Hydrodynamic Shear Stress and Mass Transport Modulation of Endothelial Cell Metabolism

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Mammalian cells respond to physical forces by altering their growth rate, morphology, metabolism, and genetic expression. We have studied the mechanism by which these cells detect the presence of mechanical stress and convert this force into intracellular signals. As our model system, we have studied cultured human endothelial cells, which line the blood vessels and form the interface between the blood and the vessel wall. These cells respond within minutes to the initiation of flow by increasing their arachidonic acid metabolism and increasing the level of the intracellular second messengers inositol trisphosphate and calcium ion concentration. With continued exposure to arterial levels of wall shear stress for up to 24 h, endothelial cells increase the expression of tissue plasminogen activator (tPA) and tPA messenger RNA (mRNA) and decrease the expression of endothelin peptide and endothelin mRNA. Since the initiation of flow also causes enhanced convective mass transfer to the endothelial cell monolayer, we have investigated the role of enhanced convection of adenosine trisphosphate (ATP) to the cell surface in eliciting a cellular response by monitoring cytosolic calcium concentrations on the single-cell level and by computing the concentration profile of ATP in a parallel-plate flow geometry. Our results demonstrate that endothelial cells respond in very specific ways to the initiation of flow and that mass transfer and fluid shear stress can both play a role in the modulation of intracellular signal transduction and metabolism.
Key words: endothelium • genetic expression • protein synthesis • shear stress • signal transduction

INTRODUCTION

Mammalian cells grown in suspension or in anchorage-dependent systems respond to the mechanical forces caused by bulk fluid motion. At sufficiently high levels of hydrodynamic stress, the cells will lyse and die. However, cells also respond to sublytic levels of stress by altering their growth rate, morphology, metabolism, and genetic expression. Several studies using bioreactors have shown that the hydrodynamic forces caused by agitation and gas sparging can reduce the viability of hybridoma or other immortalized cell types.[2,34,38] The hydrodynamic stresses experienced by a suspended cell or a microcarrier bead in a bioreactor are complicated and poorly defined since the flow field varies both in space and in time. Thus, it is difficult to obtain a quantitative relation between hydrodynamic stress and cellular viability or metabolism from studies on the response of cells in a bioreactor. Other investigators have studied the effects of shear stress on cellular metabolism in flows with uniform and well-characterized flows,[8,22,48] such as the cone and plate, concentric cylinder, or parallel-plate flow geometries. In the sublytic stress regimes of these studies, the magnitude of the shear stress correlated with the degree of metabolic alteration sustained by the cells. We have studied the mechanism by which mammalian cells detect the presence of low-level mechanical stress and convert this force into an intracellular signal which leads to altered cellular metabolism and genetic expression.

As our model system we use a specialized mammalian cell type, the endothelial cell, which in its natural environment is exposed to fluid dynamical forces. The endothelial cell monolayer lines the vasculature and forms the interface between the flowing blood and the vessel wall. The endothelial cells perform crucial roles in regulating the transport of substances through the vessel wall, maintaining vascular tone by releasing vasoactive compounds, modulating reactions involved in coagulation, thrombosis, and fibrinolysis, localizing the immune response by expressing specific receptors for leukocyte adhesion to the endothelium, and elaboration of various growth factors (such as the smooth muscle cell mitogen endothelin). Some of the bioactive molecules expressed by endothelial cells, either secreted products or membrane-associated proteins, are listed in Table I. Several of these molecules are currently used therapeutically or are under study for potential therapeutic use. With the availability of technology for the routine harvesting and culture of human endothelial cells, much research has been directed toward understanding the details of the intracellular metabolism of the endothelial cell and how this metabolism relates to the physiology of the vessel wall. Consequently, many of the pathways involved in the response of these cells to chemical signals, such as the generation and action of specific intracellular second messengers, are reasonably well understood. The goal of our studies was to determine the nature of the mechanical stress signal transduction mechanism for this mammalian cell. We

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Table I. Biomolecules expressed by endothelial cells.[a,b,c]

Molecule	Function
Secreted products	
Prostacyclin (PGI_2)[a,b]	inhibits platelet aggregation, vasodilator
Endothelial-derived relaxing factor (EDRF)	vasodilator and platelet activator
Heparin-like molecules[a]	anticoagulant; mitogenic agent
Endothelin[b]	vasoconstrictor, smooth muscle cell mitogen
Interleukin-1 (IL-1),[c] IL-6, IL-8	cytokines, immune mediators
γ-Interferon[c]	immune mediator
Platelet-derived growth factor (PDGF)[b]	growth factor, wound healing
Basic fibroblast growth factor (bFGF)[c]	growth factor, wound healing
Transforming growth factor (TGF-β)	growth factor, wound healing
Urokinase (uPA)[a]	plasminogen activator, blood clot dissolver
Tissue plasminogen activator (tPA)[a]	plasminogen activator, blood clot dissolver
Plasminogen activator inhibitor (PAI-1)[c]	inhibitor of plasminogen activators
Factor VIII: von Willebrand factor (vWF)[a]	mediator of hemostasis
Vitronectin, fibronectin, collagen, vWF[a]	matrix proteins, adhesion mediators
Angiotensin-converting enzyme (ACE)[b]	mediator of hypertension
Surface molecules/receptors	
Platelet-activating factor (PAF)[b]	platelet activator
Thrombomodulin	binds thrombin; inhibitor of coagulation
Thrombospondin	modulator of thrombin activity
Tissue factor[b]	coagulation initiator
Intracellular adherence molecule-1 (ICAM-1)[b]	leukocyte adherence
Endothelial-leukocyte adhesion molecule (ELAM)	neutrophil receptor
Granule membrane protein 140 kD (GMP-140)	leukocyte adhesion
Vascular cell adhesion molecule (VCAM-1)	adhesion mediator
tPA receptor, uPA receptor, plasminogen receptor	localization of fibrinolytic pathway
Vitronectin receptor	adhesion mediator
Tumor necrosis factor α receptor	immune mediator
Histamine (H1) receptor[b]	inflammation and permeability modulator
Bradykinin receptor[b]	inflammation and permeability modulator
Purinergic receptor	adenosine nucleotide binding
Low-density lipoprotein (LDL) receptor[b]	uptake of protein-bound cholesterol

[a] Currently used therapeutically in humans.
[b] Target for pharmaceutical intervention.
[c] Used in animal or clinical studies as potential therapeutic agent.

investigated flow-induced changes in the concentration of intracellular metabolites, such as second messengers and messenger RNA (mRNA) levels, and changes in the production rate of secreted proteins and vasoactive compounds.

MATERIALS AND METHODS

Tissue Culture

Primary, human umbilical vein endothelial cells (HUVEC) were cultured on glass slides as previously described.[14,22,43] Endothelial cells were removed from the umbilical vein lumen by a 30-min collagenase incubation. The endothelial cells were pelleted and resuspended in Medium 199 (Gibco) with 20% heat-inactivated fetal calf serum (Hyclone), 0.1 mg/mL penicillin and streptomycin, 0.2 mg/mL neomycin, and 0.30 mg/mL L-glutamine, and seeded on glass slides. Complete M199 was used for all experiments unless otherwise noted.

Exposure of Cells to Laminar Shear Stress

Three to 4 days postseeding, confluent HUVEC monolayers were placed in stationary culture as controls or exposed to steady laminar shear stress in individual parallel-plate flow systems with recirculating media driven by a constant hydrostatic pressure head as previously described[14,22,43] (Fig. 1). The cell cultures were mounted on parallel-plate flow chambers (monolayer surface area 15 cm^2; gap thickness 200 μm) and connected under sterile conditions to the flow systems, each filled with 15 mL of medium. A peristaltic pump recirculated media from the bottom reservoir to the top reservoir. Samples (1 mL) from the circulating medium or stationary cultures were frozen at −80°C for assay of metabolites. At the end of the experiments (lasting up to 24 h), HUVEC monolayers remained unaligned. Additionally, no changes in cell density, cell volume (1800 ±100 fL), or cell shape were observed relative to controls. In studies requiring lysates of endothelial cells, photographs of monolayers were used to record final confluent monolayer density prior to extraction of cells. The fluid mechanics of the parallel-plate flow chamber are well characterized in the regimes used to study endothelial cell function. The Reynolds number was less than 20 in all experiments, ensuring laminar flow. Under these conditions, entrance lengths are less than 1 mm in our system.[23] The wall shear stress was calculated according to the formula $\tau_w = 6Q\mu/(B^2W)$, where

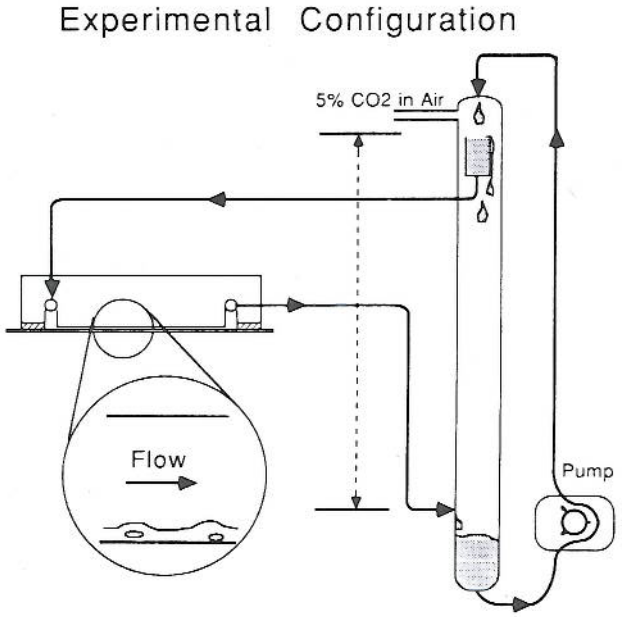

Figure 1. Schematic diagram of parallel-plate flow chamber attached to a media recirculation system. The close-up view shows the endothelial cell monolayer grown on a glass slide which formed one plate of the parallel-plate flow channel. The vertical separation between the top reservoir and bottom reservoir determined the hydrostatic pressure head. Media samples were taken from the bottom reservoir without disturbing flow through the chamber. The entire system was maintained at 37°C and pH 7.4.

flow rate Q is in cubic centimeters per second, viscosity, $\mu = 0.01$ P, total gap thickness $B = 0.02$ cm, and width $W = 2.49$ cm.

Uptake of Arachidonic Acid

Primary human umbilical vein endothelial cells were exposed to shear stress for 5 min before addition of [1-^{14}C]arachidonic acid to a final concentration of 2 μM in a total volume of 15 mL of medium in the flow loop. At the appropriate time points, the medium was removed and the cell lipids were extracted by adding 1 mL methanol to the monolayer and scraping 15 cm^2 of the slide to ensure that all cellular lipids were in solution. The cell extract was vigorously vortexed with 1 mL chloroform, then centrifuged for 15 min at 1000g. The supernatant was counted to determine total incorporated radioactivity. Cell extracts were evaporated to dryness under N_2 and resuspended in 100 μL chloroform. The distribution of labeled arachidonic acid was determined by thin-layer chromatography (TLC) as previously described.[43] The variability in the distribution of labeled arachidonic acid in each class of phospholipid for duplicate monolayers from the same cord pool was less than 5%, while the variability between separate cord pools was typically 10–20%.

Arachidonic Acid Metabolite Production

Endothelial cells were incubated with labeled arachidonic acid in stationary culture for 3 days to ensure that the distribution of labeled arachidonic acid was similar to the distribution of unlabeled arachidonic acid in the cells.[56] The production of arachidonic acid metabolites was investigated by exposing the cells to shear stress with fresh medium containing no labeled arachidonic acid. Controls were maintained in stationary culture with fresh medium. At the appropriate time points, the medium was collected and the radioactively labeled arachidonic acid metabolites were extracted using a Sep-Pak C_{18} cartridge (Waters). The eicosanoids were analyzed by reverse-phase high-pressure liquid chromatography (HPLC) with a μPAK C_{18} column (Varian). We found that only the 6-keto $PGF_{1\alpha}$ and $PGF_{2\alpha}$ peaks were dramatically altered by shear stress. The radioactivity was determined by a liquid scintillation counter. The identity of radioactivity peaks was established by comparing their retention times with those of eicosanoid standards.

Determination of Inositol-(1,4,5)-Trisphosphate

For these studies, the endothelial cell monolayer was washed 3 times with M199 (without serum) and the medium was changed to M199 (without serum) 12–24 h prior to the experiment. Initially, the slide was perfused with M199 at 0.07 dyn/cm^2 for 30 min. After this equilibration time, the shear stress was increased to either 2 or 22 dyn/cm^2 for times from 30 s to 15 min. After exposure to the elevated shear stress, the slide was removed quickly from the flow chamber and the cells were lysed by adding 1 mL ice cold 10% trichloroacetic acid (TCA) and thoroughly scraping the slide. The cell lysate was centrifuged and the TCA removed by ethyl ether extraction. The efficiency of the extraction procedure was found to be 80% as determined by extracting radioactively labeled Ins1,4,5P_3 or by using a radioimmunoassay (RIA) to determine the total mass of standard solutions of Ins1,4,5P_3. The amount of Ins1,4,5P_3 in each sample was measured with an RIA system[42] supplied by Amersham. The cross-reactivity of the binding protein with other compounds, including other phosphorylated inositides, is less than 0.5%. However, for the minor phosphorylated inositol lipid, inositol-1,3,4,5-tetrakisphosphate, the cross-reactivity was 6.4%. The cross-reactivity with the components of M199 or the TCA solution was negligible.

Measurement of Cytosolic Calcium Levels

Confluent monolayers were incubated in M199 with the fluorescent calcium-sensitive dye fura-2/AM at a concentration of 5 μM for 30 min at 37°C.[10,30] The cells were then washed 3 times with M199 and incubated in M199 for at least 30 min before mounting the cover slip on the flow chamber. The flow chamber with the attached glass cover slip was mounted on the stage of an upright Jenalumar epifluorescent microscope. The excitation light was provided by a 150-W Xe lamp

(Optiquip). The wavelength of the excitation light was alternated between 340 and 380 nm by means of a computer-controlled filter wheel (Ludl Electronics) positioned in the excitation light path. Experiments were performed using a Jena 50X fluorite objective which resulted in an image containing between 20 and 40 cells. The emission fluorescence was measured at a wavelength of 510 nm with a very low light level SIT video camera (Hamamatsu). The video signal was passed through a time base corrector (Harris) to a digital image processor (Perceptive Systems) for real-time image averaging. This digitally enhanced video image was then saved on broadcast quality $\frac{3}{4}$-in. video tape (Sony) for subsequent analysis. The positioning of the filter wheel and the operation of the image processor were coordinated by a Macintosh SE computer using software developed in our laboratory. The fluorescence ratio, which is proportional to the calcium concentration, was determined by dividing the intensity of one pixel in the image obtained with 340-nm excitation by the intensity of the corresponding pixel obtained with 380-nm excitation. Autofluorescence was subtracted prior to obtaining the ratio. Intensities less than a predetermined cutoff value were set equal to the background intensity. The fluorescence ratio was calibrated with respect to the intracellular calcium concentration by determining the fluorescence ratio of standard calcium solutions that had 25 μM fura-2 dye.

Endothelin RIA

Immunoreactive endothelin secreted by HUVEC was measured using a RIA (Amersham) employing an [^{125}I]-endothelin tracer and antiserum made against synthetic Endothelin-1. A calibration curve yielding a LOGIT plot with $r^2 > 0.99$ was generated using synthetic ET-1 added to complete medium (M199 and bovine serum) to control for nonspecific background.

tPA ELISA

A double-antibody enzyme-linked immunosorbent assay (ELISA) technique (American Diagnostica) using goat antihuman tPA immunoglobin allowed measurement of HUVEC-secreted tPA (free and inhibitor bound) as previously described.[14] Normal goat IgG was used as a nonspecific blocking agent. Soluble goat antihuman tPA IgG quenched the tPA-specific signal in the blanking well. HUVEC-conditioned media (undiluted) or antigenic standards (Bowes melanoma single-chain tPA) were added to sample wells in triplicate and to blanking wells. The ELISA was calibrated to the limit of its sensitivity (0–1500 pg/mL), yielding a linear calibration curve ($r^2 > 0.99$) with a detection limit of 50 pg/mL.

PAI-1 ELISA

In HUVEC-conditioned media, plasminogen activator inhibitor type 1 (PAI-1) exists in latent and active forms with only a very small fraction bound to tPA. An ELISA was used to measure uncomplexed (latent and active) PAI-1 antigen (American Diagnostica). Briefly, mouse antihuman PAI-1 immunoglobin was bound to a 96-well plate. The plate was washed and loaded with samples of conditioned media (diluted 1:10 and 1:25) or standards. The colorimetric reaction (measured at 490 nm) was performed using biotinylated monoclonal mouse antihuman PAI-1 immunoglobin and horseradish peroxidase conjugated streptavidin with a reaction buffer containing orthophenylenediamine and 0.04% (v/v) 30% H_2O_2.

Messenger RNA Analysis

Studies of mRNA levels using small numbers of primary human cells were carried out with a reverse transcription/polymerase chain reaction (RT/PCR) technique. Isolation of total cellular RNA from small cellular samples was accomplished with a scaled-down adaptation of the guanidine thiocyanate (GTC)/cesium chloride (CsCl) gradient method[51] as previously described.[15] Briefly, monolayers were lysed in 5M guanidine thiocyanate solution with 25 mM sodium citrate and 0.5% (w/v) sodium N-lauroyl sarcosinate, and centrifuged at 200,000g for 3 h over a 5.7M CsCl cushion. The pellet was dissolved in diethylpyrocarbonate-treated water (0.2% v/v) and extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform/isoamyl alcohol (24:1). Total RNA was precipitated with 3M sodium acetate (pH 5.4) and 100% ethanol, vacuum dried, and resuspended in 30 μL of diethylpyrocarbonate-treated H_2O. A 2.5M LiCl precipitation and resuspension were performed prior to the reverse transcription.

To provide an internal standard against experimental variations in the reverse transcription reaction or PCR amplification efficiency, simultaneous reverse transcription and coamplification of the constitutively expressed mRNA for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were carried out as previously described.[15] Simultaneous reverse transcription and coamplification of human endothelial cell RNA with primer sets[15,55] for tPA [or basic fibroblast growth factor (bFGF) or endothelin] and GAPDH produced amplification products of the predicted sizes of 368 bp for tPA, 177 bp for bFGF, 441 bp for endothelin, and 195 bp for GAPDH. Synthesis of tPA (or bFGF or endothelin) complementary DNA (cDNA) and GAPDH cDNA was carried out simultaneously in 25-μL reactions with 500 units Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT), 0.5 μg of each antisense primer, and 0.5 mM each of all four dNTPs. Each reverse transcription reaction mixture was heated at 37°C for 20 min. The cDNA products were coamplified using PCR in a total volume of 100 μL. Prior to amplification, a 75 μL volume was added containing 0.5 μg of each sense primer, 0.25 μg of each antisense primer, and 10 mM of each dNTP. Two units of recombinant Taq

DNA polymerase (AmpliTaq, Beckman) were added to initiate the PCR. Each temperature cycle consisted of 90°C for 1.5 min, 50°C for 1 min, and 72°C for 2 min. Amplified products were visualized by Southern hybridization using T4 kinase ^{32}P-end-labeled probes for tPA (or bFGF or endothelin) and for GAPDH.

RESULTS

Arachidonic Acid Uptake and Metabolism

One of the first indications that shear stress affects endothelial cell metabolism was the observation that shear stress could alter arachidonic acid (AA) metabolism.[22,28] Arachidonic acid is a 20-carbon polyunsaturated fatty acid which is found in all mammalian cells and is stored as a component of the phospholipids of the plasma membrane. In addition to performing a structural role in the membrane, arachidonic acid can be metabolized into a variety of potent biologically active compounds. In HUVEC, the primary arachidonic acid metabolites are prostacyclin (PGI_2) and $PGF_{2\alpha}$, which are both products of the cyclooxygenase pathway. PGI_2 is a strong inhibitor of platelet aggregation and can act synergistically with endothelial derived relaxing factor (EDRF) to produce vasodilation in whole-vessel preparations.[25] An increase in endothelial cell arachidonic acid metabolism is often directly related to an increase in free intracellular arachidonic acid. Thus, the flow-induced increase in PGI_2 production involves the increased liberation of arachidonic acid from intracellular stores. Arachidonic acid is released from phospholipids through at least two separate mechanisms: phospholipase A_2 can release arachidonic acid directly from the phospholipids; or phospholipase C can degrade phospholipids to produce diacylglycerol, which in turn is degraded by a diacylglycerol lipase to release free arachidonic acid.

We[43] found that the total incorporation of labeled arachidonic acid into all phospholipids was not significantly affected by shear stress when compared to stationary controls. However, the distribution of the labeled arachidonic acid among the different classes of phospholipids was altered. The increase in incorporation of arachidonic acid into the phosphatidylinositol fraction in shear-stress-stimulated cells is shown in Figure 2. The increased uptake into the phosphatidylinositol fraction was significant for times up to 2 h after the initiation of flow (25 dyn/cm^2). The incorporation of labeled arachidonic acid into the other specific classes of phospholipids was not significantly affected by shear stress.

Cells exposed to a steady arterial shear stress level of 25 dyn/cm^2 for 30 min produced approximately 4 times as much prostacyclin as $PGF_{2\alpha}$, while cells which were maintained in stationary conditions produced nearly equal amounts of these arachidonic acid metabolites.[43] In Figure 3, we show the effect of 30 min steady and pulsatile shear stress on the production of arachidonic acid metabolites by primary HUVEC that have been prelabeled for 3 days in stationary culture conditions with [1-^{14}C]arachidonic acid. The change in distribution of arachidonic acid metabolites formed by cells exposed

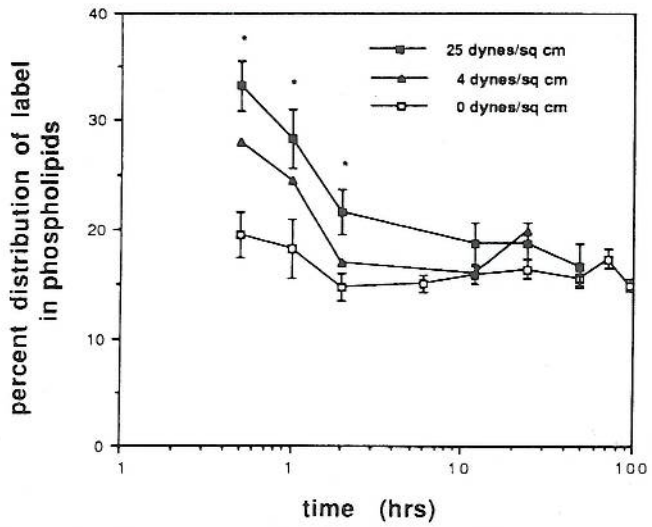

Figure 2. The time course for the uptake of labeled AA into the phosphatidylinositol fraction of HUVEC phospholipids. Cells were maintained in stationary culture or exposed to steady shear stresses of 25 or 4 dyn/cm^2. Each data point represents the average of three to six cord pools with matched slides for each level of stress. Error bars show one standard error of the mean. The asterisk signifies that cells exposed to 25 dyn/cm^2 incorporated significantly more labeled AA than cells maintained in stationary culture ($p < 0.05$ as determined by a t-test for paired samples).

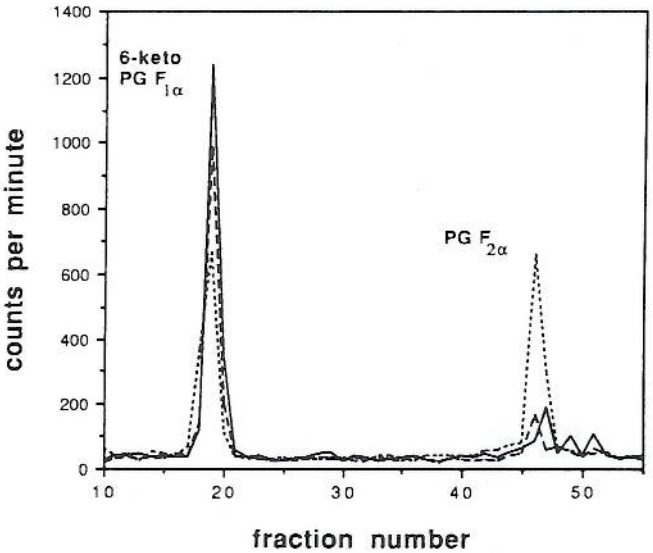

Figure 3. Typical HPLC chromatogram illustrating the production of AA metabolites by primary HUVEC exposed to a steady arterial level of shear stress (25 dyn/cm^2) and pulsatile stress (mean 12 dyn/cm^2 ±100% at 1 Hz) for 0.5 h. Cells were prelabeled for 3 days with [1-^{14}C]AA in stationary culture conditions and then exposed to flow for 0.5 h in complete medium without label. Peaks were identified by comparison with the retention times of labeled eicosanoid standards: (——) pulsatile; (– – – –) 25 dyn/cm^2; (·····) 0 dyn/cm^2. The retention time of 6-keto $PGF_{1\alpha}$, the stable hydrolysis product of prostacyclin, as determined by labeled standards was from fraction numbers 17–21 and of $PGF_{2\alpha}$ from fraction numbers 45–48.

to pulsatile shear stress (mean stress equals 12 dyn/cm^2 ±100% at 1 Hz) was similar to that formed by cells exposed to steady arterial shear stress levels.

The alterations in arachidonic acid metabolism by endothelial cells in response to the initiation of flow did not reflect a general perturbation of the cell membrane or arachidonic acid metabolism. Rather, there was a specific turnover of phosphatidylinositol-bound arachidonic acid which was selectively converted to PGI_2. Endothelial cells have a phosphatidylinositol-specific phospholipase C which is activated as one of the earliest steps in the response of the cell to external signals.[5] One possible interpretation of these results is that the increased turnover of phosphatidylinositol-bound arachidonic acid was due to the activation of this phospholipase C as an early step in the generation of intracellular second messengers in response to the initiation of flow. In order to test directly whether phospholipase C activation is occurring, we measured the level of a product of phospholipase C activity, inositol-1,4,5-trisphosphate (Ins1,4,5P_3).

Inositol-1,4,5-Trisphosphate Production

An elevated level of the intracellular second messenger Ins1,4,5P_3 has been identified as an early event in the response of endothelial cells to agonist stimulation.[7,35,49] Ins1,4,5P_3 is generated by the activation of a specific phospholipase C which breaks down the membrane phospholipid phosphatidylinositol-4,5 bisphosphate (PtIns4,5P_2).[5,41,53] The products of phospholipase C activity are diacylglycerol (DAG), which remains in the plasma membrane, and Ins1,4,5P_3, which is released into the cytosol. Ins1,4,5P_3 can bind to specific sites on the endoplasmic reticulum and release calcium from intracellular stores.[18] Elevated levels of intracellular calcium in conjunction with elevated levels of diacylglycerol in the plasma membrane may cause the translocation and activation of protein kinase C.[5]

Shear-stress-stimulated endothelial cells exhibited a rapid rise in Ins1,4,5P_3 level which peaked (2.1 ±0.2-fold stimulation) approximately 30 s (Fig. 4) after the initiation of flow (22 dyn/cm^2). After this initial peak, the level of Ins1,4,5P_3 remained significantly elevated for at least 6 min (1.6 ±0.3-fold stimulation) in cells exposed to shear stress of 22 dyn/cm^2. Monolayers exposed to a venous level of shear stress (2 dyn/cm^2) had a much smaller increase in intracellular Ins1,4,5P_3 concentration. Cells exposed to 10 μM of the inflammatory mediator histamine increased their Ins1,4,5P_3 level to 2.8 ±0.3 times the control value after 30 s exposure to the agonist, but by 4 min the Ins1,4,5P_3 level had returned to 1.3 ±0.2 times the control values.[42]

In contrast to the histamine-induced rise in Ins1,4,5P_3 concentration, the mechanical-stress-induced increase in Ins1,4,5P_3 remained elevated for several minutes after the increase in shear stress from 0.07 to 22 dyn/cm^2. This continued elevation of the Ins1,4,5P_3 level in response to a stimulus appears to be unique to shear stress stimulation since with other agonists such as histamine,[7,49] thrombin,[7,49] and bradykinin[35] the Ins1,4,5P_3 level returns to near basal levels within 2–4 min after exposure to the agonist. In fact, the time course of the response for the shear-stress-stimulated rise in Ins1,4,5P_3 level is quite similar to the time course of shear-stress-stimulated prostacyclin (PGI_2) synthesis.[22,23] After initiation of flow, PGI_2 synthesis exhibits a burst of production followed by a steady-state production rate which is lower than the initial burst but higher than the rate of PGI_2 production of cells maintained in stationary culture conditions.

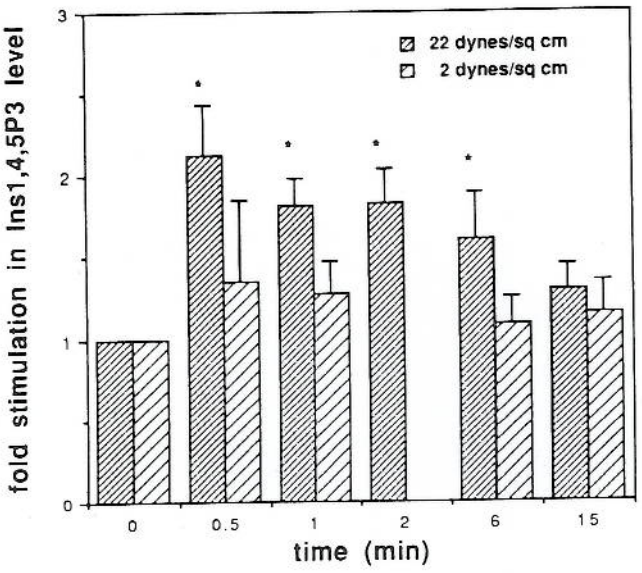

Figure 4. The intracellular level of Ins1,4,5P_3 in primary HUVEC exposed to shear stress of either 2 or 22 dyn/cm^2. Cells were exposed to 0.07 dyn/cm^2 for 30 min prior to the increase in shear stress. Each value represents the mean of results from three to six separate umbilical cord pools. The asterisk indicates that cells exposed to 22 dyn/cm^2 for 0.5, 1, 2, and 6 min had significantly greater Ins1,4,5P_3 levels ($p < 0.05$ by student's t-test for paired samples). No Ins1,4,5P_3 was detected in the media after exposure to cells.

Shear Stress Regulation of Protein Secretion by Endothelial Cells

Our studies of protein production by endothelial cells exposed to shear stress were carried out in experiments lasting over 24 h. Removal of small media samples (1 mL) from the total volume of 15 mL and replacement with fresh media were experimental requirements in order to study the time course of protein production by HUVEC monolayers. To determine the role of media dilution on cellular metabolism, stationary cultures were incubated in the batch mode (constant volume) without removal or addition of medium. Matched stationary cultures (with the same incubation volume) were maintained in a semibatch mode with 50% dilutions every 24 h. Addition of fresh medium had no effect on the production of tPA by HUVEC over a 3-day

period. Thus, complete M199 media did not stimulate or inhibit tPA production by confluent HUVEC. The continual release of tPA and PAI-1 and the absence of significant intracellular storage pools (<1.0 ng tPA_i/10^6 cells; <100 ng $PAI\text{-}1_i/10^6$ cells) indicated that the production of tPA or PAI-1 was not feedback inhibited.

During the first several hours after the onset of fluid flow, the levels of tPA in the circulating medium at all shear stress levels were the same as those of stationary control cultures (Fig. 5). Low shear stress (4 dyn/cm^2) had no effect on tPA secretion over the entire time course of the experiments. After 4–6 h of exposure to 15 or 25 dyn/cm^2, however, the level of tPA produced by shear-stressed cells exceeded that of controls (Fig. 5). The increase of tPA in the circulating medium was linear with time for over 20 h, allowing a least-squares fit to determine the steady-state secretion rate. Steady-state tPA secretion rates of cells exposed to 15 and 25 dyn/cm^2, normalized to matched controls, increased 2.06 ±0.39- ($n = 3; p < 0.015$) and 3.01 ±0.53- ($n = 3; p < 0.015$) fold over stationary cultures, respectively. The average steady-state secretion rate of tPA by HUVEC in control cultures was 0.168 ±0.053 ng tPA/10^6 cells h ($n = 3$).[14]

The secretion of tPA by control cultures was completely inhibited with cycloheximide (5 μg/mL), suggesting that new protein synthesis was required continually in order to maintain constitutive tPA release. This would indicate that the increase of tPA in the circulating medium was not due to release of intracellular stores of tPA. The greatly enhanced convective mass transport at low stresses of 4 dyn/cm^2 (compared to the natural convection of stationary cultures) had no effect on the production of tPA. Also, the cyclooxygenase inhibitor indomethacin (50 μM) had no effect on shear-stress-stimulated tPA production, indicating that the shear-enhanced tPA secretion was not mediated by a cyclooxygenase product.

Shear stress levels from 4 to 40 dyn/cm^2 caused no significant changes in the PAI-1 secretion rate relative to controls. The average steady state PAI-1 secretion rate of HUVEC in control cultures was 53 ±37 ng PAI-1/10^6 cells h ($n = 7$).[14] Measurements of PAI-1 production over the first 200 min after the onset of flow demonstrated that the PAI-1 secretion was not affected by shear stress transiently at early exposure times.

Primary HUVEC secrete about 15 ng endothelin per 10^6 cells in 24 h while in stationary culture. Secretion of endothelin was suppressed by laminar shear stress of 25 dyn/cm^2 within 4 h after the onset of flow (Fig. 6). The suppression of endothelin secretion by high shear stress (25 dyn/cm^2) continued for over 20 h during exposure to flow. In three separate experiments, endothelin production was suppressed only slightly (<15%) by low shear stress of 4 dyn/cm^2 compared to endothelin production of stationary controls. Similar results were obtained with Dulbecco's Modified Eagle (DME, Gibco) medium as the perfusion buffer (data not shown).

Genetic Expression in Endothelial Cells

To investigate the effect of laminar shear stress on mRNA levels in endothelial cells, replicate HUVEC monolayers were either maintained in stationary culture

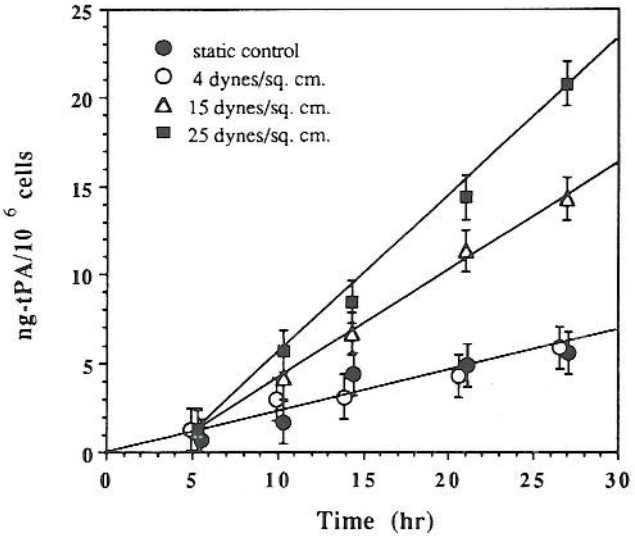

Figure 5. Cumulative production of tissue plasminogen activator (tPA) by replicate, primary confluent HUVEC monolayers maintained in stationary incubations (○) or exposed to steady laminar shear stress of 4 (●), 15 (△), or 25 dyn/cm^2 (■) using three independent flow systems. Each point is the average of triplicate ELISA determinations. Steady-state production rates were calculated for each monolayer by a least-squares fit of cumulative production between 4 and 24 h.

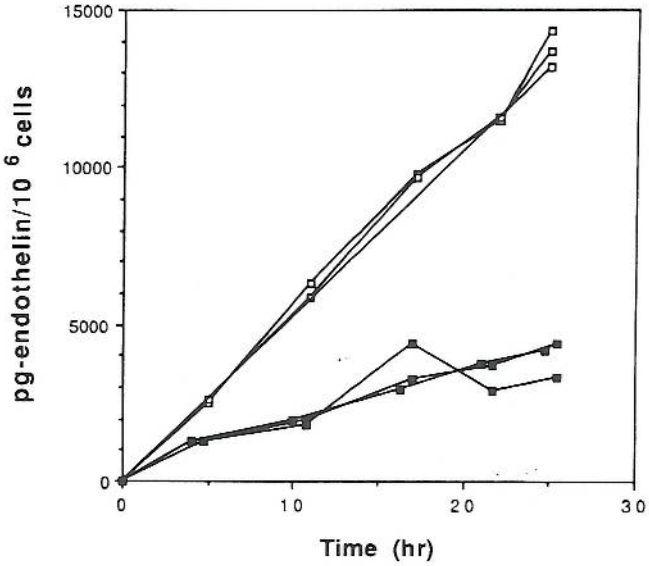

Figure 6. Cumulative production of endothelin by replicate, primary confluent HUVEC monolayers maintained in three separate stationary incubations (□) or exposed to steady laminar shear stress of 25 dyn/cm^2 (■) using three independent flow systems. Each point is the average of duplicate RIA determinations. Steady-state production rates were calculated for each monolayer by a least-squares fit of cumulative production between 4 and 24 h.

or exposed to shear stress at 25 dyn/cm^2 in individual flow systems. In each experiment, secretion of tPA, PAI-1, and endothelin (per 10^6 cells) was evaluated for the individual flow systems and the matched, stationary cultures. Changes in tPA production were observed after a lag time of several hours of exposure to shear stress (Table II) as was previously observed. Also, a reduction of endothelin secretion was found at early times in all monolayers exposed to shear stress (Table II). In each experiment, the quantity of total cellular RNA isolated from stationary and shear-stressed HUVEC cultures was not significantly different (average variation less than 10%).

Phenotypic drift is well documented in human endothelial cells during expansion of cell lines in culture.[16] We have used a coupled RT/PCR method to analyze mRNA levels in small numbers of primary human endothelial cells since Northern blotting techniques are difficult to implement with studies of only a few million cells. Using the RT/PCR method, we found that the tPA mRNA level was elevated in endothelial cells exposed to shear stress (Fig. 7, upper left). In contrast, when the same Southern blots were stripped and reprobed for the GAPDH amplification product, the transcript level of GAPDH was constant and independent of shear stress (Fig. 7, lower left). When this RNA from control and stressed monolayers was used in a similar coamplification experiment to study the effect of shear stress on levels for bFGF mRNA, no large increase in transcript levels such as that seen for tPA was noted for bFGF (Fig. 7, middle). Endothelin mRNA levels were sharply reduced in the endothelial cells exposed to 25 dyn/cm^2 for 24 h (Fig. 7, right). Again, no correspondingly sharp decrease was observed in signal strength for GAPDH product, indicating the uniformity of the coamplification reactions for bFGF and endothelin.

These findings were reproduced in an independent experiment and are unlikely to represent variation in PCR amplification efficiency because of (i) the similar cell numbers in, and RNA amounts extracted from, static and flow-subjected cultures, (ii) the comparative constancy of coamplified GAPDH transcript levels observed under different flow conditions, and (iii) the observation of increased tPA mRNA levels and increased tPA protein secretion rates detected by the RT/PCR technique in the same RNA and medium samples which showed decreased endothelin secretion and decreased endothelin mRNA levels.

Flow-Induced Cytostolic Calcium Changes

The increase of the intracellular calcium ion concentration ($[Ca^{2+}]_i$) in individual primary HUVEC with the onset of flow with M199, which contains 1 μM ATP is shown in Figure 8. This figure shows a time sequence (5–6 s between each picture) of pseudocolor images of the fluorescence ratio for the calcium sensitive dye fura-2. Flow (wall shear stress equal to 15 dyn/cm^2) was started just after the first image was acquired. Only a fraction of the cells, approximately 15–20%, responded to the increased flow rate by elevating intracellular calcium levels. The calcium level did not change when the flow was stopped, and the same cells responded when the flow was restarted. None of the cells exhibited an increase in $[Ca^{2+}]_i$ with the onset of flow if the perfusion buffer contained no ATP. The heterogeneity of the response was not due to differences in the resting levels of calcium between cells since, as shown in Figure 8a, the initial $[Ca^{2+}]_i$ was very uniform. In some cases, cells in close proximity had a coordinated response; however, in other instances neighboring cells did not respond in unison. The magnitude of the calcium response in primary HUVEC is strikingly less than that seen in multipassaged bovine aortic cells.[4,39] The time course of each responding cell was not identical. The maximum elevation in calcium occurred 5–10 s after the initiation of flow, and within 20 s the calcium concentration had

Table II. Secretion rate of tissue plasminogen activator (tPA), plasminogen activator inhibitor, type-1 (PAI-1), and endothelin (ET) by HUVEC exposed to laminar shear stress.

Experiment[a]	Cell density (10^4 cells/cm^2)	tPA secretion rate (ng tPA/10^6 cells h)	PAI-1 secretion rate (ng PAI-1/10^6 cells h)	ET secretion rate (ng ET/10^6 cells h)
1. Control	7.7 ±0.8	0.170 ±0.049 (n = 3)	70.2 ±4.97 (n = 3)	0.545 ±0.017 (n = 3)
25 dyn/cm^2	7.1 ±0.9	0.399 ±0.087 (n = 3)	47.8 ±13.6 (n = 3)	0.132 ±0.026 (n = 3)
2. Control	6.7 ±0.8	0.160 ±0.039 (n = 3)	49.3 ±3.25 (n = 3)	0.485 ±0.025 (n = 3)
25 dyn/cm^2	7.3 ±0.8	0.527 ±0.123 (n = 3)	26.5 ±3.04 (n = 3)	0.279 ±0.055 (n = 3)
3. Control	5.9 ±1.0	0.122 ±0.013 (n = 3)	53.0 ±8.71 (n = 3)	0.587 ±0.057 (n = 3)
25 dyn/cm^2	6.2 ±0.9	0.387 ±0.073 (n = 2)	58.1 ±7.60 (n = 2)	0.335 ±0.061 (n = 2)
Mean rates ±SD				
Control		0.157 ±0.036 (n = 9)	57.2 ±10.6 (n = 9)	0.549 ±0.046 (n = 9)
25 dyn/cm^2		0.453 ±0.108 (n = 8)[b]	42.6 ±15.5 (n = 8)[c]	0.239 ±0.099 (n = 8)[b]

Note: Protein secretion rates were determined by a least-squares fit of antigen production data between 6 and 24 h. ELISAs were used to quantify tPA and PAI-1 production, while endothelin production was evaluated using a RIA. Analysis of mRNA levels for experiment 1 is shown in Fig. 7.

[a] Each experiment was conducted with an independent pool of primary HUVEC.

[b] $p < 0.001$.

[c] Not significant.

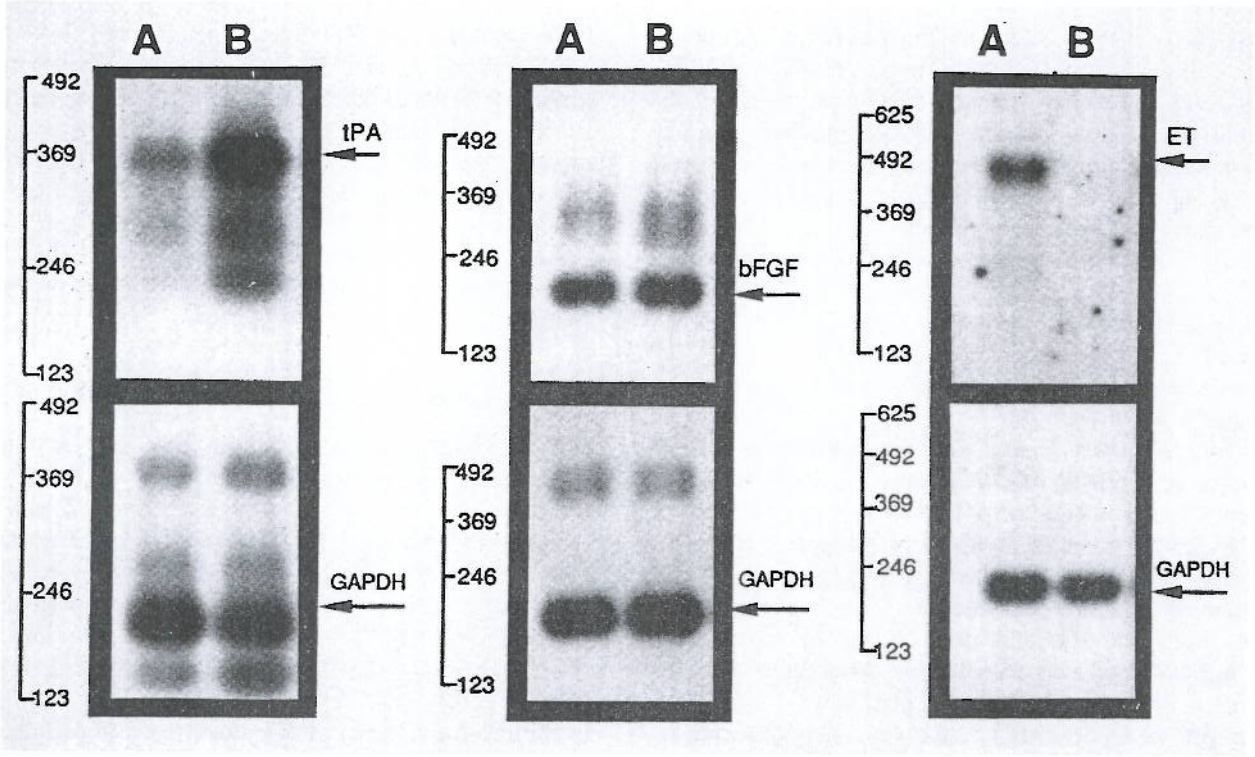

Figure 7. Analysis of mRNA levels for tissue plasminogen activator (tPA; left), basic fibroblast growth factor (bFGF; middle), endothelin (ET; right), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; bottom) using a coupled RT/PCR technique. In this experiment, total cellular RNA was isolated from stationary controls (A) or from monolayers exposed to shear stress of 25 dyn/cm^2 (B). In each PCR amplification used to study each mRNA species, GAPDH mRNA was coamplified as an internal control (bottom). Protein secretion data for this experiment is given in experiment 1 of Table II.

returned to basal levels. This time course was substantially different from the time course which has been reported for other agonists, such as histamine, thrombin, and bradykinin. With these agonists, the initial peak rise in calcium concentration is followed by a decline in $[Ca^{2+}]_i$ to a new level which is significantly higher than the initial resting calcium level.

Modeling of Solute Concentration Profiles

In order to determine if the ATP concentration near the cell surface could be dramatically altered by increasing the flow rate in the regime of physiological interest, a model was developed for the convective mass transport in our parallel-plate flow chamber. The concentration profile is governed by the convection-diffusion equation, which takes into account the convection of ATP into the flow chamber due to the flowing medium and the diffusion of ATP normal to the direction of flow. The reaction of ATP on the endothelial cell surface is assumed to proceed with first-order kinetics with a surface reaction rate constant k. A mass balance on the species of interest yields the following differential equation for the concentration c:

$$6(y^* - y^{*2})\frac{\partial c^*}{\partial x^*} = \frac{\partial^2 c^*}{\partial y^{*2}} \tag{1}$$

with the associated boundary conditions written in dimensionless form as

$$\frac{\partial c^*}{\partial y^*} = 0 \quad \text{at } y^* = 0 \tag{2a}$$

$$\frac{\partial c^*}{\partial y^*} = -\frac{ka}{D}c^* \quad \text{at } y^* = 1 \tag{2b}$$

$$c^* = 1 \quad \text{at } x^* = 0 \tag{2c}$$

where a is the height of the flow chamber (0.02 cm) and D is the diffusion coefficient for ATP (approximately 2.6×10^{-6} cm^2/s). The concentration is made dimensionless with respect to the inlet concentration y, the transverse coordinate is made dimensionless with respect to a, and x, the axial coordinate, is made dimensionless as $x^* = xD/ua^2$. Here we have assumed steady-state, two-dimensional laminar flow between parallel flat plates and have neglected axial diffusion. The first boundary condition reflects the fact that there can be no mass flux on the nonreactive wall of the flow chamber. The second boundary condition describes the rate of reaction on the cell surface. The dimensionless parameter ka/D is in the form of a Sherwood number. Coade and Pearson[9] and Pearson and Gordon[46] measured the rate of degradation of radiolabeled ATP by HUVEC, and from their data we can estimate ka/D to be approxi-

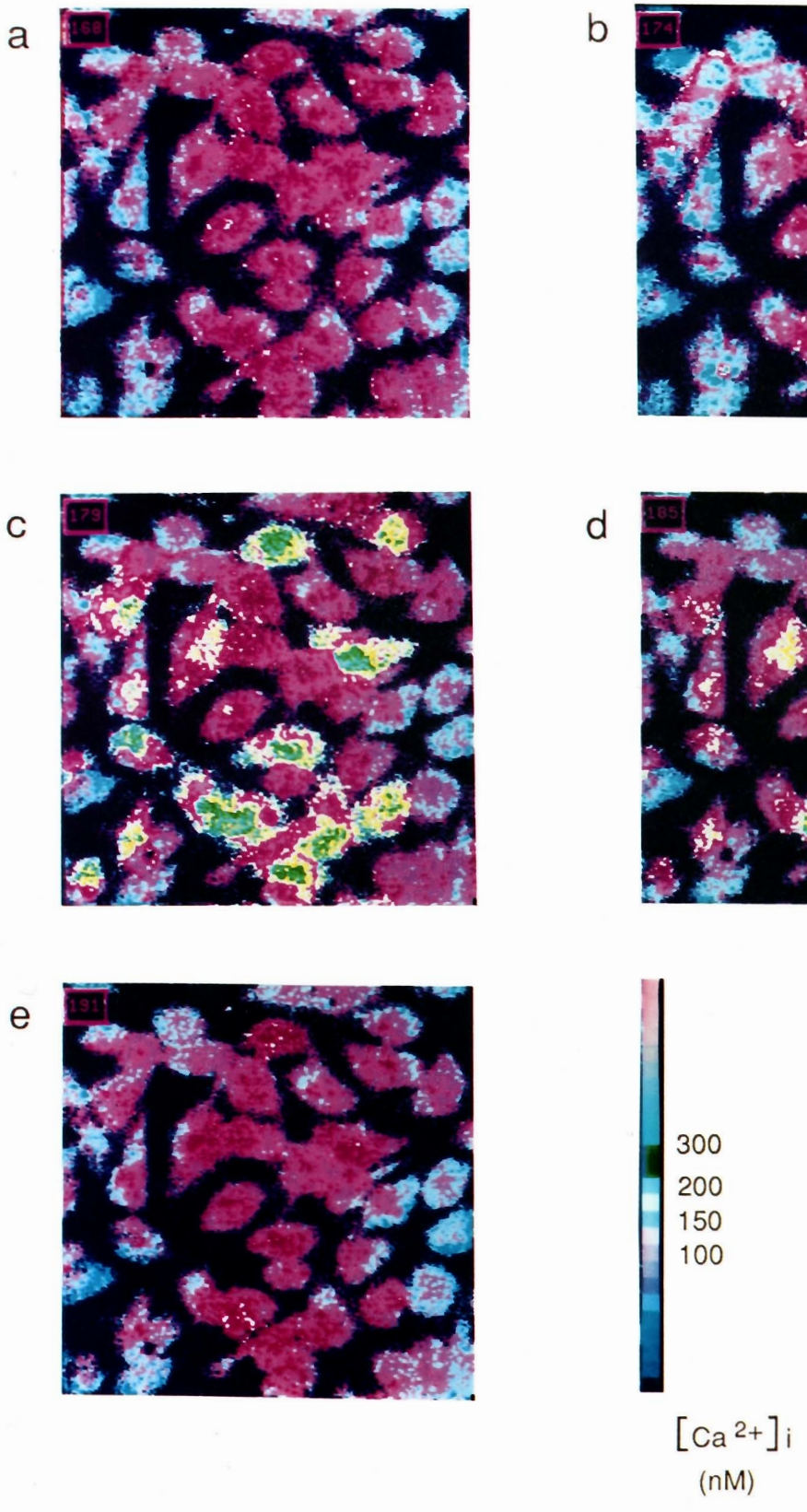

Figure 8. A time sequence of pseudocolor images of the fluorescence ratio from the calcium-sensitive dye fura-2 in primary HUVEC exposed to the initiation of flow with media containing 1 μM ATP. Flow, at 15 dyn/cm^2, was started just after image a was acquired. The number in the upper left corner of each image shows the elapsed time in seconds. The color bar (lower right) indicates the calibration for the intracellular calcium concentration as determined by the fluorescence ratios obtained from standard calcium solutions containing fura-2.

mately 1.0. However, they may underestimate the reaction rate since their experiment was performed under static conditions and thus may be diffusion limited. The final boundary condition indicates that the ATP is uniformly distributed at the inlet of the flow chamber.

The solution to a closely related problem has been obtained by Grimsrud and Babb[29] and Colton et al.[11] for moderate and large x^*. We have modified their solution technique for this problem and have solved Equation (1) and the associated boundary conditions with a Crank-Nicholson finite-difference method to obtain the concentration profile for small x^*.[44] The concentration of ATP on the endothelial cell surface 2 cm from the inlet is shown in Figure 9 as a function of the wall shear stress in the parallel-plate flow chamber used in our experiments (see Materials and Methods for details about the flow chamber). At very low shear stress, which implies very low flow rates, there is essentially no ATP near the cell surface. The rate of reaction on the cell wall, which degrades ATP, is faster than convection/diffusion of fresh ATP from upstream. At high shear stress (corresponding to high flow rates), convection of ATP exceeds the consumption of ATP by reaction, and the concentration of ATP near the cell surface is nearly equal to the inlet concentration. This model demonstrates that the calcium response of cells exposed to the initiation of flow with media containing ATP may be explained by the cells responding to the increased local concentration of ATP.

The concentration profile for other substances metabolized by endothelial cells can be determined in a similar manner. In a bioreactor, oxygen transport limitations can be a serious constraint. In our experiments, however, the use of 1 mL media/cm^2 monolayer (50,000–100,000 cells/cm^2) is 10–20 times the media volume typically used to grow exponential phase mammalian cells in a bioreactor. Oxygen consumption by contact-inhibited, confluent HUVEC is expected to be much less than the consumption rate of 0.04–0.4 mmol $O_2/10^9$ cells h for exponential phase mammalian cells.[21] Circulating medium (average residence time in chamber <2 s) is well oxygenated (0.2 mM O_2). Thus, the delivery of oxygen to the monolayer surface exceeds by several orders of magnitude the consumption of oxygen which is estimated to be less than 9×10^{-10} mmol O_2/cm^2 s. The dimensionless parameter ka/D is less than 0.003 for oxygen, resulting in concentrations near the cell surface which are greater than 95% of the bulk O_2 concentration for wall shear stresses $>10^{-3}$ dyn/cm^2. Consequently, there are no O_2 mass transfer limitations in our parallel-plate flow system.

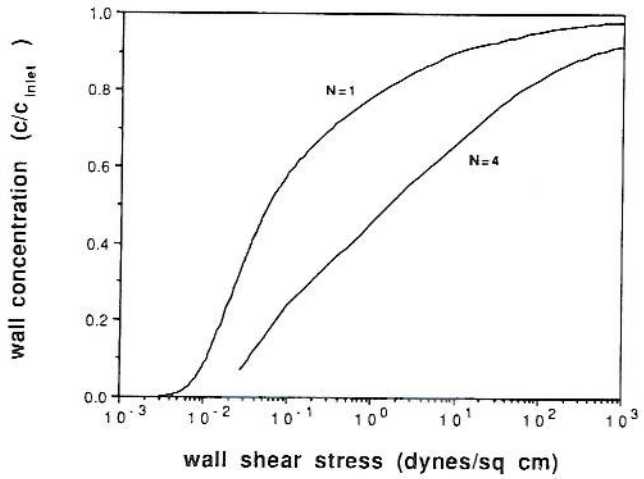

Figure 9. The steady-state concentration of ATP in the parallel-plate flow system at the endothelial cell surface is shown as a function of the wall shear stress. The axial position is fixed at 2 cm from the inlet of the flow chamber. The curves were obtained by solving Equation (1) and the associated boundary conditions for two values of the dimensionless first-order rate constant ka/D. The solution was obtained using a Crank-Nicholson finite-difference scheme with $\Delta y^* = 0.025$ and $10^{-6} < \Delta x^* < 10^{-3}$.

DISCUSSION

The production of many important bioactive substances by endothelial cells is affected by the fluid mechanical environment in which the cells reside. Arterial shear stress levels on the order of 25 dyn/cm^2 cause enhanced arachidonic acid metabolism and altered protein synthesis. The effects of altered flows on a number of functions of cultured endothelial cells are summarized in Table III. This table demonstrates that the response of the cells to flow does not reflect a general perturbation of the metabolism of the cells; rather there is selective modulation of certain metabolic pathways. In the case of protein synthesis, there is increased production of tPA, but not of PAI-1, and suppression of endothelin production. The uptake of arachidonic acid into intracellular stores is enhanced only for the phosphatidylinositol fraction. Also, there is selective enhancement of PGI_2 production relative to $PGF_{2\alpha}$.

The alteration in the metabolism of the cell in response to the initiation of flow can be characterized in terms of the time scale of the response. The most rapid changes we have identified occur on the order of seconds and involve the generation of the second messengers Ins1,4,5P_3 and $[Ca^{2+}]_i$. Increased levels of these second messengers result in the activation of specific metabolic pathways which are detectable on the order of minutes. On this time scale of minutes, we have observed increased uptake of arachidonic acid into phosphatidylinositol and increased synthesis of PGI_2. Shear stress may also activate certain protein kinases, such as protein kinase C, which have been implicated in regulating gene expression. Over a time period of 4–24 h, we find alterations in protein synthesis as well as changes in mRNA levels, indicating that there is altered genetic expression in cells exposed to shear stress. On a much longer time scale of 2–3 days, we and others (see Table III) have observed changes in HUVEC morphology and in the arrangement of the cytoskeleton.

Table III. Effect of arterial flows on endothelial cell processes.

Process	Effect	Time course	Ref.
General functions			
Morphology	alignment and elongation	24–48 h	19
Actin stress fibers	formation and alignment	3 h	24
Pinocytosis	increased	2 h	12
LDL uptake	slight increase	24 h	57
Cation channel	activated	seconds	45
DNA synthesis	no effect (if laminar flow)		13
DNA synthesis	stimulated (if turbulent flow)		13
Protein synthesis and secretion			
tPA	increase	4–24 h	14
Endothelin	decrease	24 h	55
PAI-1	no effect		14
Fibronectin	slight decrease		55
mRNA levels			
tPA	increase	24 h	15
Endothelin	decrease	24 h	55
bFGF	no effect	24 h	15
Glyceraldehyde 3-phosphate dehydrogenase	no effect	24 h	55
Arachidonic acid metabolism			
PGI_2 synthesis	large increase	5 min	22, 28
	increase	2–8 h	22, 28
$PGF_{2\alpha}$ synthesis	decrease	30 min	43
Arachidonate uptake	increase for phosphatidylinositol	0.5–4 h	43
Second messengers			
Inositol trisphosphate	increase	30 s	42
Intracellular calcium	increase (ATP mediated)	<5 s	17, 39, 44
cAMP	increase (PGI_2 mediated)		52
Diacylglycerol	increase		6

The mechanism by which the endothelial cells detect the presence of flow and convert the mechanical stress into an intracellular response, including second-messenger generation, protein activation, and modulation of genetic expression, remains poorly understood. Many agonists act on endothelial cells by causing increases in $[Ca^{2+}]_i$.[1,20] Increased levels of cytosolic calcium cause the activation of certain phospholipases and the translocation and activation of protein kinase C. Also, Ca^{2+} binds to the calcium-specific binding protein calmodulin. The Ca^{2+}–calmodulin complex activates calmodulin-dependent protein kinases leading to protein phosphorylation and altered cellular function. The resting $[Ca^{2+}]_i$ in primary HUVEC is typically 100 nM,[59] which is several orders of magnitude lower than the extracellular calcium concentration (generally in the range of 1–3 mM). The cells are able to maintain this large concentration gradient by means of calcium-specific pumps in the plasma membrane. A two- to threefold increase in $[Ca^{2+}]_i$ above this resting level of 100 nM is sufficient to cause activation of calcium-sensitive pathways. This increased level of calcium comes from two sources: release from intracellular stores and extracellular influx. Calcium is stored internally in the cell in the endoplasmic reticulum from which it can be released by elevated levels of second messengers, such as Ins1,4,5P_3.[5] Extracellular calcium can enter the cell through divalent cation channels in the plasma membrane. The opening of such channels is often regulated by the electrochemical voltage difference between the inside and outside of the cell or by specific regulatory proteins. Also, Lansman et al.[37] have identified a stretch-activated cation channel in endothelial cells.

Recently, Ando et al.[4] have investigated the calcium response of bovine aortic endothelial cells exposed to shear stress in a parallel-plate flow chamber. However, the media they used (M199, Gibco) to perfuse the cells contained micromolar quantities of ATP. ATP binds to a specific receptor on the cell surface and can elicit a calcium response when present at levels in the range of 1–10 μM.[9] Furthermore, endothelial cells have an ecto-enzyme on their plasma membrane which sequentially dephosphorylates ATP to ADP and then to AMP.[47] If the activity of this enzyme is sufficiently large, then the concentration of ATP on the cell surface can be substantially lower than the bulk concentration, particularly under low-flow or static conditions, due to diffusional limitations. The calcium response that Ando et al. observed may be due entirely to the enhanced convective mass transfer of ATP to the endothelial cell surface when the flow is initiated. Other workers[17,39] using media that does not contain ATP have not observed any change in $[Ca^{2+}]_i$ with increases in shear stress in bovine aortic endothelial cells, and we have not seen any $[Ca^{2+}]_i$ changes in HUVEC. When ATP is added back to the perfusing media, these investigators do observe an increase in $[Ca^{2+}]_i$ with the onset of flow and a decrease in $[Ca^{2+}]_i$ upon the cessation of flow. The

decrease in $[Ca^{2+}]_i$ upon cessation of flow is consistent with the rapid metabolism of ATP on the cell surface.

Even in the presence of ATP, the calcium response of primary HUVEC is small. Only 15–20% of the cells respond, and the response of these cells is not maximal. This suggests that calcium is not the most important second messenger in shear-stress-stimulated endothelial cells. Other second messengers, such as IP_3 or other intracellular ions (H^+, e.g.) may be involved. There is some evidence that shear stress causes changes in the electropotential gradient across the plasma membrane. Olesen et al.[45] showed that bovine aortic endothelial cells hyperpolarize (the cell interior becomes more negative relative to the exterior) by activating a potassium channel in response to physiological levels of shear stress. Also, Alveridou et al.[3] demonstrated that the initiation of flow causes the activation of a calcium-sensitive potassium channel in calf pulmonary artery endothelial cells. This stress-activated response can be partially blocked by a specific inhibitor, tetrabutylammonium ion (TBA), of the calcium-activated potassium channel, yet parallel studies showed no concurrent rise in intracellular calcium levels.[17,39,44] One possible interpretation which can reconcile these observations is that shear-stress-induced membrane alterations may cause a change in the affinity for calcium of the G proteins which regulate the potassium channel. It has been shown that the calcium-dependent potassium channel of smooth muscle cells can be activated at a constant calcium concentration.[50,58] Thus, there could be up-regulation of calcium-sensitive enzymes without any direct corresponding increase in bulk cytosolic calcium concentration.

Pulsatile fluid flow (±20% amplitude; 1 Hz) increases PGI_2 production even more than the enhancement by equivalent mean values of steady shear stress.[22] Davies et al.[12] found that 1-Hz oscillations in flow had no effect on fluid phase endocytosis, while step changes in flow every 15 min enhanced endocytosis. Similarly, we found that 1-Hz pulsatile shear stress had the same effect as equivalent levels of steady shear stress on the secretion of tPA, PAI-1, and endothelin.[14,15,55] The most rapid fluctuations in the magnitude and direction of shear stress can be found in turbulent flows. Turbulent flow with low levels of mean shear stress (<1 dyn/cm^2) can stimulate quiescent monolayers of bovine aortic endothelial cells into mitotic division while much higher levels of unidirectional, steady laminar shear stress have no effect on the mitotic state of these cells.[13] It should be noted, however, that turbulent flows are not commonly found under normal physiologic conditions, although unsteady secondary flows are common.

The experiments which described the flow-induced burst in arachidonic acid metabolism[22,23,43] were performed with media containing 1 μM ATP. Other workers[28] have noted a similar shear-stress-induced burst in prostacyclin production in endothelial cells exposed to flow with media that does not contain ATP (RPMI-1640). Furthermore, the results of Frangos et al.[22,23] (using media which does contain ATP) demonstrate that the steady-state production rate of prostacyclin depends on the shear stress in a dose-dependent manner, even after several hours exposure to flow, by which time the cells have depleted the ATP in the medium. Also, pulsatile flow which has the equivalent mean shear stress and mass transport as steady flow causes over a twofold increase in prostacyclin production.[22] ATP stimulation of endothelial cells is mediated by a signal transduction system which involves both release of calcium from intracellular stores and increased levels of inositol trisphosphate. It is not clear whether all of the increase in inositol trisphosphate levels can be attributed to convection of ATP since the large, flow-induced changes in inositol trisphosphate levels we observed[42] are not consistent with the relatively small increase in $[Ca^{2+}]_i$ (Fig. 8).

At low levels of shear stress (4 dyn/cm^2), the ATP concentration at the cell surface is much greater than in a stationary culture (Fig. 9). However, low shear stress of 4 dyn/cm^2 causes no significant change in tPA secretion, indicating that the increased shear forces of 15 and 25 dyn/cm^2 are responsible for inducing tPA expression rather than the increased concentration of ATP. Also, suppression of endothelin secretion by shear stress of 25 dyn/cm^2 is not observed at low shear stress.[55] Furthermore, endothelin secretion is also suppressed when ATP-free medium (DME) is used as the perfusion buffer. These observations indicate that the induction of tPA expression and the suppression of endothelin expression are predominantly mediated by laminar shear stress and not by enhanced convective mass transport.

Through expressed metabolites, the endothelium maintains a dynamic balance between activation and inhibition of blood coagulation events. The endothelium is the principal source of tPA found in the blood stream. The tPA gene and PAI-1 gene can be coordinately expressed or independently expressed, depending on the hormonal agonist. Shear stress induces the expression of tPA without stimulation of PAI-1 expression. This is distinct from thrombin, basic fibroblast growth factor, or interleukin-1 stimulation of endothelial cells, all of which increase PAI-1 secretion. The enhanced expression of tPA by endothelial cells exposed to shear stress may contribute to the nonthrombogenic nature of the cell surface. In zones of high shear stress in the cardiovasculature, this enhanced expression of tPA may protect the vessel wall from shear-induced platelet activation with thrombin generation and subsequent fibrin deposition. Additionally, enhanced fibrinolytic capacity in high shear zones may protect against plaque development.[32]

Atherosclerotic plaques in humans are localized in low-shear-stress zones adjacent to bifurcation vessels and rarely found in the high-shear zones of the flow divider.[27,33] In humans, excessive smooth muscle cell proliferation (termed *intimal hyperplasia*) and wall

thickening are inversely related to local fluid shear rates.[27,33] These observations have been ascribed previously to the prolongation in low-flow regimes of the "residence time" of platelets near the blood/vessel wall interface and possible release of platelet-derived growth factors. In regions of high flow, the concentrations of platelet-released products are reduced by convective mass transport. Our findings suggest an additional mechanism whereby reductions in intimal thickening in high-stress regions might arise from direct local suppression of endothelial cell expression of the smooth muscle cell mitogen, endothelin. Blood vessel walls exposed to high flow may have enhanced PGI_2 production,[22] enhanced fibrinolytic capacity,[14] and reduced endothelin expression, thus increasing the resistance of high-shear zones to fibrin deposition on the vessel wall and platelet-dependent and platelet-independent smooth muscle cell proliferation. That endothelial cells produce less of the matrix protein fibronectin under high flow[31] is also consistent with the in vivo finding of reduced vessel wall thickening in high-flow regions of vessels.

In addition to its mitogenicity, endothelin is an extremely potent vasoconstrictor. Suppression of endothelin release by fluid shear stress may explain the in vivo response of blood vessels to varying shear rates. The observation that blood vessels dilate in response to high flow[54] and constrict in response to decreased flow[36] might be explained by shear stress modulation of endothelin release. Similarly, dilation of diseased vessels to maintain cross-sectional area in compensation for plaque enlargement[26] may represent suppressed endothelin production in response to increased blood velocities. Our data suggest that flow-induced control of endothelin release may cooperate in a reinforcing fashion with the known release of EDRF[54] and prostacyclin[22] induced by flow.

The endothelial cell has been proposed as an ideal vector for human gene therapy since recombinant proteins can be secreted directly into the blood stream.[60] The seeding of vascular stents and grafts with endothelial cells is a well-studied technology. Several workers using animal models have successfully engineered endothelial cells to express foreign proteins (β-galactosidase) in vivo.[40] The efficacy of gene therapy using engineered endothelial cells may be affected by the intrinsic response of endothelial cells to fluid mechanical forces. The engineering analysis of cellular function and metabolism is particularly important when the hydrodynamics of blood flow may play a critical role in the success of the genetically modified tissue.

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