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Tobin NP, Henehan GT, Murphy RP, Atherton JC, Guinan AF, Kerrigan SW, Cox D, Cahill PA, Cummins PM. Helicobacter pylori-induced inhibition of vascular endothelial cell functions: a role for VacA-dependent nitric oxide reduction. Am J Physiol Heart Circ Physiol. 2008 Oct;295(4):H1403-13. doi: 10.1152/ajpheart.00240.2008. Epub 2008 Jul 25. PMID: 18660451.

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Am J Physiol Heart Circ Physiol 295: H1403–H1413, 2008.
First published July 25, 2008; doi:10.1152/ajpheart.00240.2008.

Helicobacter pylori-induced inhibition of vascular endothelial cell functions: a role for VacA-dependent nitric oxide reduction

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[1]*School of Biotechnology, Dublin City University;* [2]*School of Food Science and Environmental Health, Dublin Institute of Technology;* [3]*Wolfson Digestive Diseases Centre, University Hospital Nottingham; and* [4]*Molecular and Cellular Therapeutics, Royal College of Surgeons of Ireland, Dublin, Ireland*

Submitted 7 March 2008; accepted in final form 23 July 2008

Tobin NP, Henehan GT, Murphy RP, Atherton JC, Guinan AF, Kerrigan SW, Cox D, Cahill PA, Cummins PM. *Helicobacter pylori*-induced inhibition of vascular endothelial cell functions: a role for VacA-dependent nitric oxide reduction. *Am J Physiol Heart Circ Physiol* 295: H1403–H1413, 2008. First published July 25, 2008; doi:10.1152/ajpheart.00240.2008.—Epidemiological and clinical studies provide compelling support for a causal relationship between *Helicobacter pylori* infection and endothelial dysfunction, leading to vascular diseases. However, clear biochemical evidence for this association is limited. In the present study, we have conducted a comprehensive investigation of endothelial injury in bovine aortic endothelial cells (BAECs) induced by *H. pylori*-conditioned medium (HPCM) prepared from *H. pylori* 60190 [vacuolating cytotoxin A (Vac^+)]. BAECs were treated with either unconditioned media, HPCM (0–25% vol/vol), or *Escherichia coli*-conditioned media for 24 h, and cell functions were monitored. Vac^+ HPCM significantly decreased BAEC proliferation, tube formation, and migration (by up to 44%, 65%, and 28%, respectively). Posttreatment, we also observed sporadic zonnula occludens-1 immunolocalization along the cell-cell border, and increased BAEC permeability to FD40 Dextran, indicating barrier reduction. These effects were blocked by 5-nitro-2-(3-phenylpropylamino)benzoic acid (VacA inhibitor) and were not observed with conditioned media prepared from either VacA-deleted *H. pylori* or *E. coli*. The cellular mechanism mediating these events was also considered. Vac^+ HPCM (but not Vac^-) reduced nitric oxide (NO) by >50%, whereas *S*-nitroso-*N*-acetylpenicillamine, an NO donor, recovered all Vac^+ HPCM-dependent effects on cell functions. We further demonstrated that laminar shear stress, an endothelial NO synthase/NO stimulus in vivo, could also recover the Vac^+ HPCM-induced decreases in BAEC functions. This study shows, for the first time, a significant proatherogenic effect of *H. pylori*-secreted factors on a range of vascular endothelial dysfunction markers. Specifically, the VacA-dependent reduction in endothelial NO is indicated in these events. The atheroprotective impact of laminar shear stress in this context is also evident.

vacuolating cytotoxin A; shear stress

HELICOBACTER PYLORI is a gram-negative, spiral-shaped bacterium that colonizes the human gastric epithelium and is the causative agent in chronic gastritis, peptic ulceration, and gastric carcinoma (33). In recent years, evidence that chronic *H. pylori* infection plays a role in the extra-gastric initiation and progression of vascular diseases has been mounting (14, 16, 20, 35). Seroepidemiological and eradication studies have demonstrated a causal relationship between *H. pylori* infection and atherosclerosis (3, 16, 31). Elevated levels of homocysteine (9), asymmetric dimethylarginine (ADMA) (45), and serum lipids (25, 39), all independent risk factors for vascular disease, have also been associated with *H. pylori* infection.

Various mechanisms have been proposed to account for the contribution of *H. pylori* to vascular diseases. Molecular mimicry, oxidative modifications, bacterium-platelet interactions, and even direct plaque modification, leading to endothelial dysfunction or inflammation, have been proposed (12, 26, 43). With respect to plaque modification, perhaps the most persuasive single piece of evidence supporting this role was the identification of *H. pylori* DNA in atherosclerotic plaques. This finding was key in pointing to the direct involvement of the bacterium in this specific pathology (1, 2, 17, 36).

Endothelial dysfunction provides a crucial link by which *H. pylori*, and indeed other pathogens, may contribute to atherogenesis (55, 60). In this regard, a number of in vitro studies have previously reported *H. pylori*-dependent endothelial injury by way of reduced angiogenesis (28, 29), reduced proliferation (29, 37, 52), and elevated apoptosis (37). *H. pylori*-dependent elevations in neutrophil recruitment and transendothelial migration have also been reported (5, 15). Although this evidence points to a role for *H. pylori* in endothelial dysfunction in vivo, certain key issues remain unresolved. Many of these studies are limited to only one aspect of endothelial dysfunction and frequently lack substantial mechanistic elaboration with respect to the induction of cellular injury. Moreover, most studies were carried out using whole cell aqueous extracts of *H. pylori* (which may include unforeseen target protein modifications resulting from extract preparation) and have not directly examined the specific contribution of secreted virulence factors (using bacterially conditioned media, for example). The demonstrated translocation of the bacterium and its biochemical components from the gastric mucosa into the systemic circulation (21, 47, 54) is in agreement with the endothelium exposure to *H. pylori*-secreted virulence factors. Thus, at atherosclerotic plaque sites, these factors may reach sufficiently high levels within the vessel wall microenvironment (relative to the systemic circulation) to influence endothelial dysfunction and lesion development.

A model incorporating *H. pylori* in atherosclerosis must demonstrate clear biochemical mechanisms whereby the bacterium can cause vascular endothelial dysfunction. In the present study, we have conducted a comprehensive investigation of endothelial injury in bovine aortic endothelial cells

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(BAECs) in response to treatment with *H. pylori*-conditioned medium (HPCM). Our findings show significant effects on endothelial proliferation, tube formation, migration, and barrier properties. Moreover, we show for the first time that multiple aspects of *H. pylori*-induced endothelial dysfunction can be attributed to vacuolating cytotoxin A (VacA), a *H. pylori*-secreted virulence factor that elicits its effects by modulating plasma and mitochondrial membrane ion permeability (63) as well as altering intracellular vesicular trafficking leading to vacuole formation (57). Finally, the VacA-dependent reduction in endothelial nitric oxide (NO) is indicated in our model, as is the atheroprotective influence of laminar shear stress.

MATERIALS AND METHODS

All reagents used in this study were of the highest purity and, unless otherwise stated, were obtained from Sigma-Aldrich.

Cell Culture

BAECs were obtained from Coriell Cell Repositories (NJ-Cat No. AG08500). BAECs were routinely grown in RPMI-1640 media supplemented with 10% fetal calf serum and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin), hereafter referred to as RPMI complete media. The cells were maintained in a humidified atmosphere of 5% CO_2-95% air at 37°C. For all experiments, cells between *passages 7* and *14* were used.

Bacterial Culture and Conditioned Media Preparation

H. pylori 60190 Vac^+ (ATCC No. 49503) and Vac^- (VacA gene-deleted 60190; John Atherton, University Hospital Nottingham) strains were innoculated onto *pylori* agar plates (BioMérieux; Marcy l′Etoile) and incubated at 37°C under microaerobic conditions for 7 days. Bacterial colonies were scraped off and resuspended in 3 ml phosphate-buffered saline (PBS). The optical density of the suspension was determined at 600 nm and adjusted by dilution in PBS to a final absorbance of 1.0 (corresponding to 5×10^9 bacteria/ml) (26). Exactly 2.5 ml of bacterial suspension were added to a T25 tissue culture flask containing 40 ml of RPMI complete media and incubated at 37°C under microaerobic conditions for 24 h. The conditioned media was then sterile filtered using a 0.2 μm filter to yield HPCM. Dilutions of HPCM in RPMI complete media were routinely prepared for individual experiments (i.e., undiluted HPCM = 100% vol/vol; 1:2 dilution = 50% vol/vol; control = 0% vol/vol, etc.). Conditioned media using *Escherichia coli* (strain BL21) was also prepared, sterile filtered, and diluted in RPMI complete media in a similar manner to HPCM for inclusion as a gram-negative bacterial control in specific experiments (0.01–0.1% vol/vol). Most experiments involved the treatment of static (unsheared) BAECs with either RPMI or bacterium-conditioned media, after which changes in cell proliferation, migration, tube formation, and barrier function were monitored. For inhibition studies, 10 μg/ml jack bean urease (15), 100 μM 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB; a VacA inhibitor) (63), and 1 mM N^{ω}-nitro-L-arginine methyl ester [L-NAME; an endothelial NO synthase (eNOS) inhibitor] were included in media when required. The NO donor *S*-nitroso-*N*-acetyl-penicillamine (SNAP) was also employed for specific studies (proliferation, 0.25 μM; tube formation, 5 μM; migration, 1 μM; and permeability/immunocytochemistry, 1 μM). Bradford assay was used for protein measurement with BSA as standard (4).

BAEC Proliferation

Flow cytometry (Becton Dickinson FACSCaliber) was routinely used to monitor BAEC proliferation (and apoptosis). Cells were seeded into six-well plates (2×10^4 cells/well) and allowed to grow for 24 h. The cells were then washed once with Hanks' balanced salt solution before 1 ml of 5 μM carboxy-fluorescein diacetate succinimidyl ester (CFDA; prepared in Hanks') was added to each well for 15 min at 37°C. Following incubation, CFDA was replaced with fresh media and cells were allowed to recover for 12 h before overnight quiescence. The cells were then treated with HPCM (0–25%; 2 ml/well), harvested every 24 h for up to 5 days by trypsinization/centrifugation, and washed twice with 1 ml ice-cold PBS (containing 0.1% BSA) before fluorescence-activated cell sorting (FACS) analysis. Proliferation was also routinely monitored by cell counting using a bright line hemocytometer. For cell counting studies, proliferation was typically reported for *day 4*. For NPPB studies, proliferation was monitored over a 2-day period.

BAEC Migration

Wound heal assay was routinely used to monitor BAEC migration, as previously described (13). BAECs were seeded into 24-well plates (5×10^4 cells/well) and allowed to grow to confluency. The cells were then quiesced overnight and treated with HPCM (0–25% + 2.5 μg/ml mitomycin C; 1 ml/well) for 24 h. Following treatment, a wound or scratch was created in each well by scraping cells with a pipette tip. Cells were then washed, and HPCM was replaced. To monitor wound closure, the wound was photographed at two predefined positions every 2 h and the distance between the two wound edges was digitally measured using Macintosh Free Ruler v1.6. Migration was expressed in terms of the average decrease in wound width for the assay period.

BAEC Tube Formation

Collagen gel assay was routinely used to monitor BAEC tube formation, as previously described (66). BAECs were seeded into 24-well plates (5×10^4 cells/well) and allowed to grow to confluency. Cells were then quiesced overnight and treated with HPCM (0–25%; 1 ml/well) for 24 h. Following treatment, cells were trypsinized, resuspended in HPCM, and seeded into collagen gels (24-well format; 1.5×10^4 cells/well). Tube formation proceeded overnight (16–18 h) and was monitored by standard light microscopy with digital photography (Olympus SP-350 camera). Four random fields were photographed from each well, and tube formation was quantified by measuring the average length of the network of connected cells using imaging software for life sciences and microscopy (Olympus cell^F Image and Analysis software).

BAEC Barrier Property

Transwell permeability assay. BAECs were seeded into six-well plates (5×10^4 cells/well) and allowed to grow to confluency. Cells were then quiesced overnight and treated with HPCM (25%) for 24 h under 10 dynes/cm^2 laminar shear (i.e., to induce barrier formation), as described in *Shear Stress*. Posttreatment, cells were trypsinized and replated (2.5×10^5 cells/well) into Millipore-Clear plates with polyester membrane inserts (6-well format; 0.4 μm pore; 24 mm diameter; Millipore). At confluency, transendothelial permeability was monitored as previously described (7) using fluorescein isothiocyanate-labeled 40-kDa Dextran (FD40 Dextran). Results are given as percent transendothelial exchange of FD40 Dextran (taken as the total subluminal fluorescence at a given time expressed as percentage of total abluminal fluorescence at t = 0).

Zonnula occludens-1 immunocytochemistry. BAECs were seeded into six-well Bioflex plates (5×10^4 cells/well) (Dunn Labortechnik, Asbach, Germany) and allowed to grow to confluency. Cells were then quiesced overnight and treated with HPCM (25%) for 24 h under 10% equibiaxial cyclic strain (i.e., to induce barrier formation), as previously described (8). Posttreatment, Bioflex wells were excised with a scalpel and prepared for immunocytochemistry, again as previously described (8). The primary antibody was 0.25 μg/ml rabbit anti-zonnula occludens-1 (ZO-1) monoclonal IgG for 2 h (Zymed, San

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Francisco, CA). The secondary antibody was 1:400 Alexa 488-conjugated goat anti-rabbit IgG for 1 h (Molecular Probes, Eugene, OR). Controls included the exclusion of primary antibody and 4,6-diamidino-2-phenylindole (DAPI) nuclear staining (500 ng/ml; 3 min).

Shear Stress

With the use of an orbital rotator to apply physiological levels of laminar shear stress, as previously described (7), the impact of shear on HPCM-dependent changes in BAEC proliferation, tube formation, and migration was examined. BAECs were seeded into six-well plates (5×10^4 cells/well) and allowed to grow to confluency. Cells were quiesced overnight and treated with HPCM (25%; 4 ml/well) for 24 h at either 0, 1, or 10 dynes/cm^2 of shear. Postshear, BAECs were incorporated into proliferation, tube formation, and migration assays (as described in *BAEC Proliferation*, *BAEC Migration*, and *BAEC Tube Formation*).

Nitrite Assay

BAECs were seeded into six-well plates (5×10^4 cells/well) and allowed to grow to confluency. Cells were quiesced overnight and treated with HPCM (25%; 2 ml/well) for 24 h. Assay for nitrite in BAEC media following experimental treatments was performed by 2,4-diaminonapthalene assay, as previously described (6). NO levels were determined on a per well basis and were not normalized to cell number per well because all wells were initially seeded with an identical number of cells and no statistical difference in cells per well was observed between conditions following treatment, as determined by hemocytometer cell counting.

Western Immunoblotting

Following shearing experiments, BAECs were harvested and total lysate samples were resolved by 10% SDS-PAGE under reducing conditions according to the method of Laemmli (38). Gels were electroblotted onto nitrocellulose membranes using an ATTO semi-dry transfer system (1 h; 100 V), and membranes were blocked for 2 h in Tris-buffered saline [10 mM Tris (pH 8.0) and 150 mM NaCl] containing 5% wt/vol BSA. The preparation of BAEC lysates, protein assay, and immunostaining for eNOS have all been described previously (6, 7).

Statistical Analysis

Results are expressed as means ± SE. Experimental points were performed in triplicate with a minimum of three independent experiments ($n = 3$). Statistical comparisons between control and treatment groups were made by Student's unpaired *t*-test.

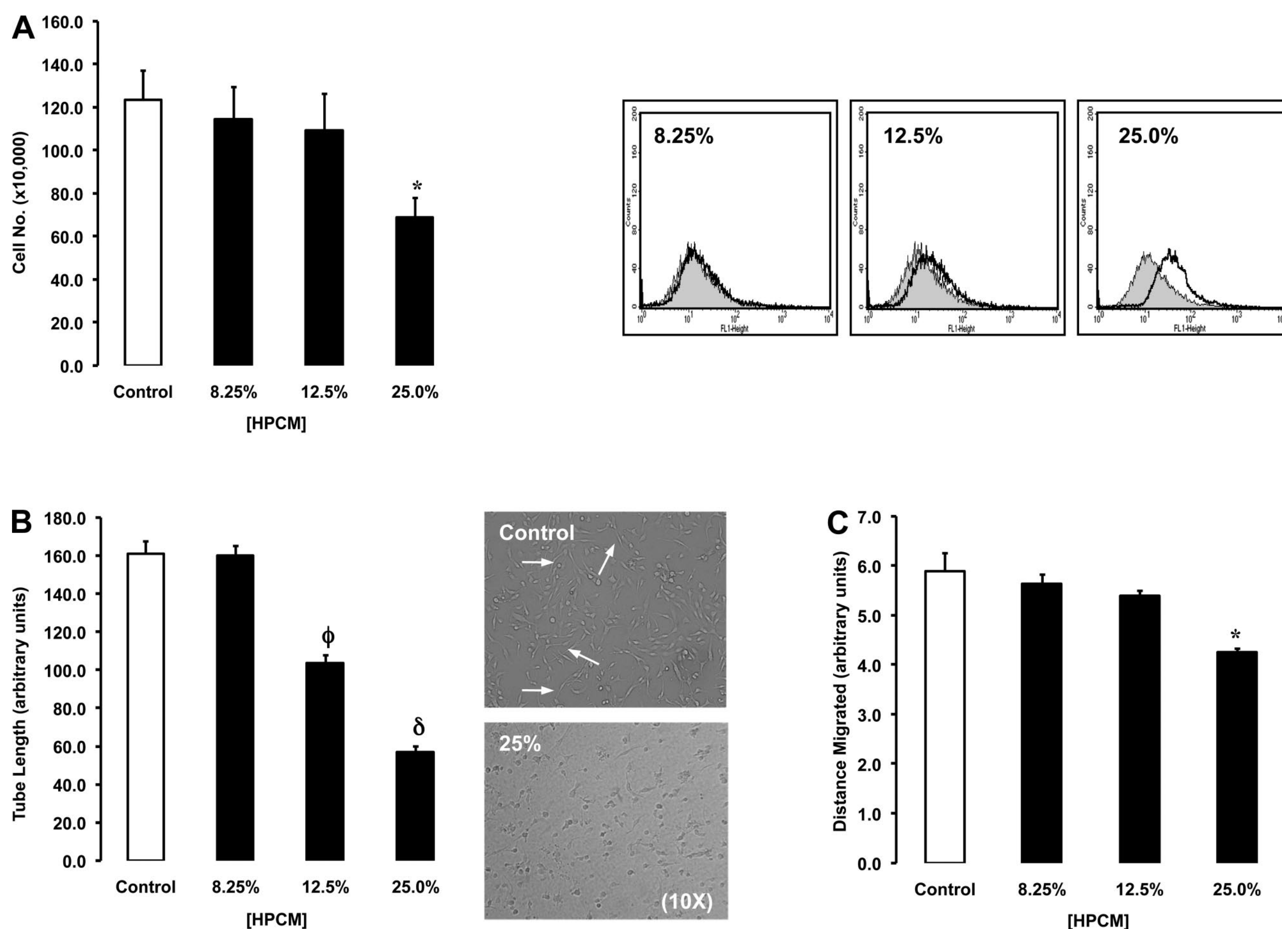

Fig. 1. Effect of *Helicobacter pylori*-conditioned medium (HPCM) on bovine aortic endothelial cell (BAEC) functions. BAECs were treated with vacuolating cytotoxin A (Vac)$^+$ HPCM (0–25%) and monitored for changes in proliferation (4 days), tube formation (24 h), and migration (24 h). Control is unconditioned RPMI-1640 complete media. HPCM treatment times are shown in parentheses. Histograms were averaged from 3 independent experiments ± SE. *A*: proliferation. $*P \leq 0.01$ vs. control. Fluorescence-activated cell sorting (FACS) analysis (*right*) shows untreated control cells (gray-shaded area) relative to HPCM-treated cells (unshaded area). *B*: tube formation. $\phi P \leq 0.001$; $\delta P \leq 0.0001$ vs. control. Representative images (*right*) show reduction in endothelial sprouting (white arrows) following treatment. *C*: migration. $*P \leq 0.01$ vs. control.

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RESULTS

HPCM Decreases BAEC Functions: Proliferation, Tube Formation, and Migration

The effect of HPCM (0–25%) on BAEC proliferation, tube formation, and migration was examined. At 25% HPCM, we observed a substantial decrease in proliferation, as determined by cell counts (Fig. 1*A*). This finding was also verified by FACS analysis (Fig. 1*A*). Significant decreases in both tube formation and migration were also noted at 25% HPCM (Fig. 1, *B* and *C*).

The effect of NPPB (a VacA inhibitor) on the above changes was next examined. In the absence of NPPB, HPCM (25%) reduced proliferation, tube formation, and migration, as described above (Fig. 2, *A–C*). These effects were completely prevented by NPPB. A baseline inhibitory effect of NPPB on proliferation and tube formation levels was also noted (44, 58).

HPCM Decreases BAEC Barrier Properties

The effect of HPCM on BAEC barrier properties was examined. Cells were treated with HPCM (25%) in the absence and presence of NPPB and stained for ZO-1 immunoreactivity. Consistent with an intact endothelial barrier, ZO-1 exhibited continuous immunolocalization along the cell-cell border in untreated controls (Fig. 2*Di*). HPCM treatment led to ZO-1 localization becoming extremely jagged and sporadic along the cell-cell border (Fig. 2*Diii*), an effect that was prevented by NPPB (Fig. 2*D*, *ii* and *iv*).

HPCM-Induced Changes in BAEC Function are Mediated by VacA

The role of VacA in these events was examined. BAECs were separately treated with HPCM (25%) prepared from either Vac$^+$ or Vac$^-$ *H. pylori* 60190 or with *E. coli*-conditioned media. The Vac$^+$ HPCM substantially decreased BAEC

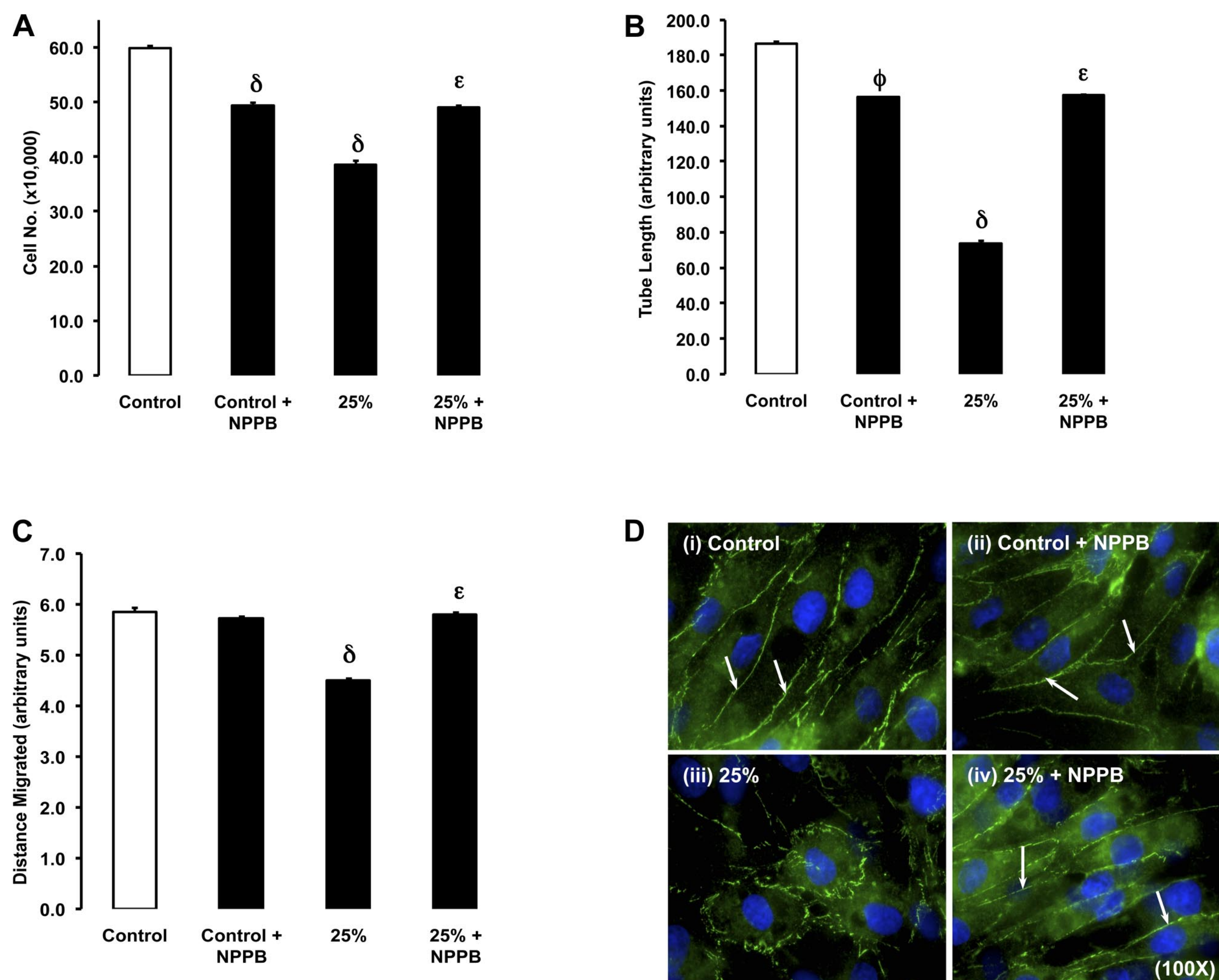

Fig. 2. Effect of 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) on HPCM-induced changes in BAEC functions. BAECs were treated with Vac$^+$ HPCM (25%) in the absence and presence of 100 μM NPPB and monitored for changes in proliferation (2 days), tube formation (24 h), migration (24 h), and barrier integrity (24 h). Control is unconditioned RPMI complete media. HPCM treatment times are shown in parentheses. Histograms were averaged from 3 independent experiments ± SE. *A*: proliferation. $\delta P \leq 0.0001$ vs. control; $\varepsilon P \leq 0.001$ vs. 25%. *B*: tube formation. $\phi P \leq 0.001$; $\delta P \leq 0.0001$ vs. control; $\varepsilon P \leq 0.0001$ vs. 25%. *C*: migration. $\delta P \leq 0.0001$ vs. control; $\varepsilon P \leq 0.0001$ vs. 25%. *D*: barrier integrity. White arrows indicate cell-cell border localization of zonnula occludens-1 (ZO-1) immunoreactivity. 4,6-Diamidino-2-phenylindole (DAPI)-stained nuclei are clearly visible in blue. Images are representative.

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proliferation, tube formation, and migration relative to untreated controls (Fig. 3, *A–C*). Neither Vac$^-$ HPCM nor *E. coli*-conditioned media, however, had any significant effects.

The impact of VacA on BAEC barrier properties was also examined using a similar treatment paradigm to above. As seen earlier, ZO-1 immunoreactivity along the cell-cell border became highly discontinuous and jagged in response to Vac$^+$ HPCM, consistent with the disruption of intercellular tight junction integrity. Moreover, Vac$^+$ HPCM treatment sharply increased FD40 Dextran transendothelial flux across BAEC monolayers (Fig. 4). Again, neither Vac$^-$ HPCM nor *E. coli*-conditioned media had any significant effects.

HPCM Decreases BAEC NO Production in a VacA-Dependent Manner

The effect of HPCM on BAEC NO levels was examined. Cells were treated with either Vac$^+$ or Vac$^-$ HPCM (25%) or with *E. coli*-conditioned media. Vac$^+$ HPCM substantially decreased NO production relative to untreated controls (Fig. 5*A*). Neither Vac$^-$ HPCM nor *E. coli*-conditioned media had any significant effect on NO levels, however, whereas L-NAME (an eNOS inhibitor) almost completely ablated NO production (Fig. 5*A*). Furthermore, 25% HPCM (Vac$^+$ or Vac$^-$) had no significant effect on eNOS total protein levels following 24 h treatment (data not shown).

HPCM-Induced Changes in BAEC Function Involve VacA-Dependent NO Reduction

The putative link between HPCM-induced change in BAEC functions and NO levels was examined. BAECs were treated with Vac$^+$ HPCM (25%) in the absence and presence of SNAP (an NO donor). In the absence of SNAP, HPCM reduced proliferation, tube formation, and migration, as described in *BAEC Proliferation*, *BAEC Migration*, and *BAEC Tube Formation*. These effects were almost completely recovered by SNAP (Fig. 5, *B–D*).

BAEC barrier properties were also examined in this context. In the absence of SNAP, Vac$^+$ HPCM (25%) increased

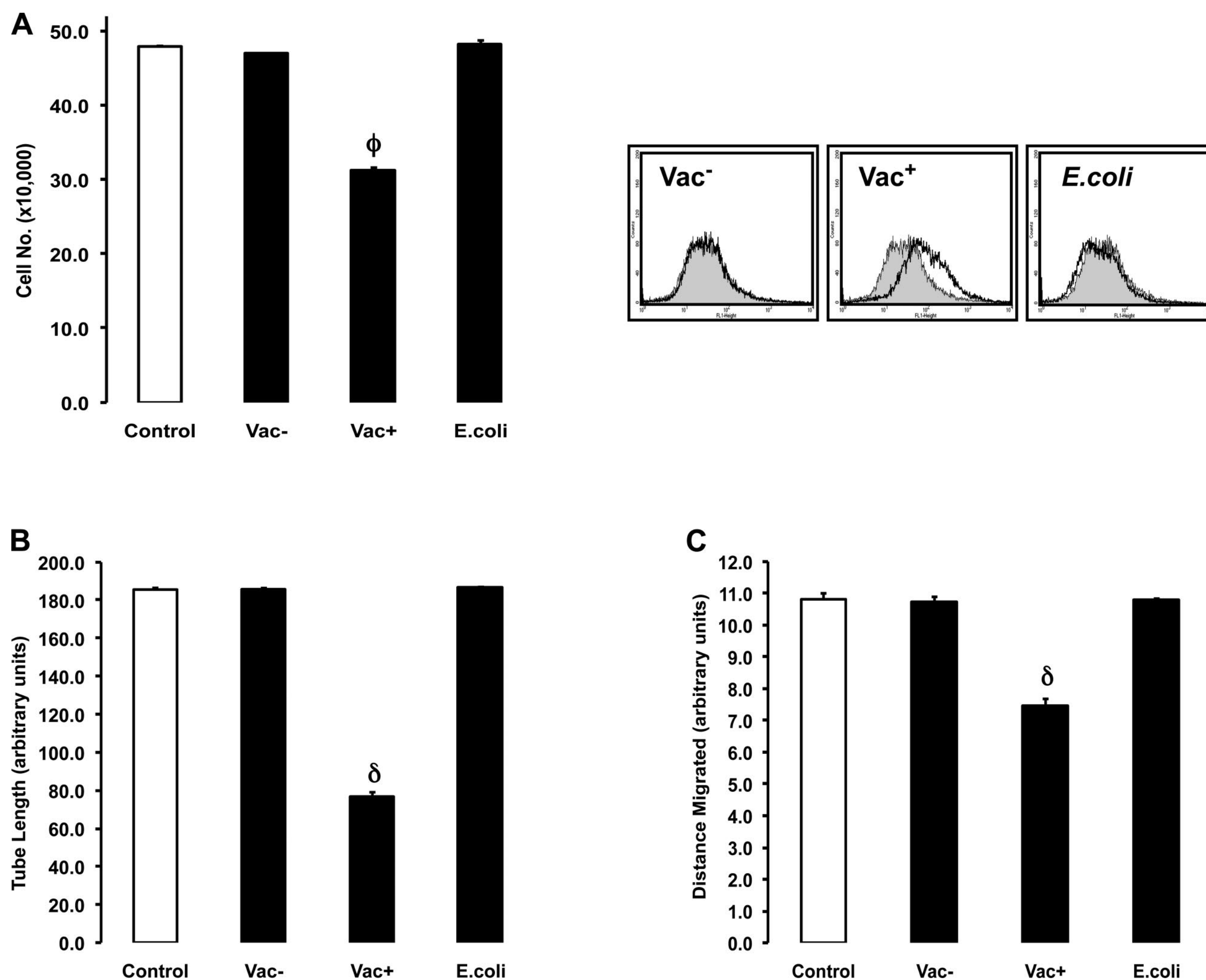

Fig. 3. Effect of VacA on HPCM-induced changes in BAEC functions. BAECs were treated with either Vac$^+$ or Vac$^-$ HPCM (25%) or *Escherichia coli*-conditioned media and monitored for changes in proliferation (4 days), tube formation (24 h), and migration (24 h). Control is unconditioned RPMI complete media. HPCM treatment times are shown in parentheses. Histograms were averaged from 3 independent experiments ± SE. *A*: proliferation. $\phi P \leq 0.001$ vs. control. FACS analysis (*right*) shows untreated control cells (gray-shaded area) relative to cells treated with conditioned media from either Vac$^-$ or Vac$^+$ *H. pylori* or wild-type *E. coli* (unshaded areas). *B*: tube formation. $\delta P \leq 0.0001$ vs. control. *C*: migration. $\delta P \leq 0.0001$ vs. control.

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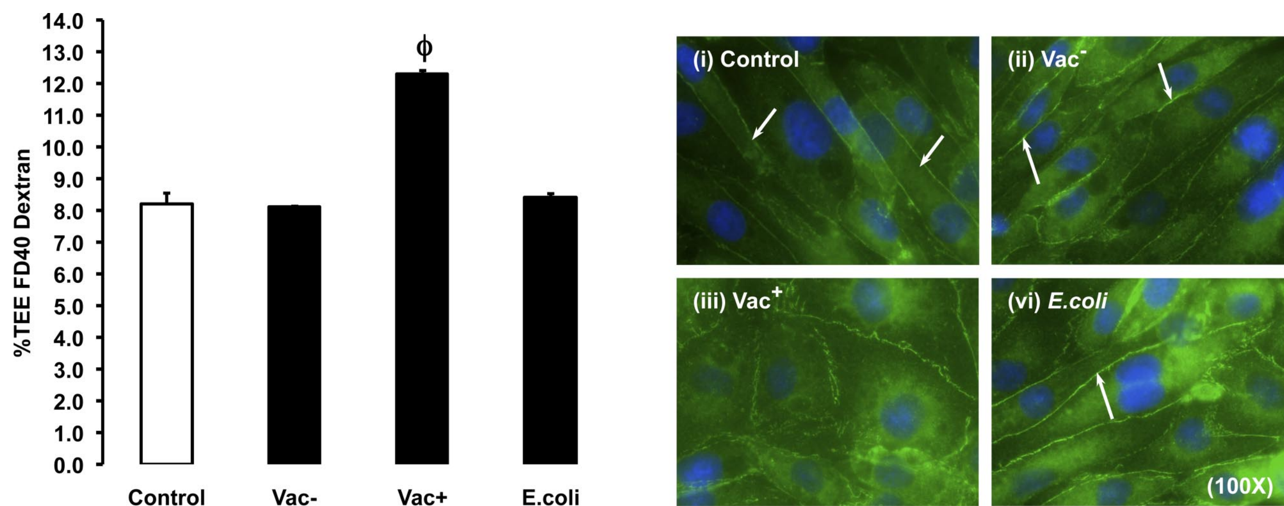

Fig. 4. Effect of VacA on HPCM-induced changes in BAEC barrier properties. BAECs were treated for 24 h with either Vac$^+$ or Vac$^-$ HPCM (25%) or *E. coli*-conditioned media, and intact monolayers were monitored for permeability to FD40 Dextran. Control is unconditioned RPMI complete media. Histogram shows percent transendothelial exchange (TEE) of FD40 Dextran as monitored by transwell permeability assay and was averaged from 3 independent experiments ± SE. $\phi P \leq 0.001$ vs. control. Posttreatment, intact monolayers were also monitored for ZO-1 immunoreactivity (*right*). White arrows indicate cell-cell border localization of ZO-1. DAPI-stained nuclei are clearly visible in blue. Images are representative.

FD40 Dextran transendothelial flux relative to untreated control (Fig. 6). Moreover, ZO-1 immunoreactivity along the cell-cell border became highly discontinuous and jagged, also consistent with endothelial barrier reduction (Fig. 6). We further observed that either effect could be recovered by SNAP.

HPCM-Induced Changes in BAEC Functions are Absent Under Shear Stress

The impact of laminar shear stress on the HPCM-induced change in BAEC functions was examined. BAECs were treated with either Vac$^+$ or Vac$^-$ HPCM (25%), or with *E. coli*-conditioned media, at a shear rate of 0, 1, or 10 dynes/cm^2. At static and low shear rates, Vac$^+$ HPCM (but not Vac$^-$ HPCM or *E. coli*-conditioned media) substantially decreased NO production (Fig. 7*A*), proliferation (Fig. 7*B*), tube formation (Fig. 7*C*), and migration (Fig. 7*D*) relative to untreated controls. At high shear rate, the inhibitory effects of Vac$^+$ HPCM on all of the aforementioned BAEC functions were recovered to the high shear baseline levels exhibited by control, Vac$^-$, and *E. coli* treatments (Fig. 7*B–D*). Under control conditions, levels of eNOS protein expression were also elevated at high shear (Fig. 7*A*, *inset*).

DISCUSSION

This comprehensive study shows, for the first time, a significant proatherogenic effect of *H. pylori*-secreted factors on a wide range of vascular endothelial dysfunction markers. Our investigations demonstrated that a chronic exposure of BAECs to 25% HPCM significantly reduced proliferation (while increasing apoptosis and vacuolation at higher concentrations; data not shown). These findings are consistent with earlier studies on *H. pylori* aqueous extracts, which report antiproliferative and proapoptotic effects on human microvascular endothelial cells and umbilical vein endothelial cells (29, 37, 52). We also observed HPCM-dependent reductions in BAEC tube formation and migration, further evidence that *H. pylori* exhibits antiangiogenic properties (28, 29). Moreover, the HPCM-induced elevation of BAEC monolayer permeability to FD40 Dextran, in conjunction with sporadic ZO-1 membrane localization, as observed in this study, verifies the barrier-lowering properties of *H. pylori*-secreted factors (7, 8), again consistent with earlier reports (5, 15).

The cytotoxin-associated gene pathogenicity island and outer membrane proteins are known determinants of *H. pylori* pathogenicity (18). However, these factors require direct bacterium-cell contact, and in the case of the former, a specialized type IV secretion system (23), likely ruling out their contribution to the HPCM effects observed in this study. Other determinants of *H. pylori* pathogenicity include urease and VacA. The former, accounting for almost 10% of total *H. pylori* protein, facilitates bacterial colonization in the acidic gastric mucosa (30). The latter functions by forming anion-selective channels in lipid bilayers, thereby modulating membrane depolarization (63). VacA can also enter cells and modulate mitochondrial membrane permeability and cytochrome C release (59). As both factors are secreted by *H. pylori*, we hypothesized a role for one or both in the HPCM-mediated effects observed.

When BAECs were treated with unconditioned media in the absence and presence of 10 μg/ml jack bean urease, an enzyme known to display many of the same properties as *H. pylori* urease (48), no significant effects on cell function were observed. The putative role of VacA was next explored. Earlier studies investigating endothelial injury do not clearly identify a role for VacA (5, 15, 28, 37, 41). The barrier-lowering (53), antiproliferative (50, 62), and proapoptotic (11) effects of *H. pylori* on other cell types (e.g., gastric epithelial cells, T-lymphocytes), however, have definitively been attributed to this virulence factor. Importantly, a number of these studies confirm the dose-dependent effects of VacA on cell functions and further demonstrate a threshold VacA concentration in the lower nM range for the induction of these effects. Use of the VacA-selective inhibitor NPPB (63) completely prevented the

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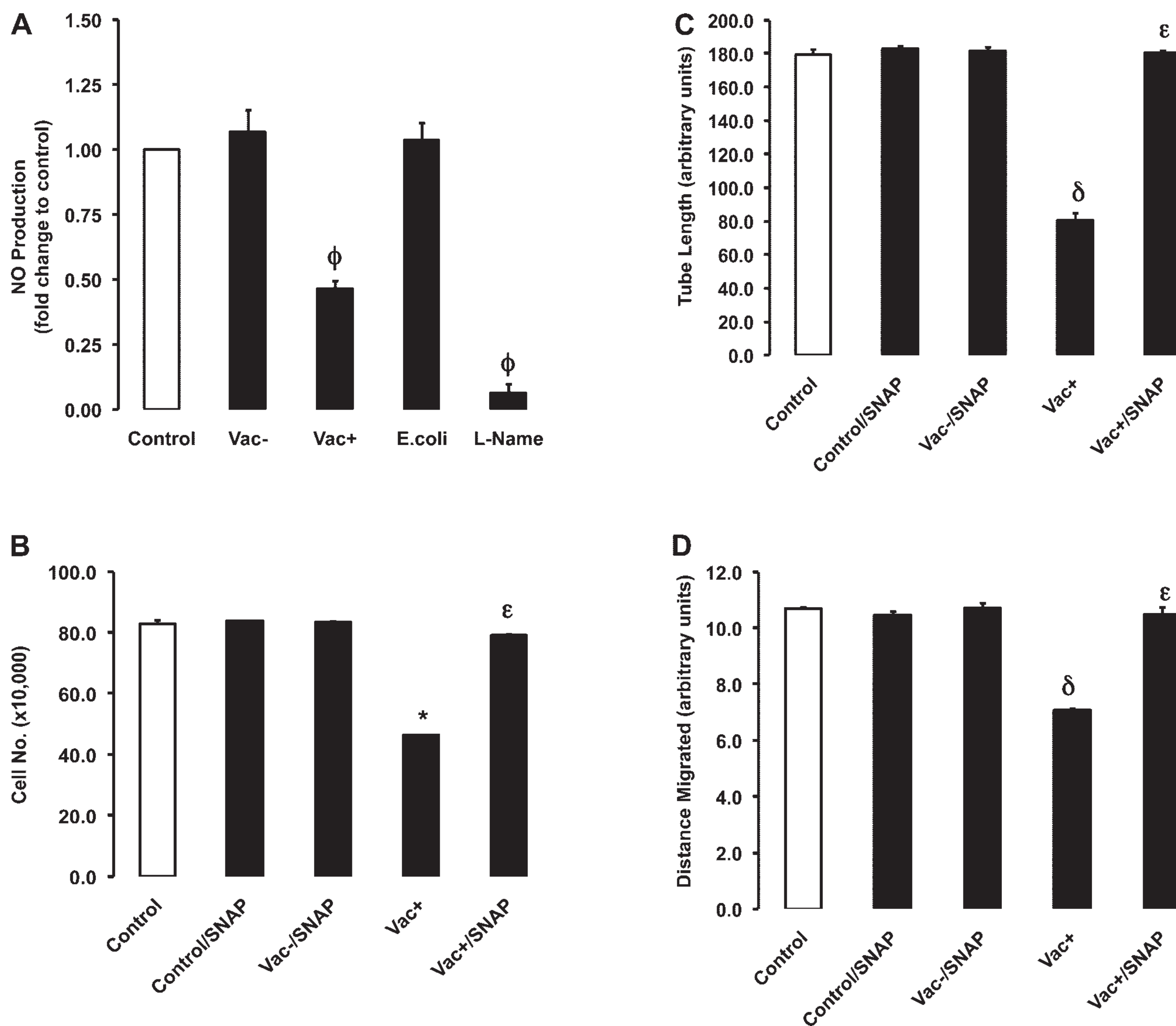

Fig. 5. Effect of nitric oxide (NO) on VacA-dependent changes in BAEC functions. BAECs were treated for 24 h with either Vac^+ or Vac^- HPCM (25%), *E. coli*-conditioned media, or N^{ω}-nitro-L-arginine methyl ester (L-NAME), and media were subsequently harvested to monitor NO production. Control is unconditioned RPMI complete media. The effects of *S*-nitroso-*N*-acetyl-penicillamine (SNAP) on HPCM-dependent changes in proliferation (4 days), tube formation (24 h), and migration (24 h) were also monitored. HPCM treatment times are shown in parentheses. Histograms were averaged from 3 independent experiments ± SE. *A*: NO production. $\phi P \leq 0.001$ vs. control. *B*: proliferation. $*P \leq 0.01$ vs. control; $\varepsilon P \leq 0.0001$ vs. Vac^+. *C*: tube formation. $\delta P \leq 0.0001$ vs. control; $\varepsilon P \leq 0.0001$ vs. Vac^+. *D*: migration. $\delta P \leq 0.0001$ vs. control; $\varepsilon P \leq 0.01$ vs. Vac^+.

HPCM-dependent effects on all BAEC dysfunction markers examined. This finding clearly points to a highly significant role for VacA in HPCM-induced endothelial injury. The lack of any effects on the aforementioned BAEC functions following treatment with conditioned media from VacA gene-deleted *H. pylori* (Vac^- HPCM) further strengthened this conclusion. Furthermore, the preparation of Vac^- and Vac^+ HPCM from the same strain (60190), in addition to the clear agreement of data from both inhibitor and mutant studies, suggests that the involvement of other bacterium-derived factors is unlikely. Finally, whereas VacA levels were not definitively quantified in our HPCM preparations, the steep concentration dependence at 25% HPCM, which was particularly notable in our proliferation study (Fig. 1*A*), possibly reflects a threshold concentration effect as outlined above.

We next considered the cellular mechanism mediating VacA involvement in this model. VacA is a multifunctional toxin that exhibits pleiotropic effects on mammalian cells (10). Its cytotoxic effects are an important feature of cellular injury and pathology. In the human gastric mucosa, for example, VacA causes extensive epithelial vacuolation, proinflammatory cytokine release, and apoptosis, leading to reduced epithelial cell viability and gastric ulceration (11, 50, 61, 67). The importance of VacA cytotoxicity to endothelial dysfunction and atherogenesis in vivo, however, is not understood. It should be noted that the Vac^+ HPCM range (0–25%) chosen for the bulk of these studies did not induce cell vacuolation (phase-contrast microscopy), and it did not reduce cell viability (trypan blue exclusion and propidium iodide incorporation assays; data not shown). This greatly reduces the possibility that the functional

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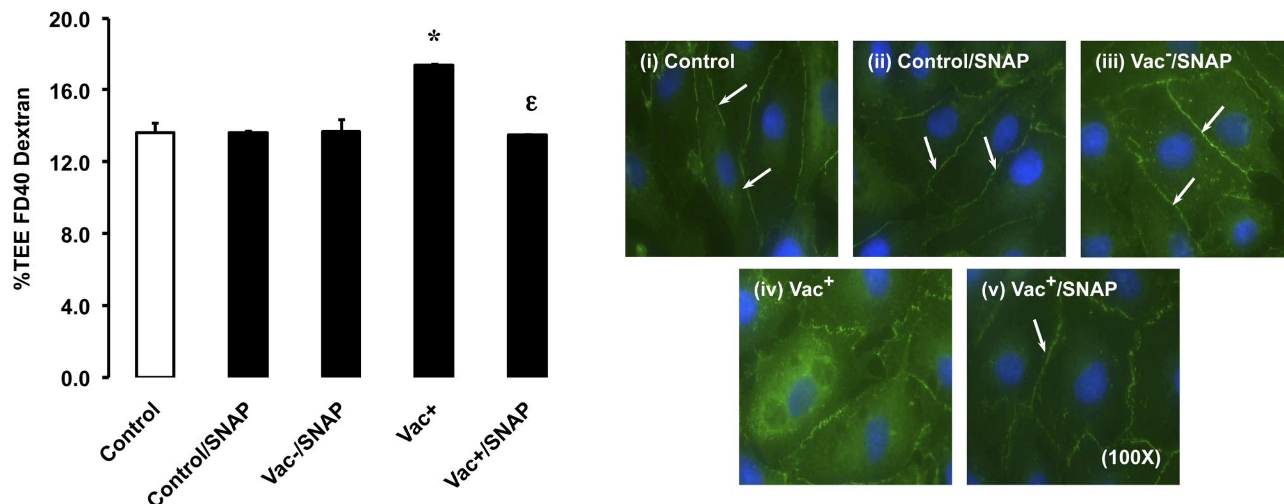

Fig. 6. Effect of NO on VacA-dependent changes in BAEC barrier properties. BAECs were treated for 24 h with either Vac$^+$ or Vac$^-$ HPCM (25%) in the absence and presence of SNAP, and intact monolayers were monitored for permeability to FD40 Dextran. Control is unconditioned RPMI complete media. Histogram shows percent TEE of FD40 Dextran as monitored by transwell permeability assay and was averaged from 2 independent experiments ± SE. $^*P \leq 0.05$ vs. control; $\varepsilon P \leq 0.001$ vs. Vac$^+$. Posttreatment, intact monolayers were also monitored for ZO-1 immunoreactivity. White arrows indicate cell-cell border localization of ZO-1. DAPI-stained nuclei are clearly visible in blue. Images are representative.

changes observed are due to cytotoxic actions of VacA and suggests that subtoxic levels of VacA can induce endothelial injury. The well-described ability of VacA to alter cell function independently of its cytotoxic actions (10, 53) is therefore relevant to our model and suggests the VacA-dependent modulation of endothelial signaling pathways.

We hypothesized that HPCM-induced endothelial injury could be mediated by VacA-dependent NO reduction. NO plays a key role in vascular homeostatic regulation (59). Endothelial dysfunction and atherogenesis are characterized by reduced NO bioavailability owing to diminished eNOS expression or activation, reactive oxygen species (ROS) overproduction, and inhibition of eNOS activity by either the overproduction/reduced clearance of endogenous ADMA or the induction of an arginase activity. Moreover, previous studies have attributed all of the above mechanisms to *H. pylori* pathogenicity in various gastric and nongastric injury models (19, 41, 45, 51). Correspondingly, treatment of BAECs with Vac$^+$ (but not Vac$^-$) HPCM reduced endogenous NO production by over 50%, whereas all of the observed Vac$^+$ HPCM-dependent changes in endothelial function could be recovered by an exogenous NO source (SNAP). Importantly, HPCM treatment (Vac$^+$ or Vac$^-$) did not appear to significantly alter total BAEC eNOS protein levels (data not shown), suggesting that the observed HPCM-induced NO reduction is not attributable to eNOS expression changes and that alternate mechanisms (i.e., eNOS activation, ROS production, etc.) are more likely associated with this phenomenon. We further demonstrated that a known stimulus for NO production in vivo, namely laminar shear stress (65), could also recover Vac$^+$ HPCM-induced changes in BAEC proliferation, tube formation, and migration. These findings confirm a central role for NO depletion in VacA-induced endothelial injury. Moreover, they further suggest that the atherogenic impact of *H. pylori* in vivo would be most acute at arterial branch points and curvatures, the principal sites of atherosclerotic lesion development, where the atheroprotective influence of laminar shear stress is attenuated (due to turbulence) and NO depletion could be further exacerbated by VacA. A recent study by Liuba et al. (41), in which the authors demonstrate that coinfection of apoE-knockout mice with *C. pneumoniae* and *H. pylori* leads to impaired bioactivity of endothelial NO and increased VCAM-1 expression at arterial branch points, supports this conclusion. Interestingly, shear stress did not appear to prevent the barrier-lowering effects of Vac$^+$ HPCM. The reasons for this are unknown, although insufficient shear-induced NO production, NO-independent effects of VacA, and/or experimental artifact are possible explanations. A more detailed investigation of the dynamic relationship between shear stress and VacA-induced endothelial injury is therefore warranted.

The precise nature of VacA signaling in vascular endothelial cells is undefined at present, although one can speculate as to the intermediates involved. As a member of the Rho-GTPase family of signaling enzymes, Rac1 integrates multiple signaling events and is known to function upstream of eNOS activation and NO production in vascular endothelial cells in various physiological contexts (27, 34, 40). Interestingly, the VacA-dependent inhibition of Rac1 has been shown to prevent repair of gastric mucosal injury and ulcer reepithelialization, an NO-dependent process (50). Studies have also recently demonstrated that VacA can induce cellular effects independently of its vacuolating function through the activation of a p38 MAPK stress signaling pathway, which leads to the activation of the transcription factors activating transcription factor-2, cAMP-response element-binding protein, and NF-κB (24, 32). Since p38 MAPK activation has also been directly linked to the superoxide-induced reduction of NO bioavailability in different vascular injury models (56, 64), this suggests the possible applicability of this signaling pathway to the HPCM-induced NO reduction observed in our BAEC model. Downstream of NO, signaling possibilities are numerous. Of note, NO is known to inhibit the SNAP receptor-mediated exocytosis of endothelial Weibel-Palade bodies, which mediate vascular inflammation and adaptive remodeling (46). Thus VacA-dependent NO reduction could ultimately lead to the excessive

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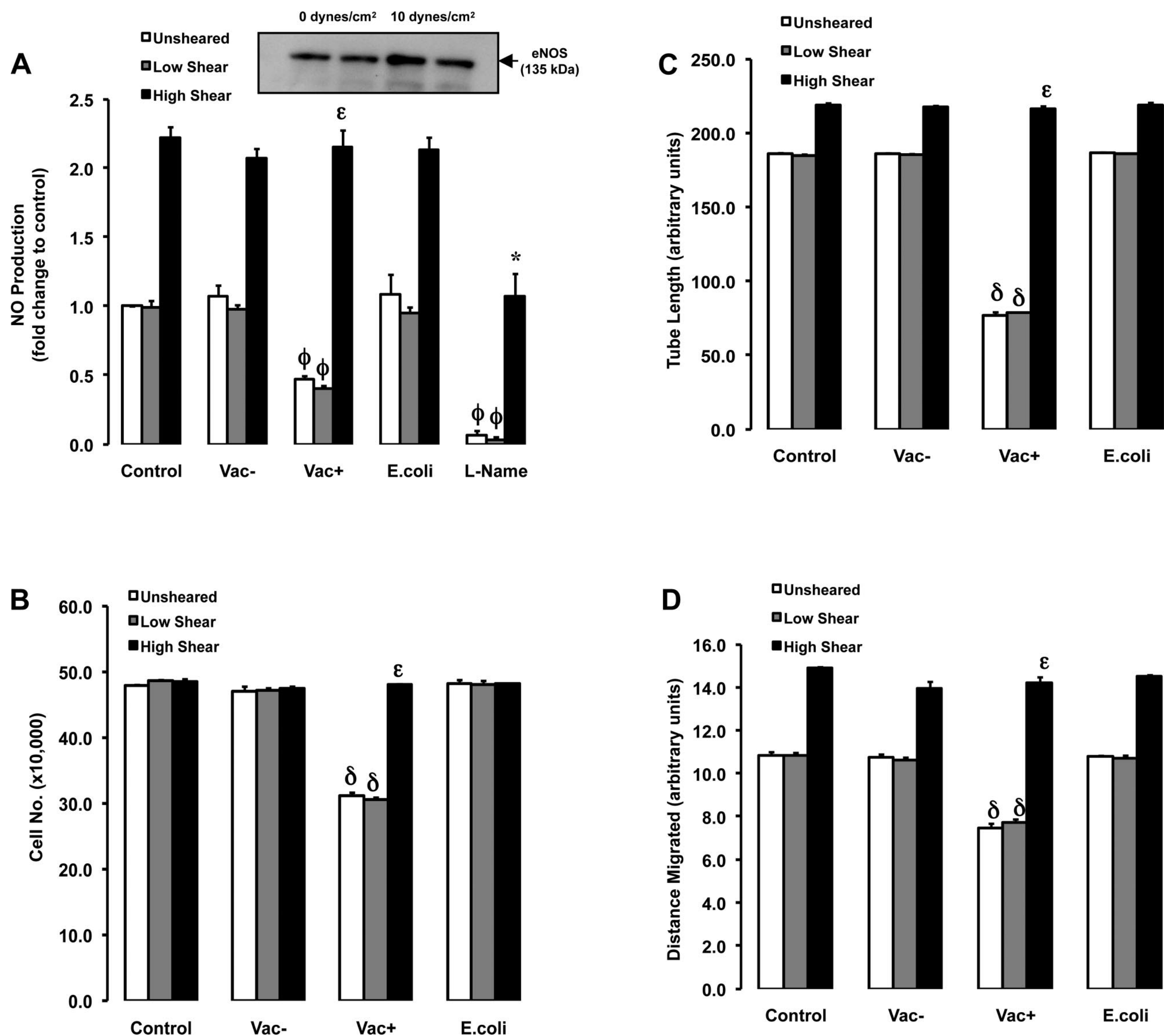

Fig. 7. Effect of laminar shear stress on VacA-dependent changes in BAEC functions. BAECs were treated for 24 h with either Vac$^+$ or Vac$^-$ HPCM (25%) or *E. coli*-conditioned media under static (0 dynes/cm^2; white), low shear (1 dyne/cm^2; gray), and high shear (10 dynes/cm^2; black) conditions. Control is unconditioned RPMI complete media. The effects of shear on HPCM-dependent changes in NO production, proliferation, tube formation, and migration were subsequently monitored as previously described. Histograms were averaged from 3 independent experiments ± SE. *A*: NO production. $\phi P \leq 0.001$ vs. control; $\varepsilon P \leq 0.0001$ vs. Vac$^+$ low shear; $*P \leq 0.01$ vs. control high shear. *Inset*: representative blot showing effect of shear stress on total endothelial NO synthase (eNOS) protein levels under control conditions. *B*: proliferation. $\delta P \leq 0.01$ vs. control; $\varepsilon P \leq 0.0001$ vs. Vac$^+$ low shear. *C*: tube formation. $\delta P \leq 0.0001$ vs. control; $\varepsilon P \leq 0.0001$ vs. Vac$^+$ low shear. *D*: migration. $\delta P \leq 0.0001$ vs. control; $\varepsilon P \leq 0.01$ vs. Vac$^+$ low shear.

release of inflammatory mediators, with predictable consequences for endothelial dysfunction.

To summarize, we have conducted a comprehensive investigation of how *H. pylori*-secreted factors play a role in vascular endothelial injury. With the use of an in vitro aortic endothelial cell model, our findings demonstrate, for the first time, the antiproliferative, antiangiogenic, and barrier-lowering properties of *H. pylori*-secreted VacA, events consistent with endothelial dysfunction. Moreover, the VacA-dependent impairment of endothelial NO bioavailability is strongly indicated in these events, as is an atheroprotective role for laminar shear stress. These findings establish a clearer vascular context in which *H. pylori* infection contributes to endothelial dysfunction and atherogenesis in vivo. Although the precise organization of the VacA signaling pathway, and indeed its dynamic relationship with shear stress, is beyond the scope of this paper, we have begun to elucidate the cellular intermediates involved and believe the present study provides an excellent foundation for further investigations in this field.

Finally, although the present study adds further weight to a causal relationship between *H. pylori* infection and atherosclerosis, one is mindful of the complex debate surrounding the contribution of the infectious burden to endothelial dysfunction and vascular disease. Indeed, researchers have provided evidence both in support of and against a role for *H. pylori* in this pathophysiological context, findings chronicled primarily through in vivo modeling studies (41, 42), epidemiological investigations (12, 20, 22, 25, 49, 55), and the identification of

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H. pylori DNA in atheromatous vascular tissue (1, 17, 36, 68). We believe that the in vitro plausibility study described in this paper makes an extremely important and timely contribution to this debate and will undoubtedly serve as an important correlate to ongoing clinical/eradication studies directly addressing this issue.

GRANTS

This research was supported in part through funding from the Health Research Board of Ireland (to G. T. Henehan and P. A. Cahill), Science Foundation Ireland (to P. A. Cahill), and the Higher Education Authority Programme for Research at Third Level Institutes (to P. A. Cahill). Additional financial support was provided through the Science Foundation Ireland, Health Research Board of Ireland, and Enterprise Ireland Basic Research Grant Programmes (to P. M. Cummins and R. P. Murphy) and through Dublin City University (to N. P. Tobin).

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