number of insect species in which the adult is the pest (*13*). Agents antagonizing JH, or stopping its biosynthesis, would be useful in controlling crop-destroying insect larvae (*14*). Knowledge of the parameters that control JH biosynthesis would assist researchers in the design of chemicals that inhibit JH biosynthesis. Access to synthetic AT should allow physiological and biochemical delineation of many of the factors controlling this vital process.

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Fluid Flow Stimulates Tissue Plasminogen Activator Secretion by Cultured Human Endothelial Cells

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Wall shear stress generated by blood flow may regulate the expression of fibrinolytic proteins by endothelial cells. Tissue plasminogen activator (tPA) and plasminogen activator inhibitor, type 1 (PAI-1) secretion by cultured human endothelial cells were not affected by exposure to venous shear stress (4 dynes/cm^2). However, at arterial shear stresses of 15 and 25 dynes/cm^2, the tPA secretion rate was 2.1 and 3.0 times greater, respectively, than the basal tPA secretion rate. PAI-1 secretion was unaffected by shear stress over the entire physiological range.

HEMODYNAMIC FORCES INFLUence several endothelial cell functions. Alignment of cells and stress fibers in the direction of fluid flow has been shown in vivo and in vitro (*1*). These morphological effects, as well as the correlation of atherosclerosis and thrombosis with regions of disturbed blood flow, suggest that shear stress may modulate the production of endothelial cell–derived products and directly affect endothelial cell function (*2*). Prostacyclin (PGI_2) production increases several-fold with the onset of laminar shear stress. Pulsatile shear stress stimulates an even greater production of PGI_2 than steady shear stress (*3, 4*). Shear stress may increase low density lipoprotein uptake, fluid endocytotic rate, and histamine-forming capacity, and induce a potassium (K^+) current (*5*).

The endothelial cell, the primary source of tPA in the blood, also secretes several plasminogen activator inhibitors that constitute about 10% of the total secreted protein of the endothelial cell in culture (*6*). Rapid increases in fibrinolytic activity and tPA antigen in the blood during venous occlusion and during exercise have been associated with release of endothelial stores of tPA (*7*). Endothelial cells in culture, however, maintain very low antigenic levels of tPA and PAI-1 intracellularly, since most (>90%) of the tPA and PAI-1 antigen synthesized is secreted (*8*).

To investigate the role of hemodynamic forces on vascular fibrinolytic mediators, we harvested human umbilical vein endothelial cells (HUVEC) by the method of Gimbrone (*9*) and seeded them on glass slides. After 72 to 86 hours, replicate primary, confluent monolayers (4.0×10^4 to 7.0×10^4 cells per square centimeter) were exposed to steady shear stresses of 4, 15, or 25 dynes/cm^2 in individual parallel-plate flow chamber systems with recirculating medium driven by a constant hydrostatic head (*10*). Replicate monolayers were also incubated under stationary conditions. Samples (1 ml) from the circulating medium were frozen at −80°C and assayed for total tPA (uncomplexed and inhibitor-bound) and uncomplexed PAI-1 (latent and active) in triplicate with separate enzyme-linked immunosorbent assays (ELISAs) (*11*). At the end of the experiment, trypan blue–excluding cells were counted by hemacytometer. No changes in cell density, cell shape, or cell volume (1800 ± 100 fL) were observed relative to controls.

During the first 4 to 6 hours after the onset of shear stress, the levels of tPA in the circulating medium at all shear stress levels were the same as those of static control cultures (Fig. 1). Low shear stress (4 dynes/cm^2) had no effect on tPA secretion during the entire time course of the experiment. After longer exposure to 15 or 25 dynes/cm^2, however, the level of tPA produced by sheared cells exceeded that of controls. The increase of tPA in the circulating medium was linear with time for more than 20 hours, allowing a least-squares fit to determine the steady-state secretion rate. Steady-state tPA secretion rates of cells exposed to 15 and 25 dynes/cm^2, normalized to matched controls, were 2.06 ± 0.39 and 3.01 ± 0.53 times that of static cultures, respectively (Table 1). The average secretion rate of tPA by HUVEC in control cultures was 0.168 ± 0.053 ng of tPA per 10^6 cells per hour (n = 3).

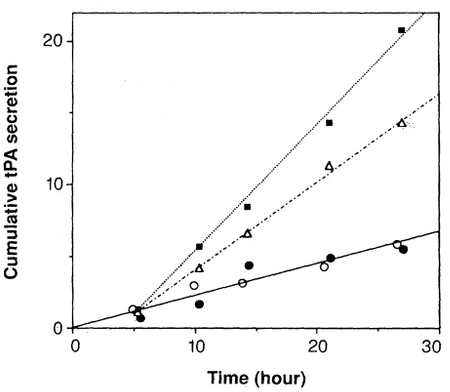

Fig. 1. Cumulative secretion (nanograms of tPA per 10^6 cells) of total tPA (uncomplexed and inhibitor-bound) by replicate, primary confluent HUVEC monolayers (6.0×10^4 cells per square centimeter; 1 ml of media per square centimeter of monolayer) maintained in static culture (●) or exposed to steady laminar shear stresses of 4 (○), 15 (△), and 25 (■) dynes/cm^2. Each point is the average of triplicate ELISA determinations. No tPA antigen was detected in the media before exposure to HUVEC. The steady-state tPA secretion rate was determined by means of a least-squares fit of cumulative secretion between 4 and 27 hours.

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Preliminary measurements indicate that pulsatile shear stress (1 Hz) does not potentiate the response. This is distinct from pulsatile stress effects on HUVEC PGI_2 synthesis (*3*) but similar to results on endocytosis (*5*). PGI_2 synthesis is probably modulated by diffusion processes over lengths of several microns with a time scale of a fraction of a second, while protein synthesis requires minutes or hours and may not be responsive to stress transients on the order of one second.

Secretion of uncomplexed PAI-1 antigen into the culture medium by controls as well as shear stressed cells was linear with time. Shear stress from 4 to 40 dynes/cm^2 caused no significant changes in the PAI-1 secretion rate relative to controls (Fig. 2). The average PAI-1 secretion rate of HUVEC in control cultures was 53 ± 37 ng of PAI-1 per 10^6 cells per hour (n = 7).

The intracellular concentrations of tPA and PAI-1 in sonicated lysates of control cells were very low (<1.0 ng of tPA per 10^6 cells and <100 ng of PAI-1 per 10^6 cells, respectively; n = 3) compared to the amount of antigen secreted over the time course of the experiments. Lactate dehydrogenase activity in the circulating media was no higher under shear stress than in the controls (<5% lysis) (*12*). Thus, the increase of tPA in the circulating medium was not due to release of intracellular stores of tPA caused by cell lysis. Furthermore, the inhibition of tPA secretion in control cultures with cycloheximide (5 μg/ml) suggested that new protein synthesis was continually required in order to maintain constitutive tPA release.

The possibility that a released intracellular component, secreted protein, or stable metabolite stimulated tPA secretion during exposure to shear stress was investigated. Fresh medium, medium from control cultures, and medium from flow systems (running at 4 dynes/cm^2 and 25 dynes/cm^2 for 6 and 12 hours) were placed on washed, primary HUVEC monolayers and incubated for 12 hours under stationary conditions. None of these media stimulated or inhibited tPA secretion compared to fresh medium. Thus, an altered level of a stable agent (for example, histamine, through histidine decarboxylase activity) in the circulating medium was not responsible for stimulating tPA secretion during exposure to shear stress.

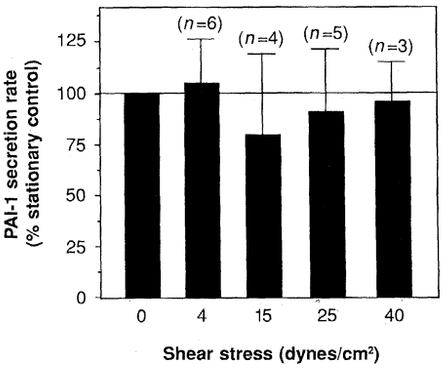

Fig. 2. Steady-state PAI-1 secretion rates (with SD) of sheared cultures were calculated from PAI-1 production between 4 and 27 hours and normalized by the PAI-1 secretion rate of matched, stationary cultures. The average PAI-1 secretion rates at venous (4 dynes/cm^2) and arterial levels of shear stress (at 15, 25, and 40 dynes/cm^2) were not significantly different from that of stationary controls. The average basal PAI-1 secretion rate of static cultures was 53 ± 37 ng of PAI-1 per 10^6 cells per hour (n = 7).

Cyclooxygenase activity is not required for tPA secretion by HUVEC (*13*). Since it is known that shear stress causes a large PGI_2 release, it was necessary to show that PGI_2 or other arachidonic acid metabolites do not stimulate tPA synthesis and secretion. PGI_2 increases intracellular adenosine 3′,5′-monophosphate (cAMP) levels in endothelial cells (*14*). Raising cAMP levels with Br-cAMP does not increase tPA secretion (*15*). Similarly, tPA secretion did not increase when HUVEC were incubated with the stable PGI_2 analog Iloprost (85 ng/ml) plus the cAMP-phosphodiesterase inhibitor 3-isobutyl 1-methylxanthine (0.5 mM) (IBMX). IBMX alone reduced tPA secretion, whereas L-epinephrine alone (50 to 500 μM) had no effect on tPA secretion. Incubation of HUVEC in indomethacin (50 μM) for 30 min before exposure to shear stress (with indomethacin in the circulating medium) had no effect on shear stress–stimulated tPA production. Indomethacin had no effect on tPA secretion by stationary cultures or PAI-1 secretion by control or sheared cultures. Thus, increased tPA secretion at arterial levels of shear stress was not mediated by a cyclooxygenase product.

Our measurements of tPA and PAI-1 secretion rates by primary HUVEC under static conditions were consistent with those of others (*8, 13, 16, 17*). The amount of PAI-1 secreted was 100 to 300 times as high as the amount of tPA secreted. Both proteins were constitutively secreted with linear kinetics over a 24-hour period, which suggests that they are not subjected to negative feedback regulation. Adjustment of the incubation volume (0.2 to 2.0 ml of medium per square centimeter of cells) of stationary cultures had no effect on the secretion rates of either tPA or PAI-1, which also suggests the lack of feedback regulation.

The increase in magnitude of tPA secretion and the time course of stimulation by shear stresses above 15 dynes/cm^2 are similar to that produced by histamine (0.1 to 1.0 μM) (*13*) or active thrombin (0.1 to 1.0 U/ml) (*17*). However, thrombin also stimulates PAI-1 production. Stimulation of endothelial cells with interleukin-1 or endotoxin (lipopolysaccharide) increases PAI production without increasing tPA secretion (*18*). Thus, the endothelial response to shear stress differs from these inflammatory responses.

Since the intracellular level of tPA in cultured endothelial cells is low, the stimulation of tPA secretion by shear stress must require either improved tPA mRNA transcription, stability, or translation. The increased tPA secretion after a lag time of 4 to 6 hours suggests that new tPA mRNA was transcribed. The selective stimulation of tPA secretion by shear stress relative to PAI-1 secretion may indicate a fibrinolytic response at the level of gene expression. Activation of protein kinase C by increased intracellular Ca^{2+} and diacylglycerol could possibly mediate this selective induction of tPA.

Arterial blood flow, possibly with higher levels of stress-induced platelet activation (*19*) and subsequent low-level thrombin production, may require a higher level of constitutive fibrinolytic activity. These studies could also implicate low hemodynamic shear stress as an additional cause of in-

Table 1. Steady-state tPA secretion rates of replicate primary, confluent HUVEC monolayers (4.0×10^5 to 7.0×10^5 cells per square centimeter) placed in static culture or exposed to steady laminar shear stress of 4, 15, or 25 dynes/cm^2. Each secretion rate was determined by a least-squares fit of tPA production between 4 and 27 hours and normalized by the secretion rate of matched static controls. The secretion rate was measured in nanograms of tPA per 10^6 cells per hour, and the average tPA secretion rate of the static controls was 0.168 ± 0.053 ng of tPA per 10^6 cells per hour (n = 3).

Condition	Normalized secretion rate			
	Experiment 1	Experiment 2	Experiment 3	Mean ± SD*
Static control	1.00 ± 0.21	1.00 ± 0.07	1.00 ± 0.23	(1.00)
Shear stress				
4 dynes/cm^2	0.92 ± 0.15	0.94 ± 0.09	0.62 ± 0.13	0.83 ± 0.18
15 dynes/cm^2	2.49 ± 0.39	1.95 ± 0.12	1.73 ± 0.30	2.06 ± 0.39†
25 dynes/cm^2	3.46 ± 0.56	3.14 ± 0.08	2.42 ± 0.40	3.01 ± 0.53†

*Each mean is the average of three independent experiments involving three separate primary cultures. A two-tailed t test with Bonferroni correction for multiple comparisons was used. †$P < 0.015$.

creased fibrin deposition in flow separations adjacent to arterial bifurcations where low shear stresses are present and atherosclerotic plaques are often seen (*20*). More generally, enhancement of the fibrinolytic activity of the endothelial cell by shear stress may contribute to the nonthrombogenic function of the endothelial cell surface.

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9. Glass slides (75 by 35 mm; Fisher) were soaked in 0.5*M* NaOH for 2 hours, rinsed with deionized water, and autoclaved. Endothelial cells from six to eight umbilical cords (obtained within 6 hours of delivery) were pooled [see M. A. Gimbrone, Jr., *Prog. Hemostasis Thromb.* **3**, 1 (1976)] and seeded at about 3×10^5 to 6×10^5 cells per slide. Medium 199 (Gibco) with 20% heat-inactivated fetal calf serum (HyClone Laboratories), glutamine (0.30 mg/ml) (Gibco), penicillin and streptomycin (0.10 mg/ml each) (Gibco), and neomycin (0.20 mg/ml) (Gibco) was used for all experiments.
10. Constant fluid flow was produced by a hydrostatic pressure head in a recirculating system [see J. A. Frangos *et al.*, (*3*)]. Confluent monolayers were washed three times with phosphate-buffered saline (at 37°C) before each experiment. The cell cultures were mounted on parallel-plate flow chambers (monolayer surface area, 15 cm^2; channel width, 200 μm) and connected under sterile conditions to the flow systems each filled with 15 ml of medium. The systems were kept at 37°C with an air curtain incubator and at *p*H 7.4 by continuous gassing with a mixture of 5% CO_2 in air.
11. Total tPA (uncomplexed and inhibitor-bound) and uncomplexed PAI-1 (latent and active) were assayed with separate enzyme-linked immunosorbent assays [American Diagnostica: Imubind-5 tPA ELISA Kit, no. 122; Imubind PAI-1 ELISA Kit, no. 822]. The tPA kit was recalibrated (0 to 1500 pg/ml), yielding a highly linear calibration curve (typically, $r^2 > 0.99$) with a detection limit of 50 pg/ml. Each tPA medium sample (undiluted; 100-μl addition per well) was used as a blank against itself to eliminate nonspecific interference. We assayed the media samples (diluted 1:10 and 1:25) for PAI-1 following kit instructions without modification (by manufacturer's standard). Sample concentrations (calculated with 95% confidence limits) were used to calculate antigen production (per 10^6 cells).
12. Lactate dehydrogenase activity of conditioned medium or sonicated cell lysates was measured with Gilford Diagnostics reagent LD-P using a Gilford 2600 spectrophotometer. The LDH activity of fresh medium was subtracted from all samples. The percent lysis was calculated by dividing the sample activity by the activity of 10^6 lysed cells.
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Direct Brønsted Analysis of the Restoration of Activity to a Mutant Enzyme by Exogenous Amines

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A true Brønsted analysis of proton transfer in an enzyme mechanism is made possible by the chemical rescue of an inactive mutant of aspartate aminotransferase, where the endogenous general base, Lys^{258}, is replaced with Ala by site-directed mutagenesis. Catalytic activity is restored to this inactive mutant by exogenous amines. The eleven amines studied generate a Brønsted correlation with β of 0.4 for the transamination of cysteine sulfinate, when steric effects are included in the regression analysis. Localized mutagenesis thus allows the classical Brønsted analysis of transition-state structure to be applied to enzyme-catalyzed reactions.

ENZYMOLOGISTS HAVE BEEN ABLE TO adapt most of the tools of the physical organic chemist to the analysis of enzyme mechanisms. One of the most important of these, the linear free energy analysis of structure-reactivity correlations, is a powerful probe of transition-state structure. The principal structure-reactivity correlations are (i) those in which the reactant is varied and one obtains a linear free energy relation between the logarithm of the rate constant (log *k*) and a quantitatively defined structural parameter of the reactants (for example, the Hammett plot) and (ii) those in which the catalyst is systematically varied and the dependence of log *k* on the electronic nature of the catalyst helps to define the structure of the transition state. The classical example of the latter correlation is the Brønsted plot, in which log *k* is plotted against the pK_a of the general acid or general base catalyst. The slope of the resulting line is frequently interpreted as a measure of the degree of proton transfer between the reactant and catalyst, or of the amount of charge development, in the transition state.

Considerable experimental difficulties are encountered in attempting to apply linear free energy probes of mechanism to the study of enzyme-catalyzed reactions. The structural variations in the substrate required for Hammett analyses often result in incongruous interactions between the modified substrate and the well-defined active site. Nonetheless, substantial success has been achieved in many cases, particularly with relatively nonspecific enzymes. [See (*1*) for a review.] It has, however, not been previously possible to generate a Brønsted plot for a general acid or general base enzyme-catalyzed reaction, because the catalytic acid or base moiety is built into the enzyme structure (*2*). We report the application of site-directed mutagenesis to overcome this latter limitation and the development of a true Brønsted correlation for an enzyme-catalyzed reaction.

The ϵ-NH_2 moiety of Lys^{258} was postulated to be the base responsible for the proton transfer (*3*), which is central to the transamination mechanism (Fig. 1). This proposal receives support from the observation that *Escherichia coli* K258A [a mutant in which Lys^{258} has been replaced by Ala (*4*)] retains

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