) followed by fluorescein isothiocyanate (FITC)–conjugated goat anti-mouse F(ab')\(_2\), fragments.

Analysis of surface labeling was performed on a Becton Dickinson FACS analyzer. Monocyte and lymphocyte populations were separated by gating on cell size; green fluorescence data were collected from 2 \(\times 10^4\) MNCs. The mean fluorescence intensity was corrected for the background fluorescence determined from FITC-labeled F(ab')\(_2\) fragments incubated alone with MNCs.

**Statistics**

The results are expressed as mean ± SEM. Since the basal and stimulated-adhesion data varied from day to day (reflecting different sources of MNCs and endothelial cells), the data for different concentrations of NO and/or PGI\(_2\) were normalized relative to the basal value for each separate experiment. The overall
effect of NO or PGI₂ on monocyte function was assessed using one-way analysis of variance; individual concentrations were compared with the relevant control using Tukey's multiple comparison procedure.¹⁹

Results

Adhesion

Monocyte adhesion to PAECs increased in the presence of fMLP at concentrations above 10⁻⁸ M (Figure 1, left panel) and was optimal for chemotaxis at 5×10⁻⁸ M (Figure 1, right panel); for the purpose of experiments involving the stimulation of MNCs, the fMLP dose chosen (final concentration, 5×10⁻⁸ M) was near optimum for both assays.

NO in the presence of SOD (15 units/ml) and M&B 22948 (1 μM) caused a significant inhibition of basal monocyte adhesion to PAEC monolayers (Figure 2, left panel). Multiple additions of NO at 10⁻⁴ M during incubation resulted in a further reduction of adhesion by 19.9% (data not shown). To observe the effects of NO on activated monocytes, the cells were stimulated with fMLP; fMLP increased basal adhesion to PAECs by 62.2±15.6%, and this adhesion was not inhibited by NO (Figure 2, right panel). PGI₂ had no effect on basal or fMLP-stimulated monocyte adhesion. SOD and M&B 22948 inhibited adhesion by 10.0±4.8% (p<0.05) in the absence of NO as compared with RPMI 1640.
NO and PGI₂ Inhibit Monocyte Function

Chemotaxis

fMLP (5×10⁻⁸ M) increased monocyte migration by 378.4±41.1% (n=4) compared with basal migration. NO caused a significant inhibition of chemotaxis (Figure 3, left panel) but only in the presence of SOD and M&B 22948. NO in the absence of SOD and M&B 22948 did not inhibit fMLP-stimulated chemotaxis. In contrast to adhesion, PGI₂ inhibited chemotaxis in a dose-dependent manner (Figure 3, right panel). Neither NO nor PGI₂ was found to be a monocyte chemoattractant. SOD and M&B 22948 did not significantly inhibit chemotaxis in the absence of NO.

Cyclic Nucleotides

NO in the presence of SOD and M&B 22948 increased cGMP levels in a dose-dependent manner (Figure 4); cAMP concentrations also rose at an NO concentration of 10⁻⁴ M, although the difference was not significant (Table 1). PGI₂ increased monocyte intracellular cAMP and cGMP concentrations, although the rise in cAMP was of a greater magnitude (Table 1).

Nitric Oxide and Prostacyclin

Experiments involving the coincubation of NO (10⁻⁷ M) with PGI₂ (10⁻⁸ M) (in the presence of SOD and M&B 22948, HL 725, or IBMX) did not find evidence for synergy between the two factors in the inhibition of monocyte adhesion to PAECs and fMLP-stimulated chemotaxis (Figure 5). Although there was no evidence for synergy between low concentrations of NO and PGI₂ with respect to cyclic nucleotide concentrations, coincubation of the two factors raised cGMP concentrations by as much as the sum of the rises caused by NO and PGI₂ independently (Table 1).

CD₁₁b Adhesion Receptor Expression

The surface expression of CD₁₁b/CD₁₈ was not altered in the presence of NO (10⁻⁷ to 10⁻⁴ M, with SOD and M&B 22948) or PGI₂ (10⁻⁹ to 10⁻₇ M) on flow cytometric analysis (data not shown).

To ensure that NO did not alter MNC function by changing extracellular pH, various concentrations of NO were added to the cells in RPMI 1640 in the

![Figure 3](image-url) Line plots showing effect of nitric oxide (log molar concentration, left panel) in the presence of superoxide dismutase (SOD, 15 units/ml) and M&B 22948 (1 μM) and prostacyclin (log molar concentration, right panel) on fMLP-induced monocyte chemotaxis. Chemotaxis was assessed in 48-well chambers and quantified as the number of monocytes that had migrated through the membrane as determined by high-power microscopic examination. Each point represents mean±SEM, n=4. *p<0.05, **p<0.01. fMLP, N-formyl-methionyl-leucyl-phenylalanine.

![Figure 4](image-url) Line plot showing effect of nitric oxide (log molar concentration) in the presence of superoxide dismutase (SOD, 15 units/ml) and M&B 22948 (1 μM) on monocyte intracellular cyclic guanosine monophosphate (cGMP) concentration (fmol/μg protein) measured by radioimmunoassay. Each point represents mean±SEM, n=4. *p<0.05, **p<0.01.
presence of SOD and M&B 22948. The extracellular pH of 7.56 fell by only 0.15 pH unit in the presence of NO at 10^{-5} M. Cell viability, assessed by Trypan Blue staining, remained in excess of 95%. Neither NO nor PGI2 altered monocyte MPO activity.

Discussion

NO inhibited unstimulated monocyte adhesion to PAECs at concentrations above 10^{-5} M, although no such inhibition of FMLP-stimulated monocyte adhesion was observed. In contrast, PGI2 had no effect on either unstimulated or stimulated adhesion. Both agents inhibited FMLP-induced chemotaxis at concentrations above 10^{-5} M and 10^{-7} M, respectively. This is the first report that NO is capable of altering leukocyte function in vitro.

Since these experiments were performed on a mixture of monocytes and lymphocytes, it is not clear whether the effects of NO and PGI2 were due to a direct effect on the monocytes or an indirect effect due to the release of factors from lymphocytes. However, the latter alternative is unlikely, since the migration and adhesion incubation times of 90 minutes and 30 minutes, respectively, were too short for lymphocyte protein synthesis; hence, any released factor would need to be stored by the lymphocytes. Additionally, there are no obvious lymphokines with such action.

The reason why high concentrations of NO (>10^{-5} M) were required to inhibit monocyte function, even in the presence of SOD and M&B 22948, is not clear but may relate to NO's very short half-life of less than 1 minute. Increased inhibition of adhesion with multiple additions of NO was observed, suggesting that to be effective, the mediator must be continuously present during the adhesion or migration process. Since the physiological concentrations of NO surrounding endothelial cells are unknown, it is also possible that NO exists locally at high concentrations or that NO is transferred directly from cell to cell.

NO in the presence of SOD (and M&B 22948), but not alone, inhibited monocyte function, suggesting that NO may be degraded rapidly by superoxide, an effect that can be partially prevented in the presence of SOD. Since FMLP stimulation of monocytes can increase their release of superoxide anion, the rate of inactivation of NO would be increased, thereby offering a possible explanation for the lack of effect of NO on stimulated monocytes. Monocyte superoxide anion production may be less in a gradient of FMLP (as in chemotaxis) than when the cells are immersed in FMLP (as in the adhesion experiments), thereby explaining NO inhibition of monocyte chemotaxis but not FMLP-stimulated adhesion to PAECs.

An alternative explanation for the absence of NO inhibition of stimulated adhesion may reside in the relation between monocyte production of and response to NO. Stimulated monocytes or macrophages synthesize NO from L-arginine, whereas NO only appears to inhibit unstimulated monocytes. These observations suggest that NO does not exert an autocrine function on monocyte activity.

Since the transduction mechanisms for the actions of PGI2 and NO on platelets and smooth muscle cells are known to involve the cyclic nucleotide second-messenger systems, it is likely that similar mechanisms apply in monocytes. Significant increases in cGMP and cAMP concentrations were observed with NO and PGI2, respectively, supporting this hypothesis. Additionally, some crossover of action was noted—PGI2 increased cGMP concentrations, while NO caused a nonsignificant increase in cAMP concentra-
tions. Similar observations have been noted in platelets, whereby PGI<sub>2</sub> increases cGMP concentrations.\textsuperscript{16}

However, the relation between NO- and PGI<sub>2</sub>-induced alterations in cyclic nucleotide concentrations and the inhibition of monocyte function is not clear, particularly since the experimental protocol for the measurement of cyclic nucleotides differed from that for the functional studies. A relatively pure population of monocytes, obtained after MNC adhesion to plastic, was used to establish the cyclic nucleotide profiles, while functional experiments were performed on MNCs to avoid further manipulations of the monocytes.

Evidence in favor of the involvement of cyclic nucleotides in the inhibition of monocyte function is supported by the observation that cyclic nucleotide concentrations rose after addition of NO and/or PGI<sub>2</sub>. However, it should be noted that cGMP concentrations peaked with an NO concentration of 10<sup>-5</sup> M, whereas there was no inhibition of chemotaxis or adhesion at this concentration. That cGMP is a second messenger for NO is supported by the requirement for M&B 22948, a specific cGMP phosphodiesterase inhibitor,\textsuperscript{23} to achieve inhibition.

The finding that increases in monocyte intracellular cGMP concentrations are associated with inhibition of chemotaxis is at variance with previous work suggesting that cGMP mediates increased migration. Ascorbic acid, carbamylcholine, and serotonin raise monocyte cGMP concentrations and stimulate chemotaxis and polarization,\textsuperscript{24-26} whereas agents that raise cAMP concentrations (such as prostaglandin E<sub>2</sub>) inhibit chemotaxis and changes in cGMP.\textsuperscript{27} The implication in these studies is that the rise in cGMP mediates the increase in cell locomotion. However, cGMP concentrations increase independently of changes in monocyte chemotaxis,\textsuperscript{25} suggesting that the above-mentioned stimulants may be acting via other mechanisms. Indeed, serotonin would appear now not to act via cGMP at all; instead, its biological effects are thought to be mediated via either adenylate cyclase, potassium channels, calcium channels, or phosphoinositide metabolism, depending on the receptor subtype involved.\textsuperscript{28}

At present, it is not possible to state confidently that the actions of NO and PGI<sub>2</sub> on monocytes are directly mediated via cGMP and cAMP, respectively; alterations in cyclic nucleotide concentrations may only be indirectly coupled, much as fMLP results in activation of neutrophils but also causes transient increases in cAMP concentration, which are known to be inhibitory.\textsuperscript{29}

NO and PGI<sub>2</sub> show functional synergy in their inhibition of platelet aggregation,\textsuperscript{30} although no such synergy exists with respect to platelet adhesion.\textsuperscript{10} There was no evidence for functional synergy between NO and PGI<sub>2</sub> on monocyte adhesion, suggesting that the monocyte and platelet adhesion processes share common features.

Monocyte adhesion to endothelium requires functional glycoprotein adhesion receptors of the CD11/CD18 family,\textsuperscript{2} particularly CD11b/CD18.\textsuperscript{3} Current evidence relating to neutrophils suggests that stimulated adhesion is accompanied by conformational changes in CD11/CD18\textsuperscript{30} rather than by an increase in surface expression; such mechanisms are also likely to occur in monocytes. Since NO and PGI<sub>2</sub> did not alter monocyte CD11b/CD18 surface expression, it would appear that these agents may only act via intracellular signals. Further experiments are required to resolve this possibility.

These studies have demonstrated that NO and PGI<sub>2</sub> inhibit fMLP-stimulated chemotaxis in vitro while NO also inhibits monocyte adhesion to PAECs. This suggests that the endothelium may have a physiological role in modulating the emigration of monocytes from the vascular compartment into the vessel wall. Impairment of such a homeostatic mechanism may lead to an imbalance in monocyte–endothelial interaction, favoring adhesion to and migration through the endothelium. An example where this may be important is atherogenesis, in which monocytes adhere to endothelium and subsequently migrate into the subintimal space.\textsuperscript{1} Risk factors for atherogenesis are associated with mechanisms that can functionally impair endothelium; cigarette smoke and elevated low density lipoprotein concentrations reduce endothelial PGI<sub>2</sub> production,\textsuperscript{31-32} while impaired EDRF and PGI<sub>2</sub> release occurs in experimental animal atherosclerosis and hypercholesterolemia.\textsuperscript{33-36} Studies in vivo of the effect of endothelium-derived NO and PGI<sub>2</sub> on monocyte adhesion to endothelium are now required. Furthermore, nitrovasodilating drugs should be examined as potential antiatherosclerotic agents.

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**References**

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