Carbon Monoxide and Nitric Oxide Mediate Cytoskeletal Reorganization in Microvascular Cells via Vasodilator–Stimulated Phosphoprotein (VASP) Phosphorylation:

Evidence for Blunted Responsiveness in Diabetes

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Objective: We examined the effect of the vasoactive agents carbon monoxide (CO) and nitric oxide (NO) on the phosphorylation and intracellular redistribution of VASP, a critical actin motor protein required for cell migration that also controls vasodilation and platelet aggregation.

Research Design: We examined the effect of donor-released CO and NO in Endothelial Progenitor Cells (EPC) and platelets from nondiabetics and diabetics and in Human Microvascular Endothelial Cells (HMEC) cultured under low- (5.5 mM) or high- (25 mM) glucose conditions. VASP phosphorylation was evaluated using phosphorylation site-specific antibodies.

Results: In control platelets, CO selectively promotes phosphorylation at VASP Ser-157, while NO promotes phosphorylation primarily at Ser-157 and also Ser-239, with maximal responses at 1 min with both agents on Ser-157 and at 15 min on Ser-239 with NO treatment. In diabetic platelets, neither agent resulted in VASP phosphorylation. In nondiabetic EPCs, NO and CO increased phosphorylation at Ser-239 and Ser-157, respectively, but this response was markedly reduced in diabetic EPCs. In endothelial cells cultured at low glucose, both CO and NO induced phosphorylation at Ser-157 and Ser-239; however, this response was completely lost when cells were cultured at high glucose. In control EPCs and in HMEC exposed to low glucose, VASP was redistributed to filopodia-like structures following CO or NO exposure, however, redistribution was dramatically attenuated in high glucose.

Conclusions: Both vasoactive gases promote cytoskeletal changes through site- and cell type-specific VASP phosphorylation and in diabetes blunted responses to these agents may lead to reduced vascular repair and tissue perfusion.
The gaseous signal molecules NO and CO exert multiple modulatory actions in regulating vascular function. While NO effects have been recognized for over a decade, similar vasoregulatory action of CO was established only recently. CO is generated by heme oxygenase (HO-1) under a wide variety of conditions (e.g. cell exposure to such stressors as hypoxia, growth factors, and cytokine stimulation) that activate the enzyme (1) (2). Unlike its highly reactive cognate NO, which participates in multiple redox reactions, CO is a relatively stable gas that exhibits extraordinary affinity for heme centers (3-5). Like NO, the signaling effects of CO rely in part on its ability to form a complex with the heme moiety of soluble guanylate cyclase (sGC), stimulating the synthesis of the diffusible second messenger guanosine 3’5’-cyclic monophosphate (cGMP) (6). The sGC/cGMP pathway plays a critical role in mediating the effects of CO on vascular relaxation and inhibition of platelet aggregation, and coagulation (7; 8).

A recently recognized property of NO is its cell type-specific facilitation or inhibition of cell migration (9), a complex process involving molecular-mechanical events that depend on extracellular signaling, actin-based motility, and cell adhesion. EPCs differentiate into endothelial cells whose function in vascular repair depends on chemokine- and growth factor-directed cell migration. The role of EPCs in endothelial repair is supported by their ability to inhibit development of atherosclerosis (10; 11) and intimal hyperplasia (12), while still promoting beneficial angiogenesis. We previously demonstrated the central role of the actin cytoskeleton in EPC migration (13), and our findings suggest that NO has a critical function within EPCs, where it regulates the distribution of vasodilator-stimulated phosphoprotein (VASP). The latter plays a pivotal role in promoting actin filament elongation at the leading edge by forming an active molecular motor complex that propels motility (14). VASP contains three distinct phosphorylation sites (Ser-157, Ser-239, Thr-278), the first of which is preferentially phosphorylated by PKA and the second by PKG. Although the exact roles of phosphorylated residues in VASP have not completely been elucidated, one idea is that a high [cAMP]/[cGMP] ratio promotes VASP-activated actin filament elongation, whereas a low [cAMP]/[cGMP] ratio favors filament capping and loss of motility (15). The following factors are known to influence VASP phosphorylation: intracellular localization in focal adhesions, filopodia, and lamellipodial; accessibility of phosphorylation sites in VASP that is complexed with other proteins; availability of specific protein kinases and/or phosphoprotein phosphatases; as well as the respective activators and inhibitors of these kinases and phosphatases (16). We previously reported that the reduced bioavailability of NO in diabetic individuals prevents VASP redistribution, resulting in the inability of EPC to form proper cytoskeletal extensions (13). We also showed that the EPC chemoattractant stromal cell derived factor-1 (SDF-1) transcriptionally activates HO-1 via the atypical PKC-ζ isoform generating CO which in turn can phosphorylate VASP in endothelial cells (17).

Because 3’,5’-cyclicGMP- and 3’,5’-cyclicAMP-stimulated protein kinases (PKG and PKA, respectively) catalyze VASP phosphorylation, and because the latter is thought to control VASP’s subcellular distribution and function (13), we directly compared the effects of NO and CO on VASP phosphorylation and redistribution in cells typically known to be dysfunctional in diabetes, namely platelets, EPC and microvascular endothelial cells. We demonstrate that both CO and NO regulate VASP phosphorylation and that pretreatment...
with either agent stimulates migration toward SDF-1. We also show that normal platelets display a modest response to exogenous CO stimulation but a greater response to NO treatment. In contrast diabetic platelets are not responsive to either CO or NO treatment (data not shown). Culturing microvascular endothelial cells at high glucose concentrations also results in reduced VASP phosphorylation. These novel findings suggest that CO regulates VASP phosphorylation and vascular cell migration under conditions of reduced NO bioavailability as observed in diabetes.

**METHODS**

**Cell Culture:** Human lung microvascular endothelial cells (HMEC) (LONZA, Walkersville, MD) were cultured to 80% confluency in EGM-2 MV medium (LONZA) in 5.5 mM or 25 mM glucose for one week. Cells were then incubated overnight in EBM-2 supplemented with 2% fetal bovine serum. Fresh medium (EBM-2 + 2% FBS) was added on the following day, and CO- or NO-containing medium was added to the designated samples for 1 or 15 minutes at 37 ºC, as described below.

**Isolation of Platelets:** Peripheral blood from healthy and diabetic volunteers giving informed consent under a protocol approved by the Institutional Review Board (IRB) was collected in cell preparation tubes with heparin (BD Biosciences). and platelets were isolated using Opti-Prep™ (Axis-Shield PoC AS, Oslo, Norway) as recommended by the manufacturer.

**EPC culture:** Peripheral blood from healthy and diabetic volunteers giving informed consent under a protocol approved by the Institutional Review Board was collected into CPT tubes (BD Biosciences, CA), and the EPCs were freed of the mononuclear cells using Endocult medium kit (Stem Cell Technologies, Vancouver, CA) using the manufacturer’s protocol as previously described (13).

**Immunohistochemistry:** EPC were cultured onto fibronectin-coated dishes with Endocult stem cell liquid media until colonies formed. These cells were then treated for 15 minutes or 4 hours with 100 μM DETA-NO (diethylenetriamine/NO) (Sigma-Aldrich, St. Louis, MO) in water or 10 μM CO-donor Ru(II)(CO)3Cl2 (Sigma-Aldrich) in DMSO. Control samples were treated with vehicle. At the end of the treatment, medium was removed, and cells were fixed overnight at 4ºC in 4% paraformaldehyde (PFA) in PBS, supplemented with calcium and magnesium ions, and adjusted to pH 7.4.

Cells were then washed in PBS and permeabilized with 0.1% Triton X-100 for 30 minutes at room temperature. After washing the samples an additional three times in PBS, cells were treated with 10% normal goat serum - 1% BSA in PBS for 1 hour at room temperature to block nonspecific antigens. Cells were then incubated with 5 μg/ml mouse anti-VASP antibody (BD Biosciences, San Jose, CA) in 5% normal goat serum overnight at 4ºC and then with FITC-labeled goat anti-mouse IgG1 (1:250 dilution) (Southern Biotech, Birmingham, AL) in 5% normal goat serum for 1 hour at room temperature. Samples were then washed, dried, and mounted with 4’,6-diamino-2-phenylindole (DAPI) (Vectashield®, Vector Laboratories Inc., Burlingame, CA) to label nuclear DNA. Samples were examined by fluorescence microscopy (Nikon Eclipse TE200), using a Nikon Plan-fluor 40X (NA=1.30) oil objective and a fluorescein filter set (520 ± 2 nm). Micrographs were captured using a SPOT™ digital camera 0.60X HRD06-NIK (Diagnostic Instruments, Inc. Sterling Heights, MI) and processed using SPOT™ Advanced software Version 2.2.1 for Windows.

**FACS:** Peripheral blood was collected into CPT tubes (BD Biosciences) and centrifuged
to obtain the mononuclear cell fraction, from which CD34\(^+\) cells were isolated using a CD34\(^+\) isolation kit (Stem Cell Technologies, Vancouver CA). Briefly, 2x10\(^7\) cells were incubated with a CD34\(^+\) selection cocktail for 15 minutes, and 50 \(\mu\)l magnetic nanoparticles were then added and incubated for another 10 minutes. The suspension volume was increased to 2.5 ml, and the particle-bound CD34\(^+\) cells were concentrated on the tube’s inner surface with the aid of a magnet. The supernatant was decanted, and the remaining CD34\(^+\) cells were resuspended in RPMI culture media (Cellgro, Herndon, VA).

The cells were then treated with 100 \(\mu\)M on DETA-NO (Sigma-Aldrich, St. Louis, MO) for either 15 minutes or 4 hours in a CO\(_2\) incubator. Following treatment, the CD34\(^+\) cells were permeabilized using a cytofix/Cytoperm kit (BD Biosciences, CA), blocked with 10% normal human serum (Jackson Immuno Research labs, West Grove, PA), and 10 \(\mu\)g antiphospho-VASP (upstate signaling, Lake Placid, NY) was added to the cells, followed by 30-minute incubation on ice. The cells were then washed with PBS and resuspended in RPMI culture media (Cellgro, Herndon, VA), and again incubated on ice for 30 minutes. The resulting cells were washed in PBS and analyzed by FACS. Anti-GFP (Molecular probes, Carlsbad, CA) was used as an isotype control.

**Preparation of DETA-NO and Ru(II)(CO)\(_3\)Cl\(_2\) Donors:** The half-lives for NO and CO release from DETA-NO and Ru(II)(CO)\(_3\)Cl\(_2\) are greatly different, resulting in the almost immediate release of CO, but very slow release of NO. Therefore, to assure that cells were exposed to comparable concentrations of NO and CO, we first calculated the amount of CO released at 1 and 15 minutes, using the integrated rate law: 
\[
[\text{CO}]_t/\text{[CO]}_{\text{initial}} = \exp (-k_{\text{CO}}t),
\]
where \([\text{CO-Donor}]_{\text{initial}}\) and \([\text{CO-Donor}]_t\) are the carbon monoxide donor concentrations at time \(t\) and initially, \(k_{\text{CO}}\) is the first-order rate constant for CO release, and \(t\) is the incubation period. We then used the corresponding rate law (i.e., \([\text{NO-Donor}]_t/\text{[NO-Donor]}_{\text{initial}} = \exp (-k_{\text{NO}}t))\) to determine the time needed to obtain a comparable extent of NO release as that observed with CO donor in 1 and 15 minutes. The CO- and NO- donor rate constants \(k_{\text{CO}}\) and \(k_{\text{NO}}\) of 0.07 \(\text{min}^{-1}\) and 0.0006 \(\text{min}^{-1}\) respectively, were determined based on the published half-lives of 10 minutes for Ru(II)(CO)\(_3\)Cl\(_2\) (18) and 18 hours for DETA-NO (19). In our experiments, we therefore separately pre-incubated the NO donor in culture medium in a sealed tube for the calculated NO times, and then transferred an appropriate aliquot to the cell. Because the partial pressure exerted by the released NO and CO is far below the solubilities in aqueous solution, and because the 1- and 15-minute exposure times were short, there was no need to maintain treated cells in a sealed culture dish.

**Immunoblot:** Following treatment with either the CO or NO donor, the CD34\(^+\) cells or platelets were washed with PBS and lysed with lysis buffer (Cell Signaling, Danvers, MA) containing protease (catalog # P8340 Sigma-Aldrich, St. Louis, MO) and phosphatase inhibitors (catalog # P2850 Sigma-Aldrich). Protein was quantified in each sample using commercially available BCA kit (Pierce, Rockford, IL) and 50 \(\mu\)g was applied to each well above the polyacrylamide gel (7.5%) (Criterion; Biorad Laboratories, Inc., Richmond, CA) at 120V for 20 minutes, followed by 140V for 65 minutes. Samples were run in duplicates for later detection of the two pVASP isoforms. The separated proteins were transferred to a nitrocellulose membrane (Biorad Laboratories, Inc., Richmond, CA) in a semi-dry transblot apparatus (Biorad Laboratories, Inc., Richmond, CA). The membranes were blocked in TBS containing 5% milk and 0.05% Tween-20 (Sigma-Aldrich) for 1 hour.
at room temperature. For the detection of phosphorylated VASP isoforms, the membranes were incubated at 4°C overnight with either a mouse monoclonal anti-pVASP Ser-239 antibody (Upstate Cell Signaling Solutions, Lake Placid NY) or a rabbit polyclonal anti-pVASP Ser-157 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), both at a 1:200 dilution. Blots were then washed with 5% milk for 5 minutes and incubated with a 1:2000 dilution of either a horseradish peroxidase (HRP)-conjugated anti-mouse antibody (Sigma-Aldrich), or an anti-rabbit (Santa Cruz Biotechnology, Inc.), respectively, for 1 hour at room temperature. After incubation with the secondary antibody, the membranes were washed twice with TBS containing 0.05% Tween-20 for 5 minutes, followed by two additional 10-minute washes with TBS. Protein bands were visualized with enhanced chemiluminescence (ECL) using a commercial western blot detection kit (Amersham Biosciences Ltd., Amersham, UK) and phospho-VASP levels were quantified using the Scionimage software (Scion Corp., Frederick, MD). Standard molecular weight markers (Biorad Laboratories, Inc.) served to verify the molecular mass of phospho-VASP (50 kDa).

After phospho-VASP detection, the membranes were reprobed for VASP using a 1:200 dilution of a goat anti-VASP antibody (Santa Cruz Biotechnology, Inc.) and for β-actin using a mouse anti-β-actin primary antibody at a dilution of 1:7000 (Sigma-Aldrich) followed by HRP-conjugated anti-goat (Santa Cruz Biotechnology, Inc.) and anti-mouse IgG antibody (Sigma-Aldrich), both at 1:2000 dilution, and finally visualized by ECL. Standard molecular-weight markers served to verify the 42 kDa molecular mass of β-actin.

**In-Cell Western Analysis:** CD34+ cells were cultured in defined serum-free medium (StemSpan SFEM; StemCell Technologies, Inc., Vancouver, Canada) to obtain an optimal number of cells. For CD34+ cell proliferation and expansion, without differentiation, we used 1ml StemSpan SFEM supplemented with a cytokine cocktail (100 ng/ml flt3 ligand, 100 ng/ml stem cell factor, 20 ng/ml interleukin-3, and 20 ng/ml interleukin-6 (StemCell Technologies) and 50 ng/ml thrombopoietin (R&D Systems, Inc., Minneapolis, MN). After determining cell number with a hemocytometer (Hausser Scientific, Horsham, PA), 10,000 cells were transferred to each well in a 96-well plate (BD Falcon, San Jose, CA). After incubation with/without CO or NO donor, cells were fixed with 4% paraformaldehyde for 20 minutes at room temperature and then harvested by centrifugation. After removing the fixation solution, cells were permeabilized with 0.1% Triton X-100 in PBS and blocked with LI-COR Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE) for 1.5 hour at room temperature with moderate shaking. Blocking buffer was removed by aspiration, and the cells were incubated with 50-μl volume of diluted primary antibody at 4°C overnight in cold room. Cells were then washed 4 times for 5 minutes in PBS containing 0.1% Tween-20 (Fisher Scientific, Pittsburgh, PA) and incubated with diluted secondary antibody. After 1-hour incubation, cells were washed 4 times (5 minutes each) with PBS containing 0.1% Tween-20, followed by final washing in PBS to remove excessive detergent. The 96-well plate was then scanned in the appropriate channels using Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE). Relative quantification was normalized and re-adjusted in cell number from well to well using DNA staining.

**Statistical analysis:** All reported data are expressed as the mean ± SEM and were obtained from at least three independent experiments. Student’s t-test and ANOVA with Student-Newman-Keuls post-test were used to compare the data.
RESULTS

The choice of the technique utilized (e.g., standard western blotting, In-Cell western analysis, and FACS) for these studies was largely governed by the number of cells available for analysis. Because CD34+ cells represented less than 1% of circulating mononuclear cells, In-Cell western (low-sensitivity) and FACS (higher sensitivity) analysis were the methods of choice; however, for more abundant cells such as endothelial cells, standard western blotting was sufficient. All the phosphorylation studies on EPCs were performed after 15 minutes treatment. We then tested whether a shorter treatment (1 minute) could still elicit a significant migratory response.

We therefore pre-treated human CD34+ endothelial progenitor cells (EPC) for 1 and 15 minutes with medium containing NO or freshly generated CO, followed by SDF-1 stimulation (13) and then assessed cell migration using Boyden chambers. As demonstrated in Figure 1, both CO and NO treatment resulted in increased EPC migration to SDF-1, although a more robust response was observed with NO at 1 minute.

Because VASP is a key component of the actoclampon molecular motors responsible for actin-based cell motility (14; 20), and because VASP was originally identified as a major substrate for 3',5'-cyclic GMP-stimulated protein kinase in platelets and endothelial cells (21), we asked whether NO and CO elicited similar effects on VASP phosphorylation and its mobilization to the leading edge of motile cells. VASP function appears to be chiefly regulated by phosphorylation at Ser-157 and Ser-239 and the availability of phosphorylation site-specific antibodies provides an unambiguous way to define both the sites and extents of signal molecule-activated VASP phosphorylation. We therefore incubated CD34+ cells for 15 minutes in the presence of NO or CO already liberated from their respective donors. As shown in Figure 2, NO treatment routinely increased the extent of VASP phosphorylation at Ser-239, whereas CO treatment consistently increased the extent of Ser-157 phosphorylation in these cells.

To confirm that the differential effects of CO and NO are not limited to CD34+ cells, we also examined platelets, which are a rich source of VASP and are the cells wherein VASP phosphorylation is best characterized (22). In platelets exposed to either NO or CO for 1- and 15-minute periods, we found that CO treatment caused a modest but yet significant VASP phosphorylation at Ser-157 while NO treatment, significantly increased VASP phosphorylation at Ser-157 with maximum response at 1 min and at Ser-239 with maximum effect at 15 min (Figure 3). These findings suggest that CO and NO elicit markedly different VASP phosphorylation responses in platelets and CD34+ cells.

Because VASP resides on the cytoplasmic face of the leading edge in motile cells (23), and because VASP phosphorylation is believed to control its interactions with VASP-docking proteins like vinculin, zyxin, and migfilin, we next examined the effects of donor-generated NO and CO on the redistribution of VASP in microvascular endothelial cells growing while firmly adhered to fibronectin-coated culture dishes. Under basal conditions (i.e., no NO or CO), VASP was mainly localized along the sides of actin filaments found throughout the peripheral cytoplasm (see Panels A and B Figure 4). However, following a 15-minute exposure to CO, VASP redistributed to the leading edge of advancing microvascular endothelial cells growing while firmly adhered to fibronectin-coated culture dishes. Under basal conditions (i.e., no NO or CO), VASP was mainly localized along the sides of actin filaments found throughout the peripheral cytoplasm (see Panels A and B Figure 4). However, following a 15-minute exposure to CO, VASP redistributed to the leading edge of advancing microvascular endothelial cells growing while firmly adhered to fibronectin-coated culture dishes. Under basal conditions (i.e., no NO or CO), VASP was mainly localized along the sides of actin filaments found throughout the peripheral cytoplasm (see Panels A and B Figure 4). However, following a 15-minute exposure to CO, VASP redistributed to the leading edge of advancing microvascular endothelial cells growing while firmly adhered to fibronectin-coated culture dishes.
In as much as diabetes-associated vascular dysfunction is frequently attributed, at least in part, to reduced levels of bioavailable NO, we sought to determine whether diabetes impacted the NO and CO effects on VASP phosphorylation within cell types typically affected by diabetes. We treated platelet samples from diabetic individuals with NO or CO under the same conditions used in Figure 1 and we observed no change in VASP phosphorylation, either at Ser-157 or Ser-239 (data not shown). In Figure 5, VASP phosphorylation is shown for two diabetic patients and one nondiabetic control. Patient 1 (gray bars) had type 2 diabetes of 5 years duration and excellent glycemic control (HgA1C = 6.4%) while patient 2 (black bars) had type 1 disease of 48 years duration and poor glycemic control (HgA1C = 11.3%). A different pattern was observed in the diabetic CD34+ cells, where NO treatment stimulated phosphorylation at VASP’s Ser-239, to a significantly less extent, when compared to the nondiabetic control. However we have observed considerable patient to patient variation in the degree of the response but all with the same pattern.

Notably, compared to control cells grown in low-glucose medium, exposure of microvascular cells to conditions mimicking aspects of diabetes (e.g., culturing these cells for one week in high-glucose medium) resulted in a failure of NO and CO to elicit changes in either VASP redistribution to the leading of the cells as shown by immunofluorescence (Compare Figure 4 to Figure 6) or phosphorylation at either Ser-157 and Ser-239 as detected by standard western blot analysis (Figure 7).

DISCUSSION

Understanding the regulation of endothelial cell migration in response to chemokines has proven to be a daunting task that requires the investigation of a manifold of factors known to affect chemotaxis (e.g., chemokine sensing by receptor-mediated signaling, cellular locomotion by receptor-mediated signaling, cellular locomotion by actin-based molecular motors, and even the cell’s energy status). Previous studies from our laboratory on CD34+ EPC showed that diabetic EPC have reduced intracellular NO concentration as well as a concomitantly reduced migratory capability. We found that, when pretreated with an NO donor, cell migration can be restored (13), and we further demonstrated that this exogenous NO exposure resulted in enhanced diabetic cell migration, with attendant changes in phosphorylation of the actin cytoskeletal protein VASP [Segal MS 2006].

Given the robust nature of the vasodilator gases CO and NO in modulating vascular function, we focused on the questions: Can CO suffice in place of NO in promoting vascular cell migration? And if so, do NO and CO exert their signaling effects on the same downstream phosphorylation target(s)? In this study, we demonstrate that CO can regulate VASP phosphorylation and in turn alter cell migration. We also directly compare the CO effects to those of NO in EPC, platelets, and microvascular endothelial cells. Our studies demonstrate that both vasoactive gases promote cytoskeletal changes through site-specific and cell type-specific VASP phosphorylation; however, these responses to NO and CO are blunted in diabetes and may be responsible for reduced vascular repair and tissue perfusion (24; 25).

The actoclampins are the force-generating motors responsible for actin-based cell crawling and vesicle motility (14). Each of these membrane surface-bound molecular motors consists of an actin filament (+)-end tracking protein, called a clampin (e.g., VASP, N-WASP, formins, etc.) and its actin filament partner. The energy for force production appears to be derived from nucleotide hydrolysis at the filament’s penultimate actin-ATP subunits, thereby promoting clampin release, translocation, and
rebinding to terminal actin-ATP subunits. Before the active motor complex is assembled, clampins must be recruited to the membrane surface, where they dock at motility sites at the tips of filopodia and lamellipodia as well as in the focal adherens complex. Rottner et al. (23), for example, showed that VASP not only co-localizes to adhesion sites with the adaptor proteins vinculin and zyxin, but is also recruited to the tips of lamellipodia in amounts that are directly proportional to the rate of lamellipodial protrusion. Tokuo and Ikebe (26) further showed that myosin X specifically transports VASP and other members of the Ena/VASP clampin protein family to the leading edge, where VASP then binds to a membrane docking protein such as vinculin, zyxin, or migfilin (27; 28). Only then can the actoclimpin motor assemble and generate the forces needed for cell migration. When viewed from this perspective, VASP phosphorylation may affect the recruitment by myosin X, VASP docking with membrane components, and/or VASP-mediated formation of an active motor that can propel cell crawling. Moreover, as cells stop moving, VASP is known to redistribute to other intracellular sites. Benz et al. (29), for example, showed that VASP interacts with II-spectrin in endothelial cells and that 3',5'-cyclic AMP protein kinase (PKA)-mediated VASP phosphorylation at Ser-157 inhibits this binding interaction. They also showed that VASP is dephosphorylated upon formation of cell-cell contacts and that, in confluent cells, II-spectrin colocalizes with non-phosphorylated VASP at cell-cell junctions (29). The exact details of how VASP phosphorylation at Ser-157 or Ser-239 controls one or more of these steps remain to be worked out. Even so, the observation that different cell types contain various clampins and their respective membrane-docking proteins is likely to explain why VASP phosphorylation can either stimulate or suppress cell crawling and actin-based cell shape changes in a manner depending on cell type and/or culture conditions.

VASP is also the most abundant platelet and endothelial protein phosphorylated by 3',5'-cyclic GMP-stimulated protein kinase (PKG) in NO signaling pathways, and, as noted above, this cytoskeletal adaptor protein is also a PKA substrate. Three Serine/Threonine phosphorylation sites within VASP play roles resulting in the inhibition of platelet aggregation and focal adhesion assembly (30; 31). In this study, we demonstrated that CO can also regulate VASP phosphorylation, and we directly compared CO and NO effects in EPCs, platelets, and microvascular endothelial cells. We found that CO pretreatment can similarly stimulate EPC migration (Figure 1) and phosphorylation of VASP’s Ser-157, whereas NO exposure results in Ser-239 phosphorylation.

While we did not perform direct measurements of CO levels in our experiments, we did use CO concentrations that are achievable in vivo in physiological conditions and previously used by investigators (32; 33). Endogenous CO has been reported to be generated in many cell types and the amount of CO released via the heme oxygenase reaction can reach up to 12 ml/day (~16 M/hour) (32). Previous studies have reported that tissues can produce 0.1-100 M CO in vivo from the HO reaction (33) and it is therefore quite reasonable to believe that sufficient levels of HO-1 are present in cells to provide sufficient levels of CO to explain our observed changes in VASP phosphorylation.

Moreover, in response to incubation of microvascular endothelial cells with NO or CO donors, VASP was readily redistributed to the peripheral membrane and filipodia (Figure 4). Thus, with regard to VASP localization, our data suggest that CO and NO may both have critical roles in vascular cell dynamics, and CO may have important contributions at
low NO bioavailability, a condition already known to occur in diabetes. Our studies clearly indicate that both vasoactive gases promote cytoskeletal changes through site-specific and cell type-specific VASP phosphorylation and that in diabetes responses to NO and CO are blunted. While the migratory deficiency seen in diabetic EPCs could be overcome by exogenous NO (13), exogenous NO did not result in phosphorylation and redistribution of VASP in mature endothelial cells cultured in high glucose conditions. This defect can be viewed as “diabetes-induced NO resistance”.

What also becomes apparent from the present study is that exposure of cells to NO or CO can greatly alter VASP recruitment to the leading edge with consequential effects on cell motility. Perhaps even more significant, in the context of diabetes, is that culturing endothelial cells at high-glucose conditions that mimic diabetes results in motility defects that can be traced, at least in part, to altered VASP phosphorylation. We therefore postulate that these defects contribute to reduced vascular repair and tissue perfusion.

Both heme oxygenases generate CO but they do so with very different kinetics (34; 35); HO-1 is induced by oxidants such as hydrogen peroxide, UV radiation, and pro-inflammatory cytokines and by growth factors, hemodynamic or shear stress, heat shock, and even by NO (3). Endothelial cells derived from either the microvasculature or the macrovasculature (36) responded equally to CO and NO, reflecting the key roles of both HO-1 and eNOS throughout in the entire vasculature. In contrast, platelets do not respond to CO, perhaps reflective of the low levels of heme oxygenase in adult platelets (37). By altering HO-1 and NOS gene expression, hypoxia potentially modulates the availability of these gaseous second messengers. Thus, fluxes in the CO and NO generation during hypoxic stress are likely to have dramatic consequences on the regulation of such vascular functions as dilation, expression of vasomodulators, inhibition of platelet aggregation, and smooth muscle cell proliferation (38). Although the findings in this report support a role for CO sufficing for NO in promoting vascular cell migration, the signaling action of CO results in the phosphorylation of different VASP sites than NO. Furthermore, we demonstrate that, while both vasoactive gases promote cytoskeletal changes through site-specific and cell type-specific VASP phosphorylation, these responses are blunted in diabetes, and may be responsible for the altered vascular repair and tissue perfusion associated with diabetic vascular complications.

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REFERENCES


**Figure 1: CO or NO treatment of EPC stimulates migration.** Because the rate constants for CO release from Ru(II)(CO)$_3$Cl$_2$ dimer and for NO release from DETA-NO are so disparate (see methods), experiments with the NO donor required prior incubation for times sufficient to release equivalent concentrations of NO and CO. Accordingly, EPC (obtained from healthy human subjects) were treated with 10 μM Ru(II)(CO)$_3$Cl$_2$ dimer (CO donor) for 1 and 15 minutes, thereby generating 0.6 and 6.5 μM CO, respectively. For the NO experiments, 100 μM DETA-NO was first incubated in a sealed tube for 2 and 28 hours (based on the calculated time required to generate 0.6 and 6.5 μM NO, respectively), and then combined with EPC for 1 and 15 minutes, respectively. Cell migration in response to SDF-1 was subsequently assessed in a Boyden chamber. Representative results from at least three independent experiments are shown. Values represent the means ± SD. An * indicates P < 0.05.
Figure 2: Time-course of NO- or CO-stimulated VASP phosphorylation at Ser-157 and Ser-239. CD34+ cells were incubated for 15 minutes in the presence of either 6.5 μM CO or NO (see legend to Figure 1 for details), and protein extracts were analyzed by InCell Western (see Methods). Note that exposure of these cells to NO resulted in increased VASP phosphorylation at Ser-239, whereas CO increased phosphorylation at Ser-157. Representative results from four independent experiments are shown. Values represent means ± SD. Reported P values indicate significance between the treated and the corresponding untreated sample.
Figure 3: Signal-mediated platelet VASP phosphorylation indicates low responsiveness to CO (A), but robust response to NO (B). Platelets (isolated from human peripheral blood as described above) were treated with either CO or NO as described in the legend to Figure 1. Platelets were then extracted with lysis buffer, and equal quantities of total protein were loaded and separated by SDS-polyacrylamide gel electrophoresis, followed by transfer to nitrocellulose for immunoblotting with VASP phosphorylation site-specific antibodies. Corrections for variations in gel loading were made by normalizing band intensities to those for β-actin. Representative results from at least three independent experiments are shown. Values represent means ± SD. An * indicates P < 0.05.
Figure 4: CO- and NO-mediated VASP redistribution in human microvascular endothelial cells (HMEC). HMEC, cultured on fibronectin-coated coverslips, were treated with CO and NO donors as described in the legend to Figure 1. (A,B): Low and high power images of untreated cells showing uniform VASP localization on actin filaments throughout the cytoplasm. (C,D): Low and high power images showing CO-induced VASP redistribution to filopodia at the leading edge of microvascular endothelial cells. (E,F): Low and high power images showing NO-induced redistribution of VASP to filopodia. Representative results from three independent experiments are shown. Green: VASP; Blue: DAPI (nuclei). (Scale bars = 25μm).
Figure 5: NO exposure results in reduced VASP Ser-239 phosphorylation in diabetic CD34<sup>+</sup> cells compared to control subject cells. The extent of phosphorylation was determined by FACS analysis on CD34<sup>+</sup> cells from two diabetic subjects (DA, DB) and one as well as cells from a normal subject (Control). Values represent means ± SD. An * indicates P < 0.05. DA: diabetic donor A; DB: diabetic donor B; Control: normal donor.
Figure 6: Cell culture under high-glucose (25mM) conditions attenuates CO- and NO-mediated VASP redistribution in HMEC. (A): Untreated cells showing uniform VASP immunoreactivity along the actin filaments distributed throughout the cytoplasm. (B): CO-induced VASP redistribution to leading-edge filopodia, albeit to an extent less than that observed at normoglycemic conditions (compare this image to that presented in Figure 3). (C): Less extensive VASP redistribution to filopodia in the presence of NO. Cells cultured for a week in 25mM glucose-supplemented medium on fibronectin-coated coverslips were either left untreated or treated NO or CO (see legend to Figure 1 for details) for 15 minutes prior to fixation and immunohistochemistry. Representative results from a minimum of three independent experiments are shown. Green: VASP; Blue: DAPI (nuclei). (Scale bar = 25μm)
Figure 7: Cell culture under high-glucose (25mM) conditions blunts CO- and NO-mediated VASP phosphorylation in HMEC.

HMEC were cultured in low (5.5mM) or high (25mM) glucose for one week, as described in the methods section, prior to treatment with NO or CO donor at 37°C for 1 or 15 min (see legend to Figure 1 for details). Cells were harvested in extraction buffer, and equal quantities of total protein were separated by SDS-PAGE, followed by transfer to nitrocellulose membrane and immunoblotting for VASP phosphorylation site determination and quantitative analysis (normalized to total VASP as a loading control). Basal levels represent no treatment in low glucose at 1 minute. Representative results from three independent experiments are shown. Values represent means ± SD. An * indicates P < 0.05.