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Does Elevated Nitric Oxide Production Enhance the Release of Prostacyclin from Shear Stressed Aortic Endothelial Cells?

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Nitric oxide (NO) enhances prostacyclin (PGI_2) production in agonist-stimulated endothelial cells, while peroxynitrite formed from NO and superoxide anion has been shown to activate cyclooxygenase. Using cultured bovine aortic endothelial cells (BAEC) exposed to arterial levels of laminar shear stress of 25 dynes/cm^2, we tested the hypothesis that NO mediated the elevated synthesis of PGI_2 by shear stressed endothelium. Shear stress caused a large and rapid burst and sustained release of NO and PGI_2 with the cumulative production at 1 hr enhanced 9.96-fold ($n = 4$, $p < 0.005$) and 9.16-fold ($n = 3$, $p < 0.005$), respectively, over stationary control production of 0.0257 nmol-NO/cm^2-BAEC and 0.0193 ng-PGI_2/cm^2-BAEC. The NO synthase inhibitors, N^G-nitro-L-arginine methyl ester (100 μM, LNAME) and N^G-nitro-L-arginine (10 μM, LNA), caused 87.5 and 65% reductions ($n = 3$, $p < 0.02$) of cumulative NO release at 1 hr, respectively, and 45 and 55% reductions ($n = 3$, $p = 0.025$) of PGI_2 release, respectively. About half of the elevated production of PGI_2 in shear stressed cells was due to NO-dependent signaling, indicating that hemodynamic control of these two dilatory molecules is partially coupled.

Vascular endothelial cells produce nitric oxide (NO) and prostacyclin (PGI_2) both of which cause potent vasodilation and inhibition of platelet activation. Hemodynamic shear stress elevates the release of NO and PGI_2 from cultured cells and vessel preparations (1-5). Receptor-mediated increases in endothelial intracellular calcium can activate constitutive endothelial nitric oxide synthase (eNOS or NOSIII) and phospholipase C and A_2 (6). Recent reports have suggested that elevated levels of NO can lead to the activation of constitutive cyclooxygenase (COX1, constitutive PGH synthase 1) (7-9). For example, treatment of cultured bovine aortic endothelial cells (BAEC) with glyceryl trinitrate, sodium nitroprusside, or 3′-morpholinosydnonimine (SIN-1, a donor of NO and O_2^-, and consequently peroxynitrite) dramatically enhanced by 4 to 6-fold the release of PGI_2 from arachidonic acid-stimulated BAEC via cGMP-independent pathways (9). In A23187-treated bovine microvessel endothelial cells, both NO and PGI_2 production were increased several fold in a cGMP-independent manner, but inhibition of NO production with LNAME attenuated the PGI_2 release by half (7). Recently, peroxynitrite generated from the reaction of NO with O_2^- was shown to activate purified ram seminal vesicle COX1 and recombinant human COX2 by serving as a substrate for the peroxidase activity of the enzymes (10). While BAEC possess soluble guanylate cyclase that can be activated by nitric oxide to elevate intracellular cGMP levels (3,4,8,11), there is some uncertainty if human umbilical endothelial cells or BAEC possess cGMP-dependent protein kinases (12). However, a more recent report has demonstrated cGMP-dependent protein kinase type I in BAEC (13). In light of the above observations, we tested the hypothesis that nitric oxide was responsible for the elevated release of PGI_2 when endothelial cells were exposed to arterial levels of shear stress.

MATERIALS AND METHODS

Cell culture and shear stress exposure. Bovine aortic endothelial cells (BAEC, passages 2-5) were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated newborn calf serum, 0.30 mg/ml glutamine, and 0.05 mg/ml gentamicin (GIBCO, Grand Island, NY) as previously described (14). Confluent monolayers were exposed to a steady laminar shear stress of 25 dynes/cm^2 in individual, parallel plate flow chamber systems with recirculating medium (20 mL) driven by a constant hydrostatic pressure head under sterile conditions as previously described (15,16) or maintained in a CO_2 incubator at 37°C. All flow experiments were

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conducted in an environmental room at 37°C. A sterile HEPES buffered saline (140 mM NaCl, 5.0 mM KCl, 1.0 mM $MgCl_2 \cdot 6H_2O$, 1.0 mM $CaCl_2 \cdot 2H_2O$, 10.0 mM D-glucose, and 10.0 mM HEPES pH 7.4) containing 1% (w/v) bovine serum albumin was used for the flow experiments and stationary controls. The perfusion media did not contain ATP or L-arginine. Samples of conditioned media were removed at various times from the control and shear stressed monolayer and stored at −80°C. In some experiments, some monolayers of replicate plated cultures were incubated in N^G-nitro-L-arginine methyl ester (LNAME, 100 μM) or N^G-nitro-L-arginine (LNA, 10 μM) (Sigma, St. Louis, MO) for 30 minutes prior to exposure of the cells to shear stress with LNAME or LNA in the incubation or perfusion media.

Nitrite assay for NO and 6-keto $PGF_{1\alpha}$ radioimmunoassay for PGI_2. Nitrite, a stable end-product of NO, was used as a measure of NO production. 2,3-diaminonaphthalene (DAN) (Aldrich, Milwaukee, WI) was reacted with nitrite under acidic conditions to form 1-(H)-naphthotriazole, a fluorescent product (17). A volume of 80 μl of freshly prepared DAN reagent (0.05 mg/ml in 0.62 M HCl) was added into each 800 μl media sample and mixed immediately. After a 10-min incubation at 20°C, the reaction was terminated with 40 μl addition of 2.8 N NaOH. Formation of 2,3-diaminonaphthotriazole was measured using a Perkin-Elmer LS50 luminescence spectrophotometer with excitation at 365 nm and emission read at 450 nm. Sodium nitrite was used as the standard for calibration of the assay between 25 and 500 nM nitrite. The DAN reagent had no cross reactivity with nitrate, LNAME, or LNA. In separate tests, nitrate in the BAEC conditioned media was converted to nitrite using 50 mU nitrate reductase from *Aspergillus Niger* (Sigma) and 40 μM NADPH (5 min at 22°C). This protocol converted over 95 % of nitrate to nitrite. However, less than 10% of the total NO-related signal in the BAEC conditioned media samples was recovered from nitrate during the conversion and the nitrate component was subsequently neglected. Radioimmunoassay (Amersham, Arlington Heights, IL) was used for the measurement of 6-keto-$PGF_{1\alpha}$, the stable breakdown product of PGI_2. The lower limit of detection was 31 pg/ml. All assays contained fresh media controls for the various agents to verify that there was no effect of the pharmacological agents on the RIA. A mass balance for volume removed and replenished during sampling was used to determine the cumulative production of NO and PGI_2 per square centimeter of monolayer (about 10^5 cells/cm^2) over time.

RESULTS

An arterial level of shear stress of 25 dynes/cm^2 stimulated a burst of NO release from BAEC after the onset of flow compared to matched stationary controls. The release of NO by shear stressed cells remained essentially constant between 5 and 60 min at a rate of 0.00175 nmol/cm^2 per minute which was 5.5-fold greater ($n = 4$, $p < 0.005$) than the NO release rate by stationary controls of 0.000321 nmol/cm^2 per minute. The treatment of BAEC with L-arginine analogs, LNAME or LNA, caused significant inhibition of NO production at every time point under conditions of shear stress (Fig. 1A). The same inhibitors also slightly reduced basal production ($n = 3$, $p < 0.1$) of NO by the stationary controls (Fig. 1B). By 1 hr, the total cumulative production of NO was 9.96-fold higher in cells exposed to shear stress and LNAME and LNA caused a 87.5 and 65 % reduction ($n = 3$, $p < 0.02$) in release of NO by shear stressed BAEC, respectively.

In concert with NO release, PGI_2 production by

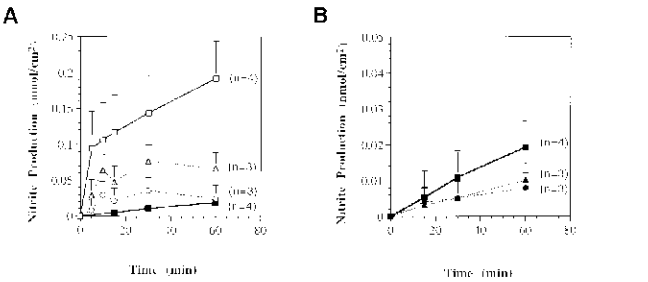

FIG. 1. The shear stress mediated increase of nitric oxide production by bovine aortic endothelial cells was inhibited by L-arginine analogs. The stable reaction product nitrite was assayed as an indicator of NO production. Cells were exposed to 25 dynes/cm^2 (open symbols) or maintained in stationary culture (closed symbols) in the absence (square) or presence of 10 μM LNA (triangle) or 100 μM LNAME (circle). Each data point is the mean ± SD of time points from 3 or 4 independent experiments. Note scale difference between panels A and B.

BAEC was also stimulated by shear stress (Fig. 2A). The time course revealed a rapid elevation of PGI_2 in response to shear stress. LNA treatment of BAEC caused between a 55% and 65% reduction of shear stress-induced PGI_2 release at each time point. The inhibitory result was similar with LNAME treatment. There was no significant effect of the inhibitors on the basal production of PGI_2(Fig. 2B). These experiments revealed that the inhibitors and consequent NO blockade significantly reduced PGI_2 production in shear stressed BAEC. Neither LNAME nor LNA had any significant inhibitory effect on basal production of PGI_2 suggesting that they were not direct inhibitors of cyclooxygenase. Although basal NO production was not completely blocked by 100 μM LNAME or 10 μM LNA and it was not possible to determine fully the effect of basal NO production on basal PGI_2 production, it appears that they were not linked under no-flow conditions.

At 1 hour, LNAME and LNA caused a 45% and 55% reduction ($n = 3$, $p = 0.025$), respectively, in PGI_2 production from sheared cells compared to PGI_2 release from cells shear stressed in the absence of the inhibitors. In the presence of LNAME or LNA, the cumulative PGI_2 production was 3 to 4-fold higher ($p < 0.02$) than the basal levels, indicating that the NO inhibition led to a blockade of about half of the PGI_2 production and that there were also NO-independent mechanisms involved in the enhancement of PGI_2 release by shear stress.

DISCUSSION

Consistent with earlier studies (1-4), shear stress was a potent inducer of NO and PGI_2 production by bovine aortic endothelial cells. In all experiments, we observed a burst of production of NO followed by a sustained elevated release of NO that was constant

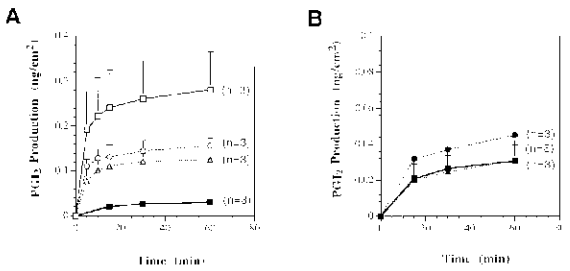

FIG. 2. Inhibition of NO production with L-arginine analogs attenuated the release of PGI_2 from shear stressed bovine aortic endothelial cells. Cells were exposed to 25 dynes/cm^2 (open symbols) or maintained in stationary culture (closed symbols) in the absence (square) or presence of 10 μM LNA (triangle) or 100 μM LNAME (circle). Each data point is the mean ± SD of time points from 3 independent experiments. Note scale difference between panels A and B.

from 5 to 60 min. Shear stress also elicited a burst of PGI_2 with a release rate that was elevated over control levels out to 15 min at which time a near steady state production rate was maintained which was only slightly greater than the basal PGI_2 production rate between 15 and 60 min. These short term experiments had no exogenous arachidonic acid or L-arginine which, in conjunction with endogenous feedback regulation, may limit the production rate of NO and PGI_2 in the presence of continual shear stress exposure after the early transitory phase.

Davidge et al. (7) tested whether NO-activated PGI_2 production required cGMP using several different approaches. Their work indicated that NO stimulation of PGI_2 production is most likely independent of cGMP levels. This is consistent with earlier reports that elevated levels of cGMP do not enhance PGI_2 production in BAEC (11).

While the COX enzymes are potential targets for NO activation because they contain an iron-heme center at their active site, Tsai et al. (18) found no activation of cyclooxygenase activity at NO concentrations ranging from micromolar to millimolar added to purified ovine seminal vesicle COX1 enzyme. This is in contrast to a report of heme-independent S-nitrosation of cysteines in the active site of COX1 (19). In a recent study (10), activation of the purified COX1 or COX2 was induced by direct addition of peroxynitrite or by in situ generation of peroxynitrite from NO and O_2^-. Furthermore, in the mouse macrophage cell line RAW264.7, lipophilic superoxide dismutase-mimetic agents decreased the prostaglandin production without affecting the level of NOS or COX or by inhibiting the release of arachidonic acid (10).

We found that under normoxic perfusion conditions without exogenous antioxidants, bicarbonate/CO_2, ATP, L-arginine, or arachidonic acid, a large portion of PGI_2 released by laminar shear stress was dependent on shear induced activation of nitric oxide synthase. This perfusion condition was consistent with the earlier reports of shear induced PGI_2 (1) and NO production (4). In an earlier study of the mechanisms of PGI_2 induction (20), intracellular calcium chelation caused a 75 to 83 % decrease in PGI_2 production while diacylglycerol (DAG) lipase inhibition caused a 66 % decrease in PGI_2 production when primary HUVEC were perfused with complete M199 media. This is not unexpected because arachidonic acid is released by phospholipases that require nominal calcium for activity. It is likely that NO production was also reduced in that study since NOS is calcium-dependent and intracellular calcium chelation has been shown to attenuate flow-induced NO production (3). Although NOS is calcium-dependent, its activity appears to be enhanced in shear stressed endothelium through a calcium-independent activation involving tyrosine kinases and integrin mediated signaling (21-23). Changes in arachidonic acid availability due to phospholipase A_2 activation (24, 25) or G-protein activation of phospholipase C and subsequent DAG cleavage by DAG lipase may be responsible (20) for the increased PGI_2 production in the presence of NO inhibition in our study. Alternatively, PGI_2 production may be further reduced in our experimental system with higher levels of L-arginine analogs to completely block NO production.

Superoxide formation by nitric oxide synthase may be elevated in the absence of exogenous L-arginine or bicarbonate/CO_2 buffering, thus favoring peroxynitrite formation (26,27). The continued elevated release of NO at longer times out to 1 hr of shear stress exposure and the rapid coupling of NO and PGI_2 production in the first 5 min of the experiment suggest that endogenous L-arginine was not fully depleted under the conditions of the experiment. The role of peroxynitrite formation in coupling flow induced NO with flow induced PGI_2 production remains an important issue of future study. Interestingly, LNAME is reported to block O_2^- production in L-arginine-free nNOS transfected kidney cells (26). It would appear that endogenous Cu/Zn-superoxide dismutase is insufficient to fully scavenge O_2^- if peroxynitrite is indeed the mediator of the observed NO-dependent PGI_2 release in our study, though such a scavenging pathway may become more effective with longer shear stress exposures (28).

There are reports of nonspecific effects of L-arginine analogs (29,30). The levels of LNAME and LNMMA used by Peterson et al. (29) to inhibit by 20 and 40 %, respectively, the reduction of purified ferric cytochrome C by ferrous iron were quite high (2.5 mM). Peterson et al. speculated that LNAME and LNMMA may inhibit prostaglandin synthesis by a putative complexation of iron. Our use of 100 μM LNAME and 10 μM LNA at a level 25 to 250-fold lower than those used in the study of Peterson would argue against a role for such an inhibition in the present study given the effect is fairly small reported by Peterson in a purified system even

at millimolar levels of L-arginine analogs. We found similar results with LNAME and an L-arginine analog lacking a methyl ester group (LNA) which would argue against nonspecific effects due to alkyl esters of arginine in our system that lacks exogenously added acetylcholine (30). Recently, LNAME at 50 μM was shown to have no effect on calcium mobilization in frog microvessels perfused with 10 μM ATP (31).

This study recreates the mass transfer environment of the cardiovasculature where high washout rates will reduce concentration boundary layers. We demonstrated using in vitro perfusion assays that shear induced NO can act autocrinically in a hemodynamic environment to elevate endothelial PGI_2 production. In terms of vessel physiology, the hemodynamic elevation of NO to accommodate elevated flows may be coordinated with the activation of COX. At a more integrative level, hemodynamic shear stress drives endothelial cell function with elevated NO and PGI_2 production and changes in endothelial cell phenotype that may promote vasodilatory and antithrombotic potential (14, 15, 32).

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