Shear Stress Elevates Endothelial cGMP
Role of a Potassium Channel and G Protein Coupling

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Background. The endothelium acts as the sensor of shear stress and as the mediator of flow-induced changes in vessel tone and structure. The purpose of this study was to delineate the signal transduction pathway of flow-induced release of endothelium-derived relaxing factor (EDRF).

Methods and Results. We used a shear stress apparatus (a modified cone-plate viscometer) to expose cultured endothelial cells to a well-defined laminar fluid flow. Confluent bovine aortic endothelial cells (BAECs) were subjected to varying levels of shear stress, and intracellular cyclic GMP (cGMP) in the BAECs was measured by radioimmunoassay. After 60 seconds of laminar fluid flow, BAEC cGMP increased by 300% from basal levels (from 0.54 to 1.70 pmol/mg protein, P < 0.05). The elevation in intracellular cGMP was proportional to the intensity of shear stress within a physiological range up to 40 dynes/cm². This increase in cGMP was abrogated by L-N-methyl-arginine (the competitive antagonist of nitric oxide [NO] synthase), indicating that the flow-induced activation of soluble guanylate cyclase was mediated by autocrine NO production. Furthermore, a potassium channel antagonist, tetraethylammonium ion (TEA [3 mmol/L]) and a G₁ or G₃ protein inhibitor, pertussis toxin (100 ng/mL) also blocked the flow-induced increase in cGMP. By contrast, calcium ionophore or atrial natriuretic peptide caused elevations of cGMP that were not affected by TEA or pertussis toxin.

Conclusions. These findings indicate that shear stress elevates endothelial cGMP via an NO-dependent mechanism. The effect of shear stress is mediated by a unique signal transduction pathway that is coupled to a pertussis toxin–sensitive G protein and that requires the activity of an endothelial potassium channel.

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Key Words • endothelium-derived factors • blood flow • pertussis toxin • GMP

As blood flow increases through a conduit vessel, the vessel dilates. This flow-mediated vasodilation is proportional to the shear stress induced by blood flow and independent of changes in luminal pressure. It is now recognized that this phenomenon requires the integrity of the endothelium and in most conduit vessels is caused by the release of an endothelium-derived relaxing factor (EDRF) with characteristics of the endogenous nitrovasodilator first described by Furchgott and Zawadzki. In some circulations, other substances (such as prostacyclin or hyperpolarizing factors) may contribute to flow-mediated vasodilation.

The mechanism by which the endothelium senses and transduces this flow stimulus remains poorly understood. Olesen and colleagues have shown that flow hyperpolarizes endothelial cells; this hyperpolarization is caused by activation of a potassium channel with the characteristics of an inward rectifier. Subsequently, we demonstrated that vortical flow induces the release of EDRF from cultured bovine aortic endothelial cells (BAECs); this flow-induced release of EDRF required the activity of a potassium channel with the characteristics of a K⁺ channel. To determine whether the same signal transduction mechanism is required for the endothelial response to laminar flow, we used a modified cone-plate viscometer to expose cultured endothelial cells to a well-defined laminar shear stress. This system has allowed us to further characterize the mechanisms by which the endothelium senses and transduces the stimulus of laminar fluid flow.

Methods

Endothelial Cell Culture

BAECs were isolated from the aortas by established techniques. These cells exhibit a typical cobblestone appearance, express angiotensin converting enzyme, and bind acetylated low density lipoprotein cholesterol. BAECs were grown in Dulbecco's modified Eagle's medium (DMEM) containing 25 mol/L HEPES and 10% calf serum. DMEM was composed of (mmol/L): NaCl, 109; KCl, 5.5; CaCl₂, 1.8; MgSO₄, 1.7; NaHCO₃, 51; NaH₂PO₄, 1.0; Fe(NO₃)₃ 9 H₂O, 3.6x10⁻²; glucose, 30; sodium pyruvate, 1.0; and phenosulfonphthalein, 4.2x10⁻². Confluent monolayers derived from passages 5 through 7 were grown on 150-mm-diameter culture dishes and, 2 days after achieving confluence, were placed in our shear stress apparatus.

Shear Stress Apparatus

We used a shear stress apparatus that allowed us to expose cultured endothelial cells to a well-defined laminar fluid flow. Our system is modified from the cone-plate viscometer device first described and characterized by Bussolari and Dewey. The modifications we

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have made allow us to expose a large number of cells to the same shear stress; this allows us to pool cells for biochemical and molecular studies. The shear stress device consists of a cone that rotates above a stationary base plate containing the cultured endothelial cells. The base plate is formed by the 150-mm-diameter polystyrene tissue culture dish. To ensure a level surface, the culture dish is secured to the underlying platform by vacuum suction (Figure 1). The methyl methacrylate (Plexiglas) cone makes an angle of 0.5° with the culture plate and is coupled to a variable motor. The angular velocity of the cone is precisely adjusted by a motor controller to expose the endothelial cells to laminar flow, inducing a physiological shear stress in the range of 0 to 40 dynes/cm².

**Experimental Protocol**

Two hours before the cells were exposed to shear stress, the medium was replaced by physiological saline solution of the following composition (mmol/L): NaCl, 118.3; KCl, 4.7; CaCl₂, 2.5; MgSO₄, 1.2; KH₂PO₄, 1.2; NaHCO₃, 25.0; calcium disodium edetate, 0.026; and glucose, 11.1. We used this physiological saline solution because, unlike standard DMEM, 1) it is devoid of L-arginine, eliminating any possibility that shear stress–induced nitric oxide (NO) production is secondary to increased delivery to the endothelial cells of the NO precursor, and 2) it does not contain ATP, which is known to modulate the endothelial response to shear stress.¹⁹,²⁰

Thirty minutes before shear stress was induced, 3-isobutyl-1-methylxanthine (10⁻³ mol/L; a phosphodiesterase inhibitor) was added to the solution bathing the cells. The tissue culture dish was then placed in the cone-plate viscometer, and the cells were subjected to shear stress from 0 to 40 dynes/cm² for 60 seconds. We established in pilot experiments that a maximal elevation by shear stress of endothelial cyclic GMP (cGMP) was achieved at this time. The tissue culture dish was then quickly removed, and cold 6% trichloroacetic acid was added to the cells. Endothelial cell lysate was prepared as previously described, and cGMP was measured by radioimmunoassay.²¹ In some experiments, the endothelial cells were stimulated by calcium ionophore A23187 (10⁻⁶ mol/L) or atrial natriuretic peptide (ANP; 10⁻⁷ mol/L) for 1 minute in the absence of shear stress and then harvested for measurements of intracellular cGMP.

**Data Analysis**

Comparisons were made with paired t tests; where multiple means were compared, an ANOVA was performed, followed by a Newman-Keuls test. A value of P<0.05 was accepted as statistically significant. Data are expressed as mean±SEM.

**Results**

Laminar fluid flow to induce endothelial shear stress in a physiological range (from 0 to 40 dynes/cm²) increased BAEC intracellular cGMP levels. At maximum levels of shear stress, the increase in cGMP was nearly threefold (Figure 2). To determine whether the shear stress–induced increase in intracellular cGMP resulted from an autocrine effect of NO released from the endothelial cells, in some experiments the cells were preincubated for 15 minutes with the NO synthase antagonist NMA (10⁻³ mol/L) before shear stress was applied. NMA abrogated the response to shear stress (Figure 3).

To determine whether the activity of an endothelial potassium channel was required for the shear stress–induced activation of soluble guanylate cyclase, tetraethylammonium ion (TEA; 3 mmol/L) was added to the cells 15 minutes before flow was induced. The nonselective potassium channel antagonist TEA completely abrogated the flow-induced increase in intracellular cGMP (Figure 3). Since many potassium channels are gated by pertussis toxin–sensitive G proteins, in some experiments pertussis toxin (IAP; 100 ng/mL) was added to the cells 2 hours before they were exposed to shear stress. Previous studies have established that this

**Drugs**

The following drugs were used: atrial natriuretic peptide, calcium ionophore A23187 (Sigma Chemical Co), L-N-monomethyl-arginine (NMA; Calbiochem), and tetraethylammonium chloride (Kodak). NMA was dissolved in 50% ethanol, and calcium ionophore was dissolved in dimethylsulfoxide (Sigma); all other agents were soluble in distilled water or physiological saline solution.
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FIGURE 2. Bar chart showing effect of shear stress on intracellular levels of cyclic GMP (cGMP). Cultured bovine aortic endothelial cells were exposed to shear stress (from 0 to 40 dynes/cm²) in the shear stress apparatus for 60 seconds. Subsequently, the cells were prepared for measurement of intracellular cGMP. Shear stress induces an elevation of endothelial cGMP.

FIGURE 3. Bar chart shows that shear stress–induced elevation of endothelial cyclic GMP (cGMP) is dependent on an endothelial potassium channel, a G protein, and NO synthase. Bovine aortic endothelial cells were exposed to shear stress (40 dynes/cm²) in the presence of vehicle (control), tetraethylammonium ion (TEA; 10⁻³ mol/L), pertussis toxin (IAP; 100 ng/mL), or L-N-monomethylarginine (NMA, 10⁻³ mol/L). Under control conditions, shear stress induced a significant increase in endothelial cGMP levels. This effect was abolished by TEA, IAP, and NMA. The numbers in each bar refer to the number of experiments performed under each condition.

FIGURE 4. Model for signal transduction of the flow stimulus. A flow-sensitive, membrane-associated structure (a) is linked to an adjacent potassium channel (b) by cytoskeletal elements. Flow deforms this structure, increasing the probability of the open state, which is also modulated by a G protein coupling mechanism (c). The subsequent potassium efflux induces a hyperpolarization that favors Ca²⁺ influx (d). Extracellular Ca²⁺ entry activates nitric oxide synthase (NOS) to produce NO, which subsequently elevates intracellular cyclic GMP (cGMP).

Exposure of BAECs to calcium ionophore (10⁻⁶ mol/L) induced a 400% elevation in intracellular cGMP. The rise in BAEC cGMP levels induced by calcium ionophore was mediated by autocrine production of NO, since it was abrogated by NMA (data not shown). However, in contrast to the shear stress–induced release of NO, the increase in BAEC cGMP induced by calcium ionophore was not affected by TEA (3 mmol/L) or pertussis toxin (100 ng/mL) (data not shown).

Discussion

In the present study, we find that shear stress elevates endothelial cGMP via an NO-dependent mechanism. The synthesis and release of NO in response to fluid flow requires the activity of a TEA-sensitive ion channel, suggesting the involvement of an endothelial potassium channel. The signal transduction pathway also requires a pertussis toxin–sensitive G protein. We speculate that this G protein may be required for activation of the flow-responsive K⁺ channel, since many other K⁺ channels are gated by pertussis toxin–sensitive G proteins.²² By contrast, we find that calcium ionophore increases endothelial cGMP via an NO-dependent mechanism that is independent of this signal transduction pathway.

Olesen and colleagues first demonstrated that fluid flow hyperpolarizes endothelial cells as a result of the activation of a potassium current.¹³ Subsequently, we demonstrated that aortic endothelial cells cultured on microcarrier beads and exposed to vortical flow released a vasodilator into the medium, detected by bioassay.¹⁴ This vasodilator had the characteristics of EDRF, because its action was antagonized by hemoglobin, methylene blue, and NMA.¹⁴,¹⁵ Antagonists of the calcium-activated potassium channel prevented the release of relaxing activity into the medium conditioned by flow-stimulated cells. These observations were consistent with the hypothesis that an endothelial potassium channel was required in the transduction of the flow stimu-
lus, whereas NO was the effector of the vasodilation. However, the conclusions derived from this experimental approach were largely applicable to the special situation of vortical flow, as occurs at flow dividers. The present experimental approach reveals that our original conclusions also apply to the endothelial mechanotransduction of the stimulus of laminar flow.

The mechanism by which the potassium channel is activated by flow has not been elucidated. The potassium channel may be the mechanosensory transducer of the flow stimulus. Conversely, a membrane glycoprotein or an endothelial calcium channel may be the mechanotransducer. Indeed, we have shown that calcium-free perfusate or perfusate containing antagonists of calcium entry does not induce flow-mediated vasodilation in the isolated, pressurized, perfused iliac artery of the rabbit. Therefore, entry of extracellular calcium through an endothelial channel is required for flow-mediated vasodilation, just as it is involved in the flow-induced release of prostacyclin. In this model, the entry of extracellular calcium would be augmented by the increased electrochemical gradient produced by the flow-activated hyperpolarizing potassium current. This is consistent with previous observations revealing that levels of intracellular calcium are partially dependent on the electronegativity of the endothelial cell.

Although the release of EDRF by flow- or receptor-mediated events appears to be critically dependent on extracellular calcium, it has been difficult to demonstrate a flow-induced increase in intracellular calcium levels in cultured endothelial cells by fluoroscopic techniques. Whereas Ando and colleagues were able to demonstrate an increase in intracellular calcium during exposure of BAECs to fluid flow, other investigators have not been able to confirm these findings in the absence of ATP added to the medium. These investigators suggest that flow increases delivery of ATP to the endothelial cell surface, disrupting a local gradient induced by endothelial ecto-apyrase. In our present study, however, we were able to observe a flow response in the absence of ATP added to the medium. Furthermore, Shen and colleagues have also been able to detect flow-induced modulation of intracellular calcium in cultured endothelial cells in the absence of ambient ATP. These observations do not exclude the possibility that flow stimulates the release of ATP from the endothelial cells to promote the autocrine release of EDRF.

The release of EDRF by serotonin, α2-adrenergic agonists, and thrombin requires a G protein to couple activation of the receptor with postreceptor events. This G protein is sensitive to pertussis toxin, which abrogates endothelium-dependent relaxation by these agonists. In the studies described in this manuscript, we provide the first evidence that the activity of a pertussis toxin-sensitive G protein is also required for an endothelial response to flow. This finding is consistent with a recent report that flow-induced expression of platelet-derived growth factor is dependent on the activity of a G protein-coupled pathway.

To conclude, we have demonstrated that flow increases endothelial cGMP by the autocrine action of EDRF. This effect of flow is mediated by a unique signal transduction pathway that is coupled to a pertussis toxin-sensitive G protein and requires the activity of an endothelial potassium channel.

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