

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

S. L. DIAMOND, S. G. ESKIN, AND L. V. MCINTIRE*

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

S. L. DIAMOND, S. G. ESKIN, L. V. MCINTIRE*

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/cm²). However, at arterial shear stresses of 15 and 25 dynes/cm², 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 (PGI₂) production increases several-fold with the onset of laminar shear stress. Pulsatile shear stress stimulates an even greater production of PGI₂ 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/cm²

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/cm²) had no effect on tPA secretion during the entire time course of the experiment. After longer exposure to 15 or 25 dynes/cm², 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/cm², 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 ($n = 3$).

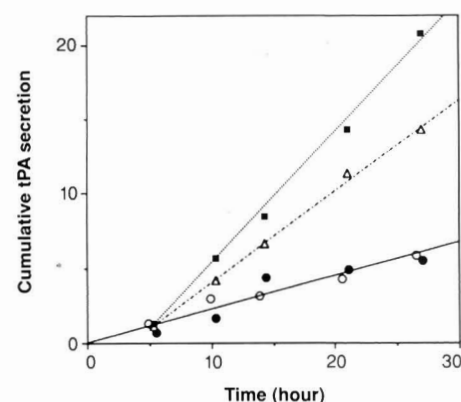


Fig. 1. Cumulative secretion (nanograms of tPA per 10^6 cells) of total tPA (uncomplexed and inhibitor-bound) by replicate, primary confluent HUVEC monolayers (6.0×10^4 cells per square centimeter; 1 ml of media per square centimeter of monolayer) maintained in static culture (●) or exposed to steady laminar shear stresses of 4 (○), 15 (△), and 25 (■) dynes/cm². 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.

S. L. Diamond and L. V. McIntire, Rice University, Biomedical Engineering Laboratory, P.O. Box 1892, Houston, TX 77251.
S. G. Eskin, Baylor College of Medicine, Department of Surgery, Houston, TX 77030.

*To whom all correspondence should be addressed.

Preliminary measurements indicate that pulsatile shear stress (1 Hz) does not potentiate the response. This is distinct from pulsatile stress effects on HUVEC PGI₂ synthesis (3) but similar to results on endocytosis (5). PGI₂ 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/cm² 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 10⁶ 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 10⁶ cells and <100 ng of PAI-1 per 10⁶ 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/cm² and 25 dynes/cm² for 6 and 12 hours) were placed on washed,

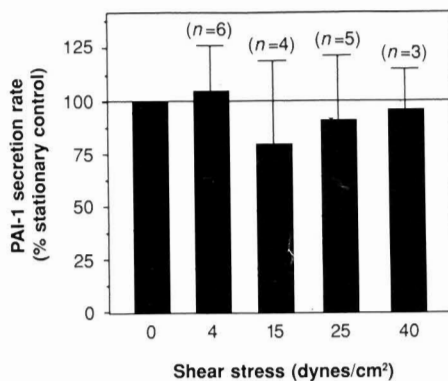


Fig. 2. Steady-state PAI-1 secretion rates (with SD) of sheared cultures were calculated from PAI-1 production between 4 and 27 hours and normalized by the PAI-1 secretion rate of matched, stationary cultures. The average PAI-1 secretion rates at venous (4 dynes/cm²) and arterial levels of shear stress (at 15, 25, and 40 dynes/cm²) 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 10⁶ cells per hour (*n* = 7).

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.

Cyclooxygenase activity is not required for tPA secretion by HUVEC (13). Since it is known that shear stress causes a large PGI₂ release, it was necessary to show that PGI₂ or other arachidonic acid metabolites do not stimulate tPA synthesis and secretion. PGI₂ 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 PGI₂ 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/cm² 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 Ca²⁺ 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/cm². 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 10⁶ cells per hour, and the average tPA secretion rate of the static controls was 0.168 ± 0.053 ng of tPA per 10⁶ 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.

REFERENCES AND NOTES

1. C. F. Dewey, Jr., S. R. Bussolari, M. A. Gimbrone, Jr., P. F. Davies, *J. Biomech. Eng.* **103**, 177 (1981); R. M. Nerem, M. J. Levesque, J. F. Cornhill, *ibid.*, p. 172; G. E. White, M. A. Gimbrone, Jr., K. Fujiwara, *J. Cell Biol.* **97**, 416 (1983); A. Remuzzi, C. F. Dewey, Jr., P. F. Davies, M. A. Gimbrone, Jr., *Biorheology* **21**, 617 (1984); S. G. Eskin, C. L. Ives, L. V. McIntire, L. T. Navarro, *Microvasc. Res.* **28**, 87 (1984); M. J. Levesque and R. M. Nerem, *J. Biomech. Eng.* **107**, 341 (1985); C. L. Ives, S. G. Eskin, L. V. McIntire, *In Vitro Cell. Dev. Biol.* **22**, 500 (1986).
2. D. L. Fry, *Circ. Res.* **22**, 165 (1968); C. G. Caro, J. M. Fitz-Gerald, R. C. Schroter, *Proc. R. Soc. London Ser. B* **117**, 109 (1971); H. L. Goldsmith and V. T. Turitto, *Thromb. Haemostasis* **55**, 415 (1986).
3. J. A. Frangos, S. G. Eskin, L. V. McIntire, C. L. Ives, *Science* **227**, 1477 (1985).
4. E. F. Grabowski, E. A. Jaffe, B. B. Weksler, *J. Lab. Clin. Med.* **105**, 36 (1985).
5. E. A. Sprague, B. L. Steinbach, R. M. Nerem, C. J. Schwartz, *Circulation* **76**, 648 (1987); P. F. Davies, C. F. Dewey, Jr., S. R. Bussolari, E. J. Gordan, M. A. Gimbrone, Jr., *J. Clin. Invest.* **73**, 1121 (1984); J. M. DeForrest and T. M. Hollis, *Am. J. Physiol.* **234**, 701 (1978); S. P. Olesen, D. E. Clapham, P. F. Davies, *Nature* **331**, 168 (1988).
6. D. C. Rijken, G. Wijngaards, J. Welbergen, *Thromb. Res.* **18**, 815 (1980); E. D. Sprengers and C. Klufft, *Blood* **69**, 381 (1987); J. A. Van Mourik, D. A. Lawrence, D. J. Loskutoff, *J. Biol. Chem.* **259**, 14914 (1984); E. G. Levin, *Blood* **67**, 1309 (1986).
7. D. Keber, M. Stegnar, I. Keber, B. Accetto, *Thromb. Haemostasis* **41**, 745 (1979); T. K. Chan and V. Chan, *Thromb. Res.* **14**, 525 (1979); B. Wiman, G. Mellbring, M. Ranby, *Clin. Chim. Acta* **127**, 279 (1983); E. Angles-Cano et al., *Thromb. Haemostasis* **58**, 843 (1987).
8. W. Speiser, E. Anders, K. T. Preissner, O. Wagner, G. Muller-Berghaus, *Blood* **69**, 964 (1987); D. C. Rijken, V. W. M. van Hinsbergh, E. H. C. Sens, *Thromb. Res.* **33**, 145 (1984); V. W. M. van Hinsbergh et al., *Arteriosclerosis* **7**, 389 (1987); I. R. MacGregor and N. A. Booth, *Thromb. Haemostasis* **59**, 68 (1988).
9. Glass slides (75 by 35 mm; Fisher) were soaked in 0.5M 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 cm²; 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 pH 7.4 by continuous gassing with a mixture of 5% CO₂ 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 10⁶ 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 10⁶ lysed cells.
13. M. Hanss and D. Collen, *J. Lab. Clin. Med.* **109**, 97 (1987).
14. A. I. Schafer, M. A. Gimbrone, Jr., R. I. Handin, *Biochem. Biophys. Res. Commun.* **96**, 1640 (1980); J. S. Makarski, *In Vitro* **17**, 450 (1981).
15. T. Kooistra et al., *Biochem. J.* **247**, 605 (1987).
16. A. J. Zonneveld et al., *ibid.* **235**, 385 (1986); V. W. M. van Hinsbergh, E. D. Sprengers, T. Kooistra, *Thromb. Haemostasis* **57**, 148 (1987).
17. E. G. Levin, U. Marzec, J. Anderson, L. A. Harker, *J. Clin. Invest.* **74**, 1988 (1984).
18. J. J. Emeis and T. Kooistra, *J. Exp. Med.* **163**, 1260 (1986).
19. C. J. Jen and L. V. McIntire, *J. Lab. Clin. Med.* **103**, 115 (1984); J. L. Moake, N. A. Turner, N. A. Stathopoulos, L. Nolasco, J. D. Hellums, *Blood* **71**, 1366 (1988).
20. D. N. Ku, D. P. Giddens, C. K. Zarins, S. Glagov, *Arteriosclerosis* **5**, 293 (1985); D. Collen and I. Juhan-Vague, *Semin. Thromb. Hemostasis* **14**, 180 (1988).
21. Supported by NIH grants HL 18672 and HL 23016, grants NAS 9-17403 and NAG 9-207 from NASA, and grant C-938 from the Robert A. Welch Foundation. Special thanks to M. Estrella and L. Navarro for laboratory assistance.

20 September 1988; accepted 27 December 1988