Nitric oxide modulation of focal adhesions in endothelial cells

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1Departments of Medicine and Physiology, State University of New York, Stony Brook, New York 11794-8152; and 2Wolfson Institute for Biomedical Research, University College London, University of London, London WC1E 6JJ, United Kingdom

Goligorsky, Michael S., Husna Abedi, Eisei Noiri, Alice Takhtajan, Sheri Lense, Victor Romanov, and Ian Zachary. Nitric oxide modulation of focal adhesions in endothelial cells. Am. J. Physiol. 276 (Cell Physiol. 45): C1271–C1281, 1999.—A permissive role of nitric oxide (NO) in endothelial cell migration and angiogenesis promoted by vascular endothelial growth factor (VEGF), endothelin, and substance P has previously been established. The present studies were designed to examine the mechanism(s) involved in the NO effect on focal adhesions. Time-lapse videomicroscopy of human umbilical vein endothelial cells (HUVECs) plated on the silicone rubber substrate revealed that unstimulated cells were constantly remodeling the wrinkling pattern, indicative of changing tractional forces. Application of NO donors reversibly decreased the degree of wrinkling, consistent with the release of tractional forces exerted by focal adhesions and stress fibers. Morphometric and immunocytochemical analyses showed that NO inhibited adhesion and spreading of HUVECs and attenuated recruitment of paxillin to focal adhesions. NO also had a profound dose-dependent effect on the formation of stress fibers by HUVECs. De novo formation of focal adhesions in HUVECs was significantly diminished in the presence of NO donors. Migration of HUVECs showed an absolute requirement for the functional NO synthase. NO donors did not interfere with focal adhesion kinase recruitment to focal adhesions but affected the state of its tyrosine phosphorylation, as judged from the results of immunoprecipitation and immunoblotting experiments. Videomicroscopy of HUVECs presented with VEGF in a micropipette showed that the rate of cell migration was slowed down by NO synthase inhibition as well as by inhibition of tyrosine phosphorylation. Collectively, these data indicate that NO reversibly releases tractional forces exerted by spreading endothelial cells via interference with the de novo formation of focal adhesions, tyrosine phosphorylation of components of focal adhesion complexes, and assembly of stress fibers.

cytoskeleton; cell migration; tyrosine phosphorylation; focal adhesion kinase; time-lapse videomicroscopy

THE DUALISTIC NATURE of cell locomotion resides in the concomitant occurrence of reciprocal processes at the leading and trailing edge of the cell: the formation of focal adhesions taking place in the former and their disassembly prevailing in the latter (reviewed in Refs. 4 and 30). Such a functional polarity of adhesive properties in a moving cell and the cellular mecha-
To gain insight into the possible mode of NO action, impedance analysis of endothelial cell monolayers subjected to NO was performed. Using a highly sensitive impedance bioprobe, we showed that micromotion in ostensibly stationary endothelial cells is elicited by NO. This spontaneous micromotion we termed podokinesis to emphasize a scalar form of cell movement, occurring in nonmigrating endothelial cells, which, in the presence of guidance cues, e.g., VEGF, is transformed to a vectorial movement (22). Thus we hypothesized that the execution of the program for a directional endothelial cell migration requires two simultaneous messages: NO-induced scalar podokinesis and a gradient for guidance cues provided by motogens like VEGF, epidermal growth factor (22, 23), or ET-1 (21) to establish the directionality of movement.

Sporadic fluctuations in endothelial resistance and their enhancement by NO, as resolved using an amplification system for miniature changes in impedance of cells grown on the surface of gold microelectrodes, are due at least in part to changes in cell-matrix adhesion. In this context, observations by Lauffenburger (13) that the rate of locomotion has a biphasic dependence on the tightness of cell-substratum adhesion, may explain the general phenomenon of NO-mediated motility. The pathways responsible for NO-induced changes in cell-matrix adhesion remain obscure. The purpose of the present study, therefore, was to determine the mechanism of NO modulation of endothelial cell adhesion and migration. We demonstrated that NO reversibly releases tractional forces exerted by spreading endothelial cells by interfering with the de novo formation of focal adhesions, tyrosine phosphorylation (PY) of focal adhesion kinase (p125FAK) and paxillin, and assembly of stress fibers.

MATERIALS AND METHODS

Cell cultures. HUVECs were obtained from Clonetics (San Diego, CA) and maintained in endothelial growth medium-2 containing 2% fetal bovine serum (FBS) supplemented with growth factors provided by the manufacturer. Cells formed typical cobblestone-appearing monolayers and expressed endothelial cell-specific markers, i.e., receptors for acetylated low-density lipoprotein and immunoreactivity of the von Willebrand factor. HUVECs were used between passages 5 and 10. Swiss 3T3 cells were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin ( Gibco BRL, Gaithersburg, MD).

Glass coverslips were coated with fibronectin, vitronectin, laminin, or collagen type I at a concentration of 10 µg/ml. After an overnight incubation, coverslips were rinsed with PBS with 0.1% BSA, dried, and sterilized by exposure to ultraviolet light.

Cell migration assay. A migration assay was performed according to the previously described technique (29) in a Boyden chemotaxis apparatus (Neuroprobe, Cabin John, MD). HUVECs were detached using 0.05% trypsin-0.53 mM EDTA (Gibco BRL) and were washed, and 10^6 cells/ml suspended in 25 µl of l-arginine-free basal medium Eagle's (BME) (Gibco) supplemented with 0.1% BSA were added to the lower chamber of the Boyden apparatus. Polycarbonate filters with 8-µm pores were coated with 10 µg/ml ProNectin F (Protein Polymer Technologies, San Diego, CA), washed twice with PBS, and positioned above the wells of the lower chemotactic chamber that contained cells. The top half of the chamber was reattached, and the chamber was incubated in an inverted position at 37°C in 95% air-5% CO2 for 2 h to allow a uniform cell attachment to the filter. The test agents L-arginine (0.1-1.0 mM) and L-NAME (400 µM to 2 mM), used in combination with VEGF165 (10 ng/ml) to promote migration and prepared in 50 µl of BME medium with 0.1% BSA, were added to the upper chambers. The chambers were wrapped with Parafilm and incubated for an additional 5 h in an upright position. After incubation, the membrane was removed from the apparatus, and cells were fixed with methanol and stained with Diff-Quick (Baxter, Miami, FL). The number of migrated cells on the upper surface of the filter was counted in six randomly chosen fields under x400 magnification and averaged. All experiments were performed in quadruplicate, and each experiment was repeated at least three times.

Adhesion of latex beads to endothelial cells. Latex beads (3.1 µm diameter) were coated with 20 µg/ml fibronectin overnight, access protein was removed, and the beads were resuspended in PBS with 0.1% BSA. Approximately 5 × 10^3 beads were applied to the wells of a 24-well cluster with HUVEC monolayers growing on 12-mm glass coverslips, according to the procedures previously developed by Plopper et al. (25) and Miyamoto et al. (17, 18). After incubation for 30–120 min in the presence or absence of NO donors or the NO synthase inhibitor L-NAME, monolayers were fixed and stained with antibodies against phosphotyrosine, as previously detailed (22).

Effect of NO on tractional forces studied in cells grown on silicone rubber. Glass coverslips were coated with phenylmethyl polysiloxane (Dow Corning 710 fluid) precisely as described (2). Briefly, a 200-µl drop of silicone rubber was placed on the surface of 25-mm coverslips and vulcanized by passing it over a Bunsen burner flame. After the coverslips were sterilized with ultraviolet light for 25 min, −5× 10^3 endothelial cells suspended in RPMI medium buffered with 25 mM HEPES and containing 10% FBS were seeded on the coverslips. Cells were allowed to attach at 37°C for 60–120 min, by which time they created adequate wrinkling of the silicone rubber. The dynamics of wrinkling pattern was examined under a Nikon Diaphot microscope equipped with a SIT camera and Universal Imaging software. Images were obtained at intervals of 5–30 min, stored, and analyzed for changes in wrinkling pattern (appearance or disappearance of preexisting wrinkles). Alternatively, the dynamics of wrinkling pattern was monitored using time-lapse video microscopy.

Fluorescence microscopy and antibodies. After they were fixed with 4% paraformaldehyde, cells were permeabilized with 0.2% Triton X-100 (10 min) and exposed for 30 min to primary antibodies, as specified in RESULTS. The following antibodies were used: monoclonal anti-vinculin (Sigma Chemical, St. Louis, MO), monoclonal anti-paxillin (Transduction Laboratories, Lexington, KY), monoclonal anti-PY20 (Upstate Biotechnology, Lake Placid, NY), monoclonal anti-FAK
(Transduction Laboratories), as well as rhodamine- or FITC-labeled phalloidin (Molecular Probes, Eugene, OR). After extensive rinsing in PBS, cells were exposed for 30–60 min to an appropriate secondary antibody, repeatedly washed in PBS, rinsed in deionized water, and mounted with Gel/Mount (Biomeda, Foster City, CA). Coverslips were viewed on a Zeiss Axiophot or a Nikon Diaphot microscope equipped for epifluorescence. Micrographs were taken on T-Max 400 film (Eastman Kodak, Rochester, NY).

A separate series of experiments was performed to monitor cell adhesion using time-lapse videomicroscopy. Cells were plated on glass coverslips in serum-containing medium, recording was performed at a rate of 1 frame/10 s, and NO donors were added 15–30 min after plating, as specified in RESULTS.

Western blot analysis. HUVECs were washed with ice-cold PBS and lysed using a buffer of the following composition (10 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.2% SDS, 2 mM orthovanadate, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 5 µg/ml leupeptin, and 5 µg/ml pepstatin) on ice for 30 min. Lysates were centrifuged at 14,000 rpm for 15 min and supernatants used for further immunoprecipitation and immunoblotting experiments after protein concentration was determined (bicinchoninic acid assay kit; Pierce, Rockford, IL). For immunoprecipitation, primary antibodies at 1 µg/ml were added to cleared lysates and incubated for 1 h at 4°C. Immunoprecipitated complexes were collected by addition of 20 µl of protein A-agarose beads (Santa Cruz Biotechnology). After 5 min of boiling in sample buffer, beads were sedimented, and supernatants containing equal amounts of protein were loaded (5–20 µg total protein) on 4–12% SDS-polyacrylamide gels (Novex, San Diego, CA) and electrophoresed. After blocking nonspecific binding and reaction with primary and secondary antibodies, labeled proteins were visualized using an enhanced chemiluminescence (ECL) kit (Amersham, Arlington Heights, IL) and were quantitated densitometrically. Proteins were transferred to Immobilon-P membranes (Millipore) in a wet transfer system (Novex, San Diego, CA). Blots were incubated in a blocking buffer (1% BSA in 10 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Tween 20) for 30 min. Tyrosine-phosphorylated proteins were visualized by incubating with horseradish peroxidase-conjugated PY20 antibodies (1:1,000 dilution) followed by ECL detection (Amersham).

Statistical analysis. Where appropriate, data are expressed as means ± SE. Mean values were compared for statistical significance using ANOVA and followed by Student’s t-test. A value of P < 0.05 was considered to be statistically significant.

Fig. 1. Serial images of human umbilical vein endothelial cells (HUVECs) plated on silicone rubber substratum: spontaneous changes in the pattern of wrinkling. Representative videomicroscopic images of HUVEC wrinkling of a flexible silicone rubber substratum. Images were collected every 2 s, but only images with an interval of 2–3 min are presented. Note subtle differences in wrinkling pattern, which occur between the frames, indicative of remodeling of tractional forces exerted by the cells on the matrix. Magnification ×100.

Fig. 2. Dynamics of wrinkling of silicone rubber substratum by HUVECs: effects of nitric oxide (NO). Bars represent integral change in number of wrinkles per cell during 30-min control period (−30 to 0) and following application of 0.1 mM sodium nitroprusside (SNP; 30-min intervals). Note that there was a net loss of wrinkles during first 30-min period after administration of NO donor. Wrinkling pattern showed recovery at 60–90 min. Black bars represent dynamics of net wrinkling per cell without SNP. *P < 0.05 difference from basal level (−30 to 0 min).
RESULTS

Videomicroscopy of HUVECs cultured on silicone rubber surfaces. During cell migration, tractional forces exerted by a cell on the adhesive interactions with the extracellular matrix provide the momentum for movement. These tractional forces can be visualized as formation of wrinkles when a substratum is represented by a flexible silicone rubber. Indeed, the time-lapse videomicroscopy of HUVECs plated on the silicone rubber surface, to visualize the dynamics of tractional forces, revealed that cells were exerting such forces by generating a “halo” of wrinkles and that cells were continuously remodeling the pattern of wrinkles, as illustrated in Fig. 1, capturing a series of consecutive images of minute variations in the position and geometry of wrinkles. Changes in wrinkling pattern of silicone rubber sheets by unstimulated cells indicated that focal adhesions and tractional forces exerted through them were constantly remodeling. Such a direct visualization of changing tractional forces provides the first demonstration of this phenomenon in a resting cell and supports our previous inference on the existence of podokinesis, a term used to denote scalar micromotion in endothelial cells (22).

In the presence of a short-acting NO donor, 100 µM sodium nitroprusside (SNP), the wrinkling pattern changed. Quantitative analysis of the wrinkling pattern demonstrated (Fig. 2) that SNP resulted in a partial net loss of wrinkles per cell within the first 30 min, coincident with the peak release of NO from SNP, followed by the recovery of wrinkling. These data suggested that NO releases tractional forces exerted through focal adhesions and stress fibers, thereby interfering with the processes of adhesion and spreading in freshly plated endothelial cells. The validity of this conclusion was next tested in the following series of experiments.

Cell spreading and formation of stress fibers. Videomicroscopy analysis of cell surface area, as a measure of cell spreading, was performed on application of different concentrations of SNP. Data presented in Table 1 demonstrate that SNP dose dependently interfered with HUVEC spreading, with the high concentrations of SNP decreasing the spreading. In contrast, inhibition of the endogenous NO production in HUVECs pretreated with L-NAME resulted in a significant increase in the cell surface area, indicating that cell spreading was enhanced. These findings were further corroborated by studies of the effect of NO on actin stress fiber formation. SNP had a profound dose-dependent inhibitory effect on the formation of stress fibers by HUVECs. Although 0.1 mM SNP did not appreciably affect cell spreading and formation of stress fibers, at 0.5 mM both were inhibited, and, at 2.0 mM SNP, cells failed to spread and to form stress fibers (Fig. 3, A-D).

Similar results were obtained in established HUVEC monolayers treated with 500 µM SNP for different periods of time, then fixed and double stained with rhodamine-phalloidin and antibodies to phosphotyrosine. As shown in Fig. 4, stress fibers and the peripheral actin band disappeared within 30 min after application of NO donor. This process was accompanied by the loss of phosphotyrosine staining (Fig. 4B), normally confined to focal adhesions, equivocal changes in the distribution of vinculin (not shown), and the retraction and dropout of some HUVECs (Fig. 4, B-D), which left behind components of focal adhesions. These changes were reversible, so that by 90 min tyrosine phosphorylation was immunodetectable at focal adhesions, and by 120 min stress fibers reappeared (Fig. 4E).

Studies employing another cell type, which is commonly used in investigations of adhesion and migration

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<th>SNP</th>
<th>Control</th>
<th>100 µM</th>
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<td>µm² ± SE</td>
<td>507 ± 21</td>
<td>484 ± 35</td>
<td>410 ± 29*</td>
<td>372 ± 39*</td>
<td>581 ± 26*</td>
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Values are means ± SE in µm²; n = 16. HUVEC, human umbilical vein endothelial cells; SNP, sodium nitroprusside; L-NAME, N^G-nitro-L-arginine. ∗P < 0.05 vs. control.

Fig. 3. Effect of NO on formation of stress fibers by freshly seeded HUVECs. Cells were seeded on fibronectin-coated glass coverslips and allowed to attach for 90 min in presence of increasing concentrations of SNP. A: no added SNP. B: 0.1 mM SNP. C: 0.5 mM SNP. D: 2.0 mM SNP. After fixation, cells were stained with rhodamine phalloidin, as detailed in MATERIALS AND METHODS. Note disorganization of stress fibers in C and especially in D. Magnification, ×630.
and which does not possess an endogenous NO-synthetic pathway (Swiss 3T3 cells), yielded similar results. Table 2 summarizes the effect of SNP and glutathione-NO on the spreading of Swiss 3T3 cells as assessed using time-lapse videomicroscopy. Although 71% of control cells were well spread by 90 min after plating, this value was reduced to ~40% when cells were treated with either NO donor. Control spread cells showed a well-developed actin cytoskeleton with prominent phalloidin-stained stress fibers and association of vinculin with focal adhesions (Fig. 5, A and B). In contrast, Swiss 3T3 cells, exposed during adhesion to either NO donor, showed reduced formation of stress fibers but relatively preserved vinculin recruitment to focal adhesions (Fig. 5, C-F). Occasionally, cellular “ghosts” could be recognized by the remnants of vinculin attached to the coverslips in the absence of any visible cells or F-actin cytoskeleton (Fig. 5, G and H, and see Discussion). A similar phenomenon was observed by Crowley and Horwitz (5) in fibroblasts detached after treatment with ATP. Time-lapse videomicroscopy of adhering and spreading Swiss 3T3 cells showed that the addition of SNP resulted in a temporal reversal of spreading (data not shown). Collectively, the data obtained in Swiss 3T3 cells confirm the ability of NO to interfere with cell spreading and suggest that this phenomenon is not limited to the NO-generating HUVECs.

De novo formation of focal adhesion complexes. The competence of HUVECs in de novo formation of focal adhesions was further evaluated using ProNectin- or fibronectin-coated latex beads attached to the established monolayers. Control cells displayed the ability to cluster tyrosine-phosphorylated components of focal adhesion complexes at the sites of cell-bead attachment, but this phenomenon was significantly diminished within the above time frame in the presence of NO donors (Fig. 6). In two separate experiments, 0.5 mM SNP decreased the number of beads with immunodetectable phosphotyrosine staining from 43% in control to 18% after 120 min incubation with SNP. The similar phenomenon of attenuation in paxillin staining of focal adhesions at sites of cell-bead attachment was observed in SNP-treated cells (not shown). Pretreatment of HUVECs with an inhibitor of the downstream signaling of NO through guanylate cyclase oxadiazolo[4,3-a]quinoxalin-1-ene (ODQ; 10 µM for 30 min), before the application of SNP, partially restored the de novo formation of focal adhesions, as judged by the abundance of immunodetectable phosphotyrosine at sites of cell-bead contacts (31% of beads showing staining). Hence high NO levels, but not the constitutively generated basal NO levels, are implicated in the inhibition of de novo formation of focal adhesions.

Effects of NO on cell adhesion and motility. The next series of experiments tested the functional role of NO in HUVEC adhesion and motility. Application of SNP resulted in a dose-dependent inhibition of HUVEC adhesion to fibronectin-coated microelectrodes, as monitored using impedance analysis (Fig. 7). The transmigration of HUVEC guided by 10 ng/ml VEGF in a modified Boyden chamber showed that the application of L-arginine, the substrate for NO synthase, accelerated the process, whereas inhibition of endogenous NO production with L-NAME inhibited it (Fig. 7). Similar results were obtained in our previously published experiments on monitoring the rate of wound healing in HUVEC monolayers (22). Finally, videomicroscopy of HUVEC migration was performed. Cells were plated onto fibronectin-coated glass coverslips and studied 2 h later. Micropipettes containing 10 ng/ml VEGF were placed in the upper corner of all chambers during the recording. Where indicated, 2 mM L-NAME was added to the 10 mM HEPES-containing culture medium. The rate of cell migration in the direction of the source of VEGF was calculated as changes in distance between consecutive images. Analysis of recorded cell migration

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<th>Table 2. Spreading of Swiss 3T3 cells</th>
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<tr>
<td>Not Spread</td>
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<tr>
<td>Control</td>
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<td>GS-NO</td>
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Values are means ± SE in percentage of 100 counted cells. GS-NO, glutathione-nitric oxide. *P < 0.05 vs. control.
Fig. 5. Double fluorescence labeling of freshly plated Swiss 3T3 cells. A, C, E, G: rhodamine phalloidin fluorescence. B, D, F, H: vinculin localization. A and B: intact cells 90 min after plating in serum-containing DMEM medium. Note substantial degree of spreading, formation of stress fibers, and clustering of vinculin to focal adhesions. C-F: different stages of spreading in Swiss 3T3 cells plated in presence of 0.5 mM SNP. Note defective formation of stress fibers. G and H: cellular ghost recognizable by remnants of vinculin (H) and absence of F-actin (G). Magnification, ×630.

Fig. 6. De novo formation of focal adhesion complexes in HUVECs presented with fibronectin-coated latex beads. HUVEC monolayers were presented with fibronectin-coated latex beads, fixed, and stained with anti-phosphotyrosine antibodies, as detailed in MATERIALS AND METHODS. A and B: images obtained from the basal and apical focal planes of intact monolayers, respectively. Note typical distribution of phosphotyrosine at focal adhesions in A and appearance of phosphotyrosine staining around the beads in B. C: bead-HUVEC adhesion reaction was conducted in presence of 0.5 mM SNP (only apical focal plane is presented). Note a dramatic decrease in immunodetectable phosphotyrosine at sites of focal contact between beads and cells. D: same as in C, but HUVECs are pretreated for 30 min with a guanylate cyclase inhibitor, 10 µM ODQ, before application of SNP. This pretreatment resulted in increased tyrosine phosphorylation of de novo formed focal adhesions. Magnification, ×630.
toward a guiding micropipette containing VEGF documented that inhibition of NO synthase by L-NAME resulted in a threefold deceleration of cell migration from 3.4 ± 0.6 µm/30 min seen in control cells to 1.1 ± 0.5 µm/30 min in cells with inhibited NO synthase (P < 0.05; n = 12 cells). Collectively, the results obtained using four independent techniques provide strong evidence implicating NO in the acquisition of a migratory phenotype by HUVECs, whereas inhibition of NO production promotes a stationary well-spread phenotype. The role of focal adhesion complexes and their tyrosine phosphorylation in these processes was examined in the next series of experiments.

NO effects on components of focal adhesion complexes. The potential involvement of focal adhesion kinase (p125FAK), a tyrosine kinase strongly implicated in adhesion-dependent signaling cascades (1, 12, 28, 32), in NO regulation of HUVEC adhesion and motility was tested next. Plating HUVECs on fibronectin resulted in a conspicuous recruitment of p125FAK to focal adhesions within 90 min (Fig. 8A). Suppression of endogenous NO production with L-NAME dramatically reduced its recruitment, resulting in a punctate staining of the entire ventral cell surface, whereas addition of SNP did not interfere with p125FAK recruitment to focal adhesions (Fig. 8B and C, respectively).

SNP caused no dramatic changes in protein expression as judged by Coomassie blue staining of proteins in HUVEC lysates (not shown). Western analysis showed that the global expression of p125FAK, p130Cas, paxillin, and vinculin did not exhibit substantial changes at 2, 10, 30, and 90 min after the application of 0.5 mM SNP to HUVECs and that SNP within the concentration range from 25 to 500 µM did not affect the expression of p125FAK, paxillin, or vinculin (data not shown). Immunoprecipitation of tyrosine-phosphorylated proteins followed by immunoblotting with anti-p125FAK antibodies revealed biphasic changes in the phosphotyrosine content of p125FAK and, to a lesser extent, paxillin (Fig. 9): a sharp increase after 2–10 min of exposure to SNP followed by a decline by 30 min.

Effect of protein tyrosine kinase inhibition on HUVECs migrating toward VEGF. To examine the functional significance of tyrosine phosphorylation events in HUVEC migration, videomicroscopy of cells migrating toward a source of VEGF (micropipette filled with 100 ng/ml VEGF) was examined in the presence and absence of genistein (20 µg/ml). As summarized in Fig. 10, the rate of HUVEC migration toward VEGF was inhibited sixfold in the presence of genistein (20 µg/ml). As summarized in Fig. 10, the rate of HUVEC migration toward VEGF was inhibited sixfold in the presence of genistein. Furthermore, when genistein alone was presented to the cells in the micropipette to generate a concentration gradient, the cells that previously displayed a random movement were repelled from the micropipette.

DISCUSSION

The data presented herein implicate endogenous NO production in the remodeling of focal adhesions, as revealed by continuous changes in the wrinkling pattern, and in supporting cell locomotion toward guidance cues. On the other hand, L-NAME inhibition of constitutive endogenous NO production by HUVECs is associated with decreased immunolocalization of p125FAK to focal adhesions, increased spreading, and...
decreased motility. The data also demonstrate the effect of exogenous NO on the inhibition of cell spreading and biphasic changes in tyrosine phosphorylation of p125FAK, as well as inhibition in the recruitment of phosphotyrosine-immunodetectable proteins to de novo formed focal adhesions. We have previously demonstrated that effects of NO on cell adhesion and locomotion can be reproduced by cell-permeant forms of cGMP (23). Murphy-Ullrich et al. (19) provided evidence that cGMP-dependent protein kinase is necessary, but not sufficient, for focal adhesion disassembly triggered by such counteradhesive matrix proteins as thrombospondin and tenasin. Collectively, these data provide the basis for a coherent framework linking the processes of assembly/disassembly of focal adhesions, their regulation by NO via the guanylate cyclase-cGMP signaling cascade, and the rate of endothelial cell migration.

Although p125FAK plays an important role in assembly of focal adhesions and their turnover, it does not seem to contribute in a major way to the process of their disassembly, at least when induced by ATP (5). However, inhibition of p125FAK signaling, either in cells obtained from mice with targeted deletion of the gene (11) or in cells loaded with a fusion protein containing the p125FAK targeting domain, which leads to the displacement of endogenous p125FAK (9), produced a phenotype that displayed enhanced focal adhesions and decreased migration rate. This was precisely the phenotype of HUVECs pretreated with L-NAME: they exhibited reduced immunolocalization of p125FAK to focal contacts and suppressed motility.

Another important observation is related to the role of NO in tyrosine phosphorylation of focal adhesion components. In vitro studies of a low-molecular-weight phosphotyrosine protein phosphatase, previously known as acid phosphatase, demonstrated that NO-generating compounds inhibit the activity of the enzyme (3). In contrast, more recent observations have established that NO increases protein tyrosine phosphatase activity (6). Tyrosine phosphorylation of focal adhesion-associated proteins like p125FAK or paxillin has been shown to be dependent on the integrity of the actin cytoskeleton. Disruption of the actin cytoskeleton using cytochalasin D causes a profound and selective inhibition of p125FAK and paxillin (31, 33), and tyrosine phosphorylation of these proteins has been suggested to stabilize interactions between focal adhesions and the cytoskeleton (1, 32), whereas their destabilization was attributed to decreased tyrosine phosphorylation of focal adhesion components (14). In this context, the phenotype of HUVECs subjected to increased concentrations of NO was that of a cell with reduced tyrosine phosphorylation of p125FAK (after the initial brief elevation of tyrosine phosphorylation), increased turnover of focal adhesions (as visual-
ized by the remodeling of wrinkles on silicone rubber), and the propensity for migration. These observations are consistent with the proposed role of NO in destabilization of contacts between focal adhesions and the cytoskeleton, thus releasing the cytoskeleton from focal adhesion complexes.

The consistently observed biphasic effect of NO on the state of tyrosine phosphorylation of p125FAK and, to a lesser degree, of paxillin is most intriguing. There is burgeoning evidence that the reduced oxygen and nitrogen species possess the ability to shift the balance between protein tyrosine kinase and phosphatase activities by acting on the redox-sensitive cysteine residues of tyrosine phosphatases and leading to their transient inactivation (reviewed in Ref. 7). The observed rapid increase in tyrosine phosphorylation of p125FAK probably reflects the process of inactivation of tyrosine phosphatases. This is followed by a decreased tyrosine phosphorylation of p125FAK, which may be coincidental with the restoration of the phosphatase activity. Although such an explanation is plausible, we have not yet studied the activity of tyrosine phosphatases during HUVEC exposure to NO. A series of videomicroscopy studies, however, provided support for the role of tyrosine phosphorylation in the directional migration of HUVECs. Inhibition of tyrosine phosphorylation of p125FAK resulted in the profound deceleration of HUVECs migrating toward a VEGF source. Furthermore, when randomly moving HUVECs were presented with a genistein gradient (micropipette filled with genistein), cells acquired a directional movement away from the pipette.

In HUVECs and Swiss 3T3 cells treated with NO-generating compounds, the observed areas containing elements of focal adhesion complexes bound to the matrix without attached cytoskeleton are of significant interest, since they buttress the previous conclusion. The phenomenon of “ripping” of integrin receptors with attached focal adhesion proteins, but not the cytoskeleton, from a trailing edge of fibroblasts has been proposed to be an important mechanism for releasing the trailing edge of the cell in the process of locomotion (26). Similarly, Nakamura et al. (20) have observed a “peeling off” phenomenon in endothelial and other cell types subjected to the stimulated neutrophils or the neutrophil-derived oxidant NH2Cl. It appears, therefore, that in all these instances, in migrating fibroblasts and in NO-treated HUVECs or in the HUVECs presented with the stimulated neutrophils, the links between adhesion complexes and the cytoskeleton become uncoupled. The observations made here in NO-treated HUVECs further emphasize the importance of assembly and disassembly of focal adhesion complexes in cell locomotion (10). Specifically, the inference to be made on the basis of data presented above ascribes to NO the function of endogenous NO production is to seek the optimal set point on the bell-shaped curve for the adhesion-to-migration ratio. When a gradient of an angiogenic agent is presented to the cell, it functions to establish the leading edge (by increasing tyrosine phosphorylation of p125FAK). Thus maneuvers directed to prevent it, e.g., the application of genistein together with VEGF, compromise the directed cell migration.

The question to be answered is whether this is a physiological or pathophysiological effect of NO. Probably two extreme situations are relevant to the observed effect of NO on focal adhesions. One is exemplified by the states of profound inhibition of NO synthase, when endothelial cells may attain a nonmotile pheno-

**Fig. 10. Effects of genistein on endothelial cell migration.** Time-lapse videomicroscopy of HUVECs presented with vascular endothelial growth factor (VEGF) in the micropipette was performed as detailed in MATERIALS AND METHODS. Number of cells (n) moving toward or away (negative values of rate of migration) from the guidance pipette and average rate of migration are presented. Note that whereas VEGF produced a consistent unidirectional migration of HUVECs, VEGF in combination with genistein resulted in a 6-fold deceleration of migration, while genistein alone increased the proportion of cells migrating away from the micropipette without changes in the rate of migration.
tye, similar to that observed in p125FAK-deficient cells (9, 11), thus explaining the reduced rate of wound healing and angiogenesis. The second is related to the conditions of NO overproduction, which may result in endothelial retraction and denudation of the vascular wall, as characteristically seen in endotoxemia (15).

In summary, although many questions remain to be answered, the above data demonstrate an important interaction of NO with focal adhesions. We have established the phenotype of HUVECs subjected to increased concentrations of NO as that of a cell with the increased turnover of focal adhesions, reversibly reduced tractional forces exerted through focal adhesions on the matrix, and propensity for migration. HUVECs with the suppressed activity of endothelial NO synthase, in contrast, exhibit a gradual decrease in the expression of p125FAK, inability to recruit it to focal adhesions, increased ability to spread, and increased ability to migrate.

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