Shear-Induced Increase in Hydraulic Conductivity in Endothelial Cells Is Mediated by a Nitric Oxide–Dependent Mechanism

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Abstract—This study addresses the role of nitric oxide (NO) and its downstream mechanism in mediating the shear-induced increase in hydraulic conductivity (Lp) of bovine aortic endothelial cell monolayers grown on porous polycarbonate filters. Direct exposure of endothelial monolayers to 20-dyne/cm² shear stress induced a 4.70±0.20-fold increase in Lp at the end of 3 hours. Shear stress (20 dyne/cm²) also elicited a multiphasic NO production pattern in which a rapid initial production was followed by a less rapid, sustained production. In the absence of shear stress, an exogenous NO donor, S-nitroso-N-acetylpenicillamine, increased endothelial Lp 2.23±0.14-fold (100 μmol/L) and 4.8±0.66-fold (500 μmol/L) at the end of 3 hours. In separate experiments, bovine aortic endothelial cells exposed to NO synthase inhibitors, N⁶-monomethyl-L-arginine and N⁶-nitro-L-arginine methyl ester, exhibited significant attenuation of shear-induced increase in Lp in a dose-dependent manner. Inhibition of guanylate cyclase (GC) with LY-83,583 (1 μmol/L) or protein kinase G (PKG) with KT5823 (1 μmol/L) failed to attenuate the shear-induced increase in Lp. Furthermore, direct addition of a stable cGMP analogue, 8-bromo-cGMP, had no effect in altering baseline Lp, indicating that the GC/cGMP/PKG pathway is not involved in shear stress–NO–Lp response. Incubation with iodoacetate (IAA), a putative inhibitor of glycolysis, dose-dependently increased Lp. Addition of IAA at levels that did not affect baseline Lp greatly potentiated the response of Lp to 20-dyne/cm² shear stress. Finally, both shear stress–induced and IAA-induced increases in Lp could be reversed with the addition of dibutyryl cAMP. However, additional metabolic inhibitors, 2 deoxyglucose (10 mmol/L) and oligomycin (1 μmol/L), or reactive oxygen species scavengers, deferoxamine (1 mmol/L) and ascorbate (10 mmol/L), failed to alter shear-induced increases in Lp. Our results show that neither the NO/cGMP/PKG pathway nor a metabolic pathway mediates the shear stress–Lp response. An alternate mechanism downstream from NO that is sensitive to IAA must mediate this response. (Arterioscler Thromb Vasc Biol. 2000;20:35-42.)

Key Words: shear stress • nitric oxide • hydraulic conductivity • endothelial cells
that NO can alter endothelial transport properties in a variety of blood vessels.

It is widely believed that NO affects transport through a downstream pathway involving cGMP.\(^{18}\) Another downstream effect of NO that could impact the endothelial transport barriers is its alteration of cellular energy metabolism. For example, Salzman et al\(^{19}\) demonstrated that NO reduces the transepithelial resistance of cultured Caco-2BBE intestinal epithelial monolayers by a mechanism involving ATP depletion, not the alteration of cGMP. In endothelial cells, Padgett and Whorton\(^{20,21}\) have shown that NO inhibits a key glycolytic enzyme, GAPDH. However, to date, the role of NO as a mediator of shear-induced increase in endothelial monolayer \(L_p\) has not been evaluated. Therefore, the objective of the present study was to elucidate the role of NO and its downstream effectors in mediating the response of \(L_p\) to acute changes in shear stress.

### Methods

#### Chemicals

The following chemicals were obtained from Sigma Chemical Co: Hanks’ balanced salt solution, BSA (fraction V, 30% solution), minimal essential medium (MEM), FBS, glutamic acid, sodium bicarbonate, gelatin, fibronectin, penicillin G, streptomycin sulfate, \(N^\circ\)-monomethyl-L-arginine (L-NMMA), \(N^\circ\)-nitro-L-arginine methyl ester (L-NAME), \(\beta\)-nicotinamide adenine dinucleotide phosphate, \(N\)-(1-naphthyl)ethylenediamine, flavin adenine dinucleotide, sulfanilamide, nitrate reductase, dibutyryl cAMP (DBcAMP), 8-bromo-cGMP, 2-deoxyglucose (DOG), oligomycin, iodoacetate (IAA), deferoxamine, and ascorbate. \(N^\circ\)-Monomethyl-L-arginine (D-NMMA), LY-83,583, and KT5823 were purchased from Calbiochem. S-Nitroso-N-acetylpenicillamine (SNAP) was obtained from Research Biochemicals Intl. 1,1\(^\text{2}\)-Dioctadecyl-3,3,3\(^\text{2}\),3\(^\text{2}\)-tetramethyl-indocarbocyanine perchlorate-acetylated LDL was obtained from Biomedical Technologies. Polycarbonate filters (Transwell chambers, 0.4-μm pore size, 24.5-mm diameter) were obtained from Corning Costar. Trypsin was obtained from Gibco-BRL. Finally, high vacuum grease was obtained from Dow Corning.

#### Cell Culture

Primary bovine aortic endothelial cells (BAECs) were harvested from bovine thoracic aortas and subsequently maintained in MEM–10% FBS as described previously.\(^{22}\) Cells were plated at a density of 2.5×10\(^4\) cells/cm\(^2\) on polycarbonate membrane Transwell filters pretreated with gelatin (5 mg/L, type A from porcine skin) and fibronectin (30 μg/mL). Cells between passages 6 and 12 were used in experiments.

#### Experimental Protocol

### Measurement of Water Flux Under Shear Stress

A detailed description of the experimental apparatus used to measure water flux under shear stress has been presented by Sill et al.\(^6\) Briefly, a polycarbonate membrane Transwell filter containing the endothelial monolayer was sealed between 2 pieces of polycarbonate assembly separating the luminal and abluminal compartments. The compartments were continuously provided with positive-pressure gassing of 5% \(CO_2/95%\) balanced air to maintain the pH of the medium. The abluminal compartment was connected to a reservoir via Tygon tubing and borosilicate glass tubing. The reservoir could be lowered to a desired height to create the hydrostatic pressure gradient required to drive water flux across the cell monolayer. To eliminate the oncotic pressure gradient, the same medium (MEM–1% BSA) was added to both the luminal and the abluminal compartments. The luminal compartment contained a cylindrical disk, which was rotated by a motor drive to produce a defined shear stress that varied linearly from zero at the center to a maximum value at the edge (mean value was two thirds of the maximum value). All subsequent values of shear stress will be given as the maximum shear value. It should be noted, however, that if the dependence of \(L_p\) on shear stress is nonlinear, the spatial variation of shear stress in the apparatus will provide some distortion in the measured shear dependence based on the maximum (or mean) value.

To measure fluid flux across the monolayer, a bubble was inserted into the borosilicate glass tubing and tracked with a spectrophotometer mounted on a screw rod, which was driven by a stepper motor. This traveling spectrophotometer was interfaced to a computer, and the bubble position was displayed as a function of time on the computer screen. The bubble displacement was then converted to fluid volume flux (\(J_v\)) through a calibration equation. \(L_p\) could be calculated by the following equation: \(L_p = J_v / S \cdot \Delta P\), where \(S\) is the surface area of the monolayer, and \(\Delta P\) is the hydrostatic pressure differential across the monolayer (10 cm H\(_2\)O). There may have been a slight excess of protein near the luminal surface because of concentration polarization driven by the volume flux, but this is expected to have a negligible oncotic effect at a protein concentration of 1%.\(^7\)

### Response of \(L_p\) to SNAP

Experiments were conducted in the presence of an exogenous NO donor, SNAP, at 100 and 500 μmol/L without shear stress. After establishment of baseline \(L_p\) for 1 hour, SNAP was added to the luminal compartment, and \(L_p\) was measured for an additional 3 hours.

#### Nitrite/Nitrate Determination

Endothelial monolayers grown to confluence on polycarbonate filters in Transwell chambers were rinsed twice with MEM+1% BSA. Then 2 mL of MEM+1% BSA was pipetted into the luminal side of the chamber, and the filter, while still attached to the Transwell chamber, was mounted onto a glass slide and sealed with high vacuum grease. Finally, the monolayer was exposed to defined shear stress (20 dyne/cm\(^2\)) with or without the NOS inhibitor, L-NMMA, by using the rotating disk apparatus. Cell perfusate samples (500 μL) were taken and replaced with fresh experimental media at 0, 5, 30, 60, 120, and 180 minutes after addition of shear stress. The concentrations of the stable products, NO\(_x\), and NO\(_2\), were determined as previously.\(^8\)

#### Effect of NOS Inhibitors on Shear-Induced Increase in \(L_p\)

In separate experiments, the endothelial cell monolayers were preincubated for 1 hour with L-NMMA (10, 50, and 100 μmol/L), L-NAME (100 μmol/L), or D-NMMA (100 μmol/L), then exposed to a shear stress differential of 10 cm H\(_2\)O without shear stress for 1 hour to establish the baseline \(L_p\), and subsequently subjected to 20-dyne/cm\(^2\) shear stress for 3 hours. \(L_p\) was measured continuously during the 1-hour preshear period and the 3-hour shear period.

#### Mechanism Downstream From NO: cGMP/PKG Pathway

Endothelial cell monolayers were exposed to 20-dyne/cm\(^2\) shear stress in the presence of inhibitors of guanylate cyclase (GC) and protein kinase G (PKG). In separate experiments, monolayers were incubated with LY-83,583 (10 μmol/L, a selective GC inhibitor) or KT5823 (1 μmol/L, a PKG inhibitor) for 30 minutes before the addition of shear stress. In addition, the response of \(L_p\) after direct exposure to the stable cGMP analogue, 8-bromo-cGMP, was examined. After the establishment of baseline \(L_p\), 8-bromo-cGMP (1 mMol/L) was added onto the monolayer, and \(L_p\) was measured for 3 hours.

#### Mechanism Downstream From NO: Metabolic Pathway

First, either IAA (10 μmol/L, a putative GAPDH inhibitor) or DOG (10 μmol/L, a selective inhibitor of glycolysis) was added just before the onset of 20-dyne/cm\(^2\) shear stress. Endothelial monolayer \(L_p\) was then measured for 3 hours. In similar sets of experiments, monolayers were preincubated with DOG (10 μmol/L) and/or oligomycin (1 μmol/L, an inhibitor of mitochondrial ATP synthesis) in a glucose-free medium before the addition of 20-dyne/cm\(^2\) shear stress.

Because peroxynitrite (OONO\(^-\)) can inhibit metabolic and other enzymes, experiments were conducted in which monolayers were preincubated with scavengers of reactive oxygen species, deferoxamine (1 mMol/L) or ascorbate (10 mMol/L), for 30 minutes before...
the addition of 20-dyne/cm² shear stress. Endothelial monolayer Lₚ was then measured for 3 hours.

Data Presentation and Statistical Analysis

As described previously, on application of the 10-cm H₂O pressure head, there was an initial decrease in Lₚ, which stabilized over a period of 30 to 50 minutes. Therefore, a 1-hour period was allotted to establish a baseline Lₚ before further intervention. Experiments with baseline Lₚ values <5.0×10⁻⁷ cm·s⁻¹·cm H₂O⁻¹ were used for evaluation. About 10% of the experiments were rejected because of elevated baseline values associated with incomplete monolayer formation.

Five-minute average Lₚ values were calculated, normalized with respect to the established baseline Lₚ, and presented as mean±SEM. Significant differences between group means were performed every 30 minutes after establishment of baseline Lₚ and analyzed by a 2-way (time and treatment) ANOVA with the use of statistical analysis software (SAS). Time was the repeated factor, and planned pairwise comparisons were performed with the Bonferroni correction. A level of P<0.05 was considered significant for the statistical analysis.

Results

Figure 1 illustrates the basic response of cultured bovine aortic endothelial monolayer Lₚ to a physiological shear stress of 20 dyne/cm². As reported previously in our laboratory, shear stress induced a significant increase in Lₚ: 4.70±0.20-fold after 3 hours of shear stress.

Shear stress of 20 dyne/cm² alone (n=5) elicited a time-dependent increase in nitrite/nitrate (NOₓ) production (Figure 2). Within 5 minutes of the onset of shear, there was a significant (P<0.02) and dramatic increase in NOₓ concentration to a level of 17.8±2.1 nmol/mg protein (compared with 8.7±0.5 nmol/mg protein at time 0). This burst was followed by a less rapid, sustained production for the next 55 minutes. Between 60 and 120 minutes, there was an acceleration of production, albeit at a lower rate than observed for the first 5 minutes, followed by a final phase (120 to 180 minutes) in which production was less vigorous. At the end of 3 hours of shear stress, NOₓ rose to 85.7±19.1 nmol/mg protein, whereas stationary controls (n=8) produced only 18.4±3.7 nmol/mg protein (4.8±1.3 nmol/mg protein at time 0). Similar to stationary controls, NOₓ produced by monolayers exposed to L-NMMA alone (n=4) increased to only 11.3±3.3 nmol/mg protein (3.4±0.3 nmol/mg protein at time 0) for the same time period, which did not prove to be significantly different from control values (P>0.40). This indicates that L-NMMA had no significant effect on basal production of NOₓ. As expected, the shear-induced increase in NOₓ could be blocked by the addition of L-NMMA. At the end of 3 hours, cumulative NOₓ concentration in the presence of 20-dyne/cm² shear stress and L-NMMA (n=4) was 28.42±2.44 nmol/mg protein (6.8±1.4 nmol/mg protein at time 0), which was not significantly different from control values (P>0.25). It was also not significantly different from L-NMMA alone (P>0.09).

As shown in Figure 3, 100 μmol/L SNAP (n=6) significantly increased endothelial monolayer Lₚ by 2.23±0.14-fold (P<0.01) 3 hours after the addition of this pharmacological agent. Moreover, 500 μmol/L SNAP elicited an even greater increase in Lₚ after 3 hours (4.8±0.66-fold).

Figure 4 depicts the direct effect of the NOS inhibitor L-NMMA in the absence of shear. L-NMMA at 10 μmol/L increased Lₚ by 1.36±0.19-fold after 3 hours, whereas L-NMMA at 50 μmol/L increased Lₚ by 1.90±0.19-fold after 3 hours. Neither response was significantly different from control values (P>0.15). However, for 100 μmol/L L-NMMA, Lₚ increased by 2.71±0.33-fold after 3 hours, and this was significantly greater than control values (P<0.002).

The marked increase of Lₚ in response to shear stress (Figure 1) was highly attenuated (P<0.001) when endothelial cells were treated with 50 or 100 μmol/L L-NMMA (Figure 5). The response to 20-dyne/cm² shear stress in the presence of 100 μmol/L L-NMMA was an increase in Lₚ of only 2.40±0.32-fold at 3 hours, and in the presence of 50 μmol/L L-NMMA was an increase in Lₚ of only 2.20±0.24-fold at 3 hours.
L-NMMA, the response was an increase in $L_p$ of only $2.30 \pm 0.22$-fold after 3 hours. At $10 \mu M/L$, L-NMMA did not significantly alter the shear-induced increase in $L_p$, which was $3.87 \pm 0.54$-fold after 3 hours ($P > 0.20$).

To determine whether attenuation of the shear response of $L_p$ was in fact due to NOS inhibition and not a side effect of the drug, another set of experiments (n = 4) was performed with the use of an inert enantiomer, D-NMMA (100 $\mu M/L$). The baseline $L_p$ in the presence of D-NMMA and 20-dyne/cm$^2$ shear stress was $4.07 \pm 0.36 \times 10^{-7}$ cm $s^{-1}$ cm H$_2$O$^{-1}$. D-NMMA, unlike L-NMMA, did not attenuate the shear-induced increase in $L_p$. At the end of 3 hours of 20-dyne/cm$^2$ shear stress plus D-NMMA, $L_p$ increased by $4.01 \pm 0.45$-fold, which was not significantly different from the response to 20-dyne/cm$^2$ shear stress alone ($P = 0.20$).

To further confirm the role of NOS in the shear stress response of $L_p$, we examined the effect of another NOS inhibitor, L-NAME (100 $\mu M/L$), in the presence of 20-dyne/cm$^2$ shear stress. Similar to 100 $\mu M/L$ L-NMMA, 100 $\mu M/L$ L-NAME (baseline $L_p$ $4.71 \pm 0.19 \times 10^{-7}$ cm $s^{-1}$ cm H$_2$O$^{-1}$, n = 5) highly attenuated the shear-induced increase in $L_p$. At the end of the third hour, normalized $L_p$ was elevated only $2.25 \pm 0.12$-fold, which was significantly lower than $L_p$ in response to 20-dyne/cm$^2$ shear stress alone ($P < 0.001$).

To examine the role of the GC/cGMP/PKG pathway, endothelial cell monolayers were exposed to 20-dyne/cm$^2$ shear stress in the presence of a GC inhibitor, LY-83,583 (1 $\mu M/L$, $K_i$ 2 $\mu M/L$). The inhibitor did not significantly alter the shear-induced increase in $L_p$. At the end of 3 hours, 20-dyne/cm$^2$ shear stress in the presence of LY-83,583 (baseline $L_p$ $2.94 \pm 0.33 \times 10^{-7}$ cm $s^{-1}$ cm H$_2$O$^{-1}$, n = 4) induced an increase in endothelial $L_p$ of $3.27 \pm 0.47$-fold, which was not significantly different from the shear-induced increase in the absence of the inhibitor ($P > 0.15$). Consistent with the LY-83,583 data, addition of a cell-permeant stable analogue of cGMP, 8-bromo-cGMP, had no significant effect ($P > 0.50$) in altering baseline $L_p$ ($2.96 \pm 0.63 \times 10^{-7}$ cm $s^{-1}$ cm H$_2$O$^{-1}$, n = 6). At the end of 3 hours of exposure, 8-bromo-cGMP (1 mmol/L) increased $L_p$ by $1.58 \pm 0.39$-fold, whereas...
In Lp of 4.11 ± 0.44-fold, which was not significantly different from the response in the absence of the inhibitor (P > 0.25). These data indicate that the GC/cGMP/PKG pathway does not play a significant role downstream from NO in BAECs.

Another pathway that is known to be affected (inhibited) by NO in BAECs is glycolysis, through the enzyme GAPDH. It is clear in Figure 6 that use of the putative glycolysis inhibitor IAA led to a dose-dependent increase in endothelial Lp. At the lowest dose (10 μmol/L), IAA did not increase Lp above control levels after 3 hours of exposure (P > 0.10). IAA (30 μmol/L) was required to elicit a significant increase in Lp at the end of 3 hours (3.31 ± 0.11-fold, P < 0.01), which was comparable to that induced by shear stress. This IAA-induced increase in Lp could be reversed by addition of the cell-permeant stable analogue of cAMP, DBcAMP (1 mmol/L). Within 30 minutes of the addition of DBcAMP, Lp returned to original baseline levels. A similar return of Lp to baseline within 30 minutes of the addition of DBcAMP (1 mmol/L), P > 0.05 for 20-dyne/cm² shear stress compared with IAA plus 20-dyne/cm² shear stress. Data are presented as mean ± SEM.

Figure 6. Response of endothelial Lp to the glycolysis inhibitor IAA. Addition of IAA led to a dose-dependent increase in Lp; 10 μmol/L (1.85 ± 0.22-fold) and 30 μmol/L (3.31 ± 0.11-fold). Baseline Lp values for 10 μmol/L and 30 μmol/L IAA were 3.58 ± 0.22 × 10⁻² cm²·s⁻¹·cm H₂O⁻¹ (n = 4) and 4.01 ± 0.57 × 10⁻² cm²·s⁻¹·cm H₂O⁻¹ (n = 4), respectively. The increase in Lp induced by 30 μmol/L IAA could be reversed within 30 minutes with the addition of DBcAMP (1 mmol/L). *P < 0.05 for IAA compared with stationary control. Data are presented as mean ± SEM.

Discussion

The present study addresses the role of NO in mediating the shear-induced increase in Lp of BAEC monolayers. We observed that an exogenous NO donor, SNAP, increased endothelial Lp in a dose-dependent manner and that the response of Lp to 500 μmol/L SNAP was comparable in magnitude to that elicited by 20-dyne/cm² shear stress, although the dynamics of the responses were different (Figures 1 and 3). The differences in dynamics of the Lp response to SNAP and shear stress were probably the result of differing
rates of NO release associated with the 2 mechanisms. We also demonstrated that shear stress of 20 dyne/cm² caused a multiphasic NO release that was greatly attenuated by the NOS inhibitor L-NMMA (Figure 2). These results are similar to those in human umbilical vein endothelial cells reported by Kuchan and Frangos.8

Another important finding was that NOS inhibitors blocked the shear-induced increase in Lp. When Figures 4 and 5 are compared, it is apparent that after 3 hours, the increase in Lp, induced by 20-dyne/cm² shear stress in the presence of 100 μmol/L L-NMMA is not significantly different from the increase in Lp in response to 100 μmol/L L-NMMA alone (P>0.50). Therefore, the attenuation observed in response to 100 μmol/L L-NMMA is a total inhibition of the shear-induced response. Even at 50 μmol/L L-NMMA, there is total inhibition of the shear-induced response (P>0.20). These data support the central role of NO in mediating shear-induced increases in endothelial monolayer Lp. It should also be noted that Ranjan et al 9 showed that in BAECs, shear stress alters constitutive NOS within 3 hours but has no effect on inducible NOS levels. This suggests that constitutive NOS has been inhibited in our experiments.

Our results are consistent with the observations of Meyer and Huxley,12 who superfused the NO donor sodium nitroprusside into frog mesenteric venular capillaries and recorded 2.6-fold elevation in Lp. Subsequently, Rumbaut et al.13 using a similar experimental protocol, demonstrated that microvascular Lp decreases when vessels are superfused with the NOS inhibitor L-NMMA. In related experiments dealing with soluble transport, Yuan et al.5 measured the permeability coefficient of albumin in isolated porcine coronary venules at various intraluminal perfusion velocities and observed a 48% increase in permeability when velocity increased from 7 to 13 mm/s. The flow-induced permeability change was completely abolished by addition of L-NMMA. Investigators who examined the effect of NOS inhibition on agonist-mediated endothelial transport properties also observed significant attenuation of agonist-induced increases in permeability. Hughes et al14 showed that L-NMMA and L-NAME inhibited substance P–induced edema formation in the dorsal skin of male Wistar rats. Mayhan15 also demonstrated in the hamster cheek pouch that L-NMMA significantly decreased the formation of histamine-induced leaky sites. Similarly, Noel et al16 reported that inhibition of NOS with L-NMMA attenuated the platelet activating factor–induced and histamine-induced increase in permeability of FITC-dextran in the hamster cheek pouch.

The studies cited above indicate that NO increases endothelial transport rates, but there are studies that exhibit an opposite action of NO. For example, Oliver50 demonstrated that the bradykinin-induced increase in sucrose permeability was enhanced when endothelial cells were exposed to NOS inhibitors. Kubes51 and Kurose et al11 also showed an enhancement of albumin permeability when vessels were exposed to L-NAME in feline small intestinal microvessels and in mesenteric venules of male Sprague-Dawley rats, respectively. Even in our own system, opposing results were observed. L-NMMA (100 μmol/L), which was expected to have an action opposite that of SNAP, actually elicited an increase in Lp (Figure 4), albeit of lesser magnitude than that induced by SNAP (Figure 3). When shear stress was present to stimulate higher levels of NO, the addition of L-NMMA (Figure 5) attenuated Lp. Thus, the effects of NO on endothelial transport properties are somewhat controversial. They appear to depend on the specific species and vascular origin of the endothelium under consideration and possibly the level of NO concentration within the cells.

Although we have demonstrated that NO mediates shear-induced increases in endothelial Lp, the mechanism downstream from NO is of great interest. It has been well established that NO stimulates soluble GC, which elevates the level of cGMP.31 Meyer and Huxley12 have shown that prusside into frog mesenteric venular capillaries and recorded 2.6-fold elevation in Lp. Subsequently, Rumbaut et al.,13 using a similar experimental protocol, demonstrated that microvascular Lp decreases when vessels are superfused with the NOS inhibitor L-NMMA. In related experiments dealing with soluble transport, Yuan et al.5 measured the permeability coefficient of albumin in isolated porcine coronary venules at various intraluminal perfusion velocities and observed a 48% increase in permeability when velocity increased from 7 to 13 mm/s. The flow-induced permeability change was completely abolished by addition of L-NMMA. Investigators who examined the effect of NOS inhibition on agonist-mediated endothelial transport properties also observed significant attenuation of agonist-induced increases in permeability. Hughes et al14 showed that L-NMMA and L-NAME inhibited substance P–induced edema formation in the dorsal skin of male Wistar rats. Mayhan15 also demonstrated in the hamster cheek pouch that L-NMMA significantly decreased the formation of histamine-induced leaky sites. Similarly, Noel et al16 reported that inhibition of NOS with L-NMMA attenuated the platelet activating factor–induced and histamine-induced increase in permeability of FITC-dextran in the hamster cheek pouch.

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Our data show that inhibition of PKG with KT5823 does not attenuate the shear-induced increase in Lp after 3 hours, indicating that the cGMP/PKG pathway is not involved. Furthermore, direct exposure to a stable analogue of cGMP, 8-bromo-cGMP (at a relatively high concentration), had no effect on baseline Lp and inhibition of GC with LY-83,583 did not alter the shear stress–Lp response, confirming that the cGMP/phosphodiesterase pathway is also inoperative. These findings are consistent with a recent study by Gooch et al,35 who reported that BAECs (received from our laboratory) actually release very low basal levels of cGMP. Moreover, stimulation of these cells with shear stress of 22 dyne/cm² or with spermine–NO complex (30 μmol/L) had no effect in altering the production of cGMP. In contrast, the endothelial cells derived from human umbilical veins released substantially higher (∼20-fold) basal levels of cGMP, and this level could be increased 20-fold with the addition of 5 μmol/L spermine–NO complex and 2.5-fold with application of 22-dyne/cm² shear stress.36 Taken together, our studies as well as the findings of Gooch et al provide strong evidence that the NO/GC/cGMP pathway does not play a significant role in the shear stress–Lp response in BAECs. An alternate mechanism downstream from NO must mediate this response.

Previously, we have shown that the shear-induced increase in BAEC Lp can be highly attenuated by elevating the level of cAMP through exogenous (DBcAMP) and endogenous (3-isobutyl-1-methylxanthine) means.6 The effects of cAMP analogues in preventing permeability increases in response to a variety of chemical agonists have been reported previously.33,34
A novel pathway linking NO and cAMP was suggested by recent studies showing that NO leads to reversible inhibition of a key glycolytic enzyme, GAPDH, via S-nitrosylation of the active site cysteine residue in intact bovine pulmonary artery endothelial cells and BAECs. Because endothelial cells rely primarily on glycolysis for the production of ATP, inhibition of glycolysis leads to a reduction in the intracellular concentration of ATP and, thus, cAMP. To test for the role of glycolytic activity in the shear stress–Lp response, we used a putative inhibitor of glycolysis, IAA. Our results (Figure 6) show that addition of IAA leads to a dose-dependent increase in endothelial Lp. Moreover, we observed that in the presence of 10 µmol/L IAA (a concentration at which the baseline Lp was not altered), the effect of 20-dyne/cm² shear stress on endothelial Lp was greatly potentiated (Figure 7) and that this potentiated response could be reversed by the addition of DBcAMP. Thus, even though IAA by itself did not significantly alter endothelial Lp, it had a synergistic effect in enhancing the shear-induced increase. This strongly suggests that shear stress and IAA mediate their effects on Lp through the same pathway, potentially via inhibition of glycolysis.

It is important to note, however, that IAA is a potent acetylation agent that can nonselectively inhibit susceptible sulfhydryl groups within a cell. To further explore the metabolic pathway, more selective inhibitors were incorporated. DOG (a selective inhibitor of glycolysis) and oligomycin (an inhibitor of the citric acid cycle) failed to alter the shear stress–Lp response, indicating that the metabolic pathway may not mediate the physiological response. Peroxynitrite represents another intermediate species through which NO can inhibit ATP synthesis. NO forms OONO⁻ by reacting with a superoxide anion radical (O₂⁻), and OONO⁻ has been shown to inhibit ATP synthesis by inhibiting aconitase, the rate-limiting enzyme in the citric acid cycle. However, consistent with the results obtained with DOG and oligomycin, scavengers of reactive oxygen species (deferoxamine or ascorbate) also failed to alter the shear-induced increase in Lp. Taken together, these data confirm that the metabolic pathway does not play a role in mediating the shear stress—NO−Lp response.

Our findings are consistent with those of Bolin et al., who demonstrated that the IAA-induced increase in vascular permeability of isolated rabbit lung was independent of ATP synthesis. Similarly, Wilson et al. showed that low glucose and DOG, as well as DOG plus antimycin-A (a metabolic inhibitor), can greatly inhibit ATP but not affect albumin or dextran permeability in pulmonary arterial endothelial cells and that H₂O₂ will also inhibit ATP but does increase albumin permeability.

In conclusion, the present study has shown that NO is a key signaling molecule mediating the shear stress–Lp response. In addition, we have demonstrated that neither the GC/cGMP/PKG pathway nor the metabolic pathway plays a prominent role in mediating the shear stress–Lp response. We do, however, show that endothelial Lp is sensitive to inhibition by IAA. Moreover, shear stress and IAA may mediate their effects on Lp through the same pathway. The exact nature of this downstream pathway remains to be explored but may involve a direct inhibition of adenylate cyclase, as has been suggested recently.

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References


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