Elevated Intraluminal Pressure Inhibits Vascular Tissue Plasminogen Activator Secretion and Downregulates Its Gene Expression

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Abstract—We recently discovered that patients with essential hypertension have a markedly impaired capacity for stimulated release of tissue plasminogen activator (tPA) from vascular endothelium. This defect may reduce the chance of timely spontaneous thrombolysis in case of an atherothrombotic event. We now investigated whether increased intraluminal pressure as such may depress vascular tPA release or downregulate its gene expression. Segments of human umbilical veins were studied in a new computerized vascular perfusion model under steady laminar flow conditions for 3 or 6 hours. Paired segments were perfused at high or physiological intraluminal pressure (40 versus 20 mm Hg) under identical shear stress (10 dyne/cm²). Quantitative immunohistochemical evaluation of cellular tPA immunoreactivity was performed on paraffin-embedded 5-μm vascular sections. tPA mRNA in endothelial cells was quantified with reverse transcription real-time TaqMan polymerase chain reaction with GAPDH as endogenous control. Secretion of tPA into perfusion medium was evaluated with SDS-PAGE and Western blotting, followed by densitometric quantification. High-pressure perfusion downregulated tPA gene expression with a 38% decrease in tPA mRNA levels \((P<0.01)\) compared with vessels perfused under normal intraluminal pressure. tPA release into the perfusion medium was markedly suppressed by high pressure \((P<0.01 \text{ ANOVA})\). The intracellular storage pool of tPA was reduced after 6 but not 3 hours. Thus, elevated intraluminal pressure downregulates tPA gene and protein expression and inhibits its release from the endothelium independently of shear stress. The defective capacity for stimulated tPA release that we demonstrated in patients with essential hypertension might thus be an effect of the elevated intraluminal pressure per se. (Hypertension. 2000;35:1002-1008.)

Key Words: endothelium • plasminogen activators • fibrinolysis • gene expression

In the healthy blood vessel, vasodilatory, antiaggregatory, and fibrinolytic mechanisms act in concert to maintain thromboprotective properties of the vascular endothelial surface. Secretion from the endothelium of the key enzyme of the fibrinolytic system, tissue plasminogen activator (tPA), plays a pivotal role in this defense. In addition to a low-rate constitutive secretion, the activation of platelets and the coagulation cascade may induce a rapid and massive acute release of tPA from its intracellular endothelial storage pool. Hereby, stimulated tPA secretion appears to be an important counterregulatory mechanism to prevent a clotting process, for instance when initiated by a plaque rupture, from progression into an occlusive thrombus. If the capacity for acute tPA release is defective, the likelihood of timely, spontaneous thrombolysis may be reduced.

In patients with essential hypertension, the risk of coronary thrombosis cannot be fully ameliorated with antihypertensive treatment. This fact suggests that the hypertensive condition might be associated with defective antithrombotic properties of the vascular wall. Interestingly, we recently found that otherwise healthy patients with established essential hypertension have a markedly impaired capacity for stimulated tPA release from the vascular endothelium. This defect, which may reduce resistance to atherothrombotic events, was not related to metabolic aberrations or atherosclerosis but rather appeared to be related to the blood pressure elevation as such.

In the present study, we investigated whether elevated intraluminal pressure could depress vascular tPA secretion or gene expression. However, the investigation of this hypothesis is complicated by the fact that in an intact vessel, changes in perfusion pressure invariably lead to changes in wall shear stress, which by itself may increase tPA gene expression. To overcome this problem, we used a new computerized biomechanical ex vivo perfusion model that we developed, in which intraluminal pressure and shear stress can be controlled independently of each other. Our results show for
the first time that elevated intraluminal pressure per se inhibits tPA secretion from endothelial cells and downregulates its gene expression in intact human conduit vessels.

Methods

Vascular Perfusion System

A detailed description of the computerized perfusion system was recently provided elsewhere. Briefly, fresh human umbilical veins are perfused antegrade with Tyrode’s saline solution containing (in mmol/L) NaCl 146, KCl 6, CaCl2 3, MgCl2 0.5, KH2PO4 0.3, NaHCO3 20, and glucose 5.6, pH 7.4, in 2 parallel gravity-fed circuits (Figure 1). Medium is continuously pumped to an upper reservoir, of which the height is regulated with a computer-controlled motor unit. Flow rate in each circuit is measured in-line and controlled with a proportioning solenoid valve. The 37.0±0.1°C medium is bubbled with a computer-controlled amount of gas mixture of 90% N2:5% O2:5% CO2 to maintain target pH (7.4), PO2, and PCO2. Pressure is monitored through upstream and downstream pressure catheters. Shear stress is calculated by the following relation:

\[ \tau = \frac{1}{2} \left( \frac{\Delta P}{L} \right) \left( \frac{8 \eta Q L}{\pi} \right) \]

where \( \tau \) is wall shear stress, \( \Delta P \) is pressure drop over the vessel, \( L \) is the vessel length, \( \eta \) is viscosity of the fluid, and \( Q \) is flow rate. Pressure and flow are automatically adjusted to maintain target combinations of predefined hydrodynamic parameters (ie, mean intraluminal pressure and flow) are automatically adjusted to maintain target combinations of predefined hydrodynamic parameters (ie, mean intraluminal pressure and flow rate). Reynolds’ number is monitored to ensure laminar flow conditions.

Experimental Protocol

Umbilical cords were obtained immediately after full-term vaginal deliveries. Vessel segments were divided into 2 parts (~200 mm) and randomized to parallel high- and low-pressure perfusion. A 30-minute equilibration period with 10 mL/min flow at 20 mm Hg mean perfusion pressure was allowed before experimental conditions were established. Paired umbilical vein segments were perfused at high (target 40 mm Hg) or low (20 mm Hg) intraluminal perfusion pressure at a shear stress of 10 dynes/cm2 for 3 (n = 7) or 6 (n = 10) hours. tPA protein secretion into perfusion medium was measured at baseline and after 1, 2, 4, and 6 hours (n = 10). Immunohistochemical staining for tPA was performed after 3 (n = 4) and 6 (n = 4) hours of perfusion in 2 perfusion series. The study was approved by the Ethics Committee of Göteborg University.

 SDS-PAGE Protein Electrophoresis and Western Blotting

Perfusate samples were immediately frozen and stored at −70°C. Before the assay, samples were concentrated and purified by filtration through highly selective OMEGA 10K ultrafiltration mem-
Quantitative Reverse Transcriptase-Real-Time Polymerase Chain Reaction

**Isolation of Total RNA**

After perfusion, endothelial cells were explanted through incubation with 0.1% collagenase for 12 minutes at 37°C. The cell suspension was centrifuged for 10 minutes at 2600 g, and the cell pellet was resuspended in denaturing solution (4 mol/L guanidinium thiocyanate, 25 mmol/L sodium citrate, pH 7, 0.5% sarcosyl, and 0.1 mol/L 2-mercaptoethanol). Total cellular RNA was extracted with the guanidinium thiocyanate–phenol-chloroform method according to Chomczynski and Sacchi. Total RNA concentration was determined with a GeneQuant II RNA/DNA calculator (Amersham Pharmacia Biotech Ltd). The purity of precipitated total RNA was determined with 1% agarose gel electrophoresis during denatured conditions. Reverse transcription (RT) of 1 μg total RNA was performed in a total volume of 20 μL containing 2.5 μL L RNA-guard, and 2.5 U AmplErase UNG, 15 pmol of each primer, and 5 pmol probe in 0.5 U AmpErase UNG, 15 pmol of each primer, and 5 pmol probe in a final volume of 50 μL. Moloney murine leukemia virus (MuLV) reverse transcriptase. Samples were incubated at 20°C for 10 minutes, 42°C for 15 minutes, 99°C for 5 minutes, and finally 5°C for 5 minutes.

**Principle of RT–Polymerase Chain Reaction Assay**

Relative quantification of mRNA was performed with a TaqMan real-time RT–polymerase chain reaction (PCR) assay on an ABI PRISM 7700 Sequence Detector (Perkin-Elmer Applied Biosystems Inc). Briefly, this method uses the 5’ nucleic acid of Taq polymerase to cleave a reporter dye from a nonextendable hybridization probe during the extension phase of the PCR. The fluorogenic probe is labeled with a reporter dye (6-carboxy-fluorescein) at the 5’ end and a quencher dye (6-carboxy-tetramethyl-rhodamine) at its 3’ end via a linker arm nucleotide. During the extension phase, the reporter dye is released and the increase in dye emission is monitored via a linker arm nucleotide. During the extension phase, the reporter dye is released and the increase in dye emission is monitored.

**PCR Conditions**

Oligonucleotide primers and TaqMan probes were designed with use of the Primer Express 1.0 software (Perkin-Elmer Applied Biosystems Inc) from the GenBank database as follows: tPA (accession number L00140 J00278) and GAPDH (accession number M31397) (Table). Each primer pair was selected so the amplicon spanned an exon junction to avoid the amplification of genomic DNA (tPA exons 12 and 13, GAPDH exons 1 and 2). PCR products were 68 bp for tPA and 70 bp for GAPDH. RT-PCR products of the 2 targets were verified by sequencing on an ABI Prism 310 Genetic Analyzer (Perkin-Elmer Applied Biosystems Inc.). Amplifications were performed with the ABI PRISM 7700 Sequence Detector (Perkin-Elmer Applied Biosystems Inc). For amplification of the 68-bp tPA product, 1 μL cDNA diluted 1:8 was added to the PCR mixture consisting of TaqMan buffer A, 5 mmol/L MgCl₂, 0.2 mmol/L dNTP mix (20 mmol/L dUTP and 10 mmol/L concentration of dATP, dCTP, and dGTP), 1.25 U Taq Gold polymerase, 0.5 U AmpErase UNG, 15 pmol of each primer, and 5 pmol probe in a final volume of 50 μL. For amplification of the 70-bp GAPDH product, 1 μL cDNA diluted 1:4 was added to the PCR mixture. Thermal cycling conditions were 2 minutes at 50°C and 10 minutes of initial denaturation at 95°C to activate Taq Gold polymerase, followed by 50 cycles of 2-step PCR consisting of 15 seconds at 95°C and 1 minute at 60°C. All samples were amplified in triplicate.

**Methodological Validation**

The average amount of extracted RNA from the endothelial cells was ~5 μg/200-mm umbilical vessel. The quantity of total cellular RNA extracted was similar in vessels exposed to high or low pressure. Transcript levels of the endogenous control GAPDH were independent of pressure stimulation. In addition, no effect of stimulation was observed when GAPDH mRNA was expressed relative to β-actin (data not shown). Variation coefficients for Cₜ values for triplicate reactions were excellent (1.0% and 0.6% for tPA and GAPDH, respectively).

**Materials**

Unless otherwise stated, all reagents were purchased from Sigma Chemical Co. PCR consumables were supplied by Perkin-Elmer Applied Biosystems Inc.

**Statistical Analysis**

Standard statistical methods were used. Data are given as mean±SEM. Parametric methods (ANOVA and t test) were used for
the evaluation of responses to the different perfusion conditions. Responses to perfusions were evaluated with 2-way (condition and time) and 1-way (time) ANOVAs for repeated measures. Contrast analysis was used when the overall ANOVA indicated a significant main effect of treatment or interaction. Effects on mRNA expression were evaluated with \( t \) test after logarithmic transformation of the tPA-to-GAPDH ratio. A significance level of \( P < 0.05 \) was chosen (2-tailed test).

**Results**

**Hemodynamics**

During the 6-hour perfusion period, mean intraluminal pressure was maintained at an average of 39.2 ± 0.8 and 19.5 ± 0.5 mm Hg in the high- and low-pressure circuits (\( P < 0.0001 \)), respectively (Figure 2). The corresponding pressure levels during 3-hour perfusions were 39.3 ± 6.8 and 19.8 ± 3.3 mm Hg (\( P < 0.0001 \)), respectively. Mean shear stress was maintained at 10.0 ± 0.1 and 9.9 ± 0.1 dyne/cm² in the 6-hour perfusion series and at 10.0 ± 1.6 and 9.9 ± 1.6 during the 3-hour perfusion series (\( P = \text{NS} \) throughout). Average fluid flow across the entire 6-hour experiment was 67.7 ± 7.8 and 55.9 ± 6.0 mL/min in the high- and low-pressure systems, respectively (\( P = \text{NS} \)). During 3-hour perfusions, average flow was maintained at 78.0 ± 17.5 and 53.6 ± 11.8 mL/min (\( P = \text{NS} \)), respectively.

**Quantification of tPA mRNA Expression**

Figure 3 illustrates the results of the real-time RT-PCR analysis of tPA relative to GAPDH cDNA performed after 3 and 6 hours of perfusion. In vessels perfused at high intraluminal pressure (40 mm Hg) for 6 hours, tPA gene expression was significantly depressed, and the tPA-to-GAPDH ratio was reduced by 38 ± 11% (log \( t \) test, \( P = 0.01 \)) compared with the vessels perfused under low-pressure conditions. After 3 hours of high-pressure perfusion, there was a slight and nonsignificant reduction in the tPA-to-GAPDH ratio by 9 ± 17% (\( P = \text{NS} \)). Transcript levels of the endogenous control GAPDH were independent of pressure stimulation in both perfusion series (data not shown).

**Quantitative Immunohistochemistry**

The cellular content of tPA in vessel wall as evaluated with quantitative immunohistochemistry was similar in vascular sections from vessels perfused under high and low intraluminal pressure for 3 hours. However, in vessels perfused for 6 hours, 3 of the 4 paired vascular sections had a decreased immunostaining for tPA in high- compared with low-pressure sections, whereas the staining patterns were similar in the fourth vessel pair. On the average, tPA immunostaining was reduced by \( \approx 30\% \) in vessels perfused under high-pressure conditions for 6 hours. Typical photomicrographs are shown in Figure 4. A positive staining for tPA was mainly observed in the endothelium. Hematoxylin counterstaining revealed intact structure of the vessel wall in all stimulated umbilical veins in both high- and low-pressure vessels.

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**Figure 2.** Left, Trend curves of hemodynamics during the 6-hour high (■) - and low (□)-pressure perfusions. Intraluminal pressure and shear stress were maintained at target levels with high precision throughout the experiment. Right, Summary of hemodynamics during 3 (\( n = 7 \)) and 6 (\( n = 10 \)) hours of perfusion at high (target 40 mm Hg) and low (20 mm Hg) intraluminal pressure. Shear stress levels were maintained at similar levels (target 10 dyne/cm²) in both series.

**Figure 3.** Expression of tPA cDNA normalized to GAPDH for vessels exposed to high and low pressure at identical levels of shear stress during 3 (\( n = 7 \)) and 6 (\( n = 10 \)) hours of perfusion. tPA gene expression was significantly decreased in endothelial cells perfused for 6 hours at high compared with low intraluminal pressure. Significance levels refer to \( t \) test after logarithmic transformation of tPA-to-GAPDH cDNA ratios.
6-hour perfusion
20 mmHg  40 mmHg

Figure 5 shows cumulative perfusate concentrations of tPA during 6-hour high and low intraluminal pressure perfusions. The amount of tPA accumulated in the perfusion medium differed significantly between pressure conditions (ANOVA, condition \times time interaction $P = 0.002$). During the 20 mm Hg perfusion, there was a marked increase in the amount of tPA in the medium during the first 4 hours of stimulation (ANOVA, $P < 0.0001$), whereas there was no significant increase in the medium concentration of tPA during high-pressure perfusion (ANOVA, $P = \text{NS}$). Contrast analysis revealed that the difference in secretion occurred between 1 and 2 hours of perfusion ($P < 0.005$).

**Discussion**

To our knowledge, the present study is the first to demonstrate an inhibitory effect of high intraluminal pressure on endothelial cell tPA production in an intact conduit vessel. The observation that the pressure-induced decrease in tPA secretion appeared earlier than the downregulation of mRNA levels indicates that pressure exerts dual and distinct inhibitory effects on the secretory machinery and tPA gene expression. In the normal blood vessel wall, secretion of tPA from the endothelium plays a pivotal role to maintain its thromboprotective properties, and the induction of acute tPA release is an important defense mechanism to prevent the formation of occlusive intraluminal clots. Thus, our findings suggest that exposure of the vessel wall to increased intraluminal pressure may reduce the antithrombotic capacity of the endothelium.

In 1994, we described a model to study basal and stimulated vascular tPA release in vivo by using intra-arterially infused secretagogues. Using this approach, we recently discovered that patients with established essential hypertension have a markedly impaired capacity for stimulated tPA release from the vessel wall. Despite careful matching between groups regarding metabolic and other potentially confounding variables, the total amount of tPA released across the forearm vasculature in hypertensive patients was approximately one third of that observed in normal subjects. Because we found no reduction in vascular tPA secretion in subjects with mild borderline hypertension, it is conceivable that a certain level of blood pressure elevation must be reached before the defect becomes apparent. Hence, the observations of the present study support the hypothesis that elevated intraluminal pressure as such may suppress tPA synthesis and release.

tPA can be secreted from the vascular endothelium via both a constitutive and a regulated pathway. Acute regulated release is important for rapid elevation of the local tPA concentration in response to the activation of platelets or plasma coagulation. Under basal conditions in vivo, the...
steady state plasma concentration of tPA is mainly maintained through constitutive secretion,\(^7\) although a low-degree activation of the stimulated pathway (eg, by catecholamines\(^8\) or other humoral agonists\(^9\)) may contribute to the basal secretion rate. It is not known which pathway mediates tPA secretion during \(\text{in vivo}\) perfusion conditions. Although agonist-stimulated secretion is unlikely to occur with blood-free perfusion, it is possible that the regulated pathway could be activated by hemodynamic stress. In vitro studies have shown that shear stress can enhance tPA secretion.\(^7\) However, this stimulatory effect has a slow onset with a lag phase of 8 to 10 hours.\(^7\) and there is no experimental evidence that shear can induce acute tPA release. Rather, the delayed response suggests that shear increases the constitutive secretion secondary to stimulation of tPA de novo synthesis.\(^4\) This inference is supported by our recent finding that the secretion rate of tPA was unaltered when isolated conduit vessels were exposed to high shear stress for 6 hours, despite a pronounced increase in synthesis and massive intracellular accumulation of tPA.\(^5,6\)

Hence, it is likely that the pressure-induced suppression of tPA secretion was due to an inhibitory effect on the constitutive secretory pathway. However, the decrease in tPA mRNA levels indicates that high-pressure perfusion also downregulates de novo synthesis, which is supported by the diminution of the intracellular tPA content observed after 6 hours of perfusion. Thus, it is likely that prolonged exposure to elevated intraluminal pressure reduces the intracellular storage pool of tPA, and thereby the amount of tPA available for acute release. This assumption is in line with our recent finding of a reduced capacity for stimulated tPA release in hypertensive patients.\(^3\)

Previous studies have used various stretch devices to induce mechanical deformation on cultured cell monolayers.\(^21–24\) The few studies that investigated the effect of stretch on tPA production have shown inconsistent results. Carosi and McIntire\(^25\) observed no change in the production of tPA when cultured first-passage human umbilical vein endothelial cells were exposed to 10% cyclic strain for 24 hours. However, Iba et al\(^6,26,27\) observed a significant increase in both expression and release of tPA in \(\text{in vivo}\) passage human saphenous vein endothelial cells grown on flexible membranes cyclically deformed by vacuum. The stimulatory effect was evident already after 24 hours but increased further after 3 and 5 days. Apart from differences in the duration of stimulation, there are some important differences between our experimental set-up and those used in in vitro studies. In cultured endothelial cells, subcultivation induces phenotypic changes in the expression of tPA,\(^28\) which may alter responses to external stimuli. Also, in contrast to cell monolayers in mechanical devices, deformation forces developed within vascular cells of a living vessel are dependent not only on its elastic properties but also on myogenic and vasomotor responses to stimulation. Furthermore, the potential for cross-talk between various cell populations in the intact vessel may modify the responses induced by the mechanical stress as such.

In accordance with previous data from cell culture experiments,\(^5\) we recently showed that shear stress increases tPA expression.\(^5,6\) In this case, the agreement between results obtained in different experimental models may reflect the fact that shearing forces in an intact vessel are solely taken up by endothelial cells. However, the contrasting effects of shear and pressure on tPA expression underscore the importance of the use of an experimental model in which the 2 major hemodynamic forces are controlled independently of each other.\(^7\) Because available data suggest that shearing and tensile deformation stimuli use partially overlapping signaling pathways, the effects of pressure may be different when studied in a context of physiological shear stress. Unfortunately, combinations of the 2 forces are difficult to simulate in cell culture-based systems.

In conclusion, the present study demonstrates that high intraluminal pressure exerts an inhibitory effect on tPA production in vascular endothelial cells. The inhibition is due to a dual effect on both secretory and synthetic mechanisms. Taken together with our recent finding that hypertensive patients have a markedly impaired capacity for stimulated tPA, the present observations suggest that the depression of vascular thromboprotective mechanisms is an effect of elevated pressure as such.

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