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

Lena Selin Sjögren, Roya Doroudi, Li-ming Gan, Lennart Jungersten, Thórdís Hrafnkelsdóttir, Sverker Jern

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.\(^1\) 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.\(^4\)–\(^6\) To overcome this problem, we used a new computerized biomechanical ex vivo perfusion model that we developed,\(^7\) 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 vessels are perfused antegradely 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°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 intraluminal pressure and shear stress. Reynolds’ number is monitored with an equal volume of electrophoresis sample buffer (62.5 mmol/L Tris-glycine, 2% SDS, 25% glycerol, 0.01% bromphenol blue, 5% 2-mercaptoethanol) and electrophoresed on gradient gels (4% to 15% Tris-HCl polyacrylamide gel; Bio-Rad Laboratories AB) in running buffer (25 mmol/L Tris, 192 mmol/L glycine, and 0.1% [wt/vol] SDS, pH 8.3) at 200 V for 1 hour. Resolved proteins were transferred by blotting onto Hybond-P polyvinylidene fluoride membranes (Amersham Pharmacia Biotech Ltd) in transfer buffer (25 mmol/L Tris, 192 mmol/L glycine, and 20% [wt/vol] methanol, pH 8.3) at 100 V for 1 hour. To minimize nonspecific binding, membranes were placed in 25 mL blocking solution (5% fat-free dried milk in PBS–Tween 20% solution). Membranes were incubated for 1 hour with primary antibody, 10 μL to 15 mL PBS–Tween 20% (1:1500, anti-tPA monoclonal, mouse antibody [B10]; IMCO Corp), and thereafter with secondary antibody (anti-mouse Ig, horseradish peroxidase linked; Amersham Pharmacia Biotech Ltd) on a microparticle shaker at room temperature. Proteins were visualized by chemiluminescence with the ECL revealing kit (Amersham Pharma-acia Biotech Ltd). Autoradiographic images were densitometrically analyzed (NIH Image) after background subtraction. Samples were analyzed in duplicate, and paired experiments were analyzed within 1 gel. Results are given in relative optical density units.

#### Immunohistochemistry

After perfusion, 10-mm vascular segments were cut from the middle part of each umbilical cord and fixed in formalin at room temperature for 24 hours. Standard histological methods of dehydration were used in ascending grades of ethanol, clearing in xylene, and paraffin infiltration; tissues were transferred in paraffin wax blocks to present a luminal cross section. Then, 5-μm sections of matched vessel pairs were mounted on Superfast Plusglass (Menzel: Merck Ltd). Sections were deparaffinized in xylene, rehydrated in a graded alcohol series, and placed in distilled water. Antigen retrieval was performed with incubation in an epitope recovery buffer (0.01% Protease type XXIV) in 0.05 mol/L Tris buffer (pH 7.6), with a 0.01% calcium chloride). Together with coverplates, the slides were placed in cassettes with Cadenza buffer (Shandon Life Sciences). Universal streptavidin/biotin immunoperoxidase detection system with diaminobenzidine as chromogen (OmniTags Plus; Immunon) was used. Endogenous peroxidase activity was quenched with a 5-minute incubation in 3% hydrogen peroxide, and vessel sections were treated with a protein-blocking agent to reduce nonspecific antibody binding. Slides were incubated with 1:5 and 1:10 dilutions of anti-tPA monoclonal mouse antibody (B10; IMCO Corporation Ltd) or nonimmune mouse serum as negative control (Immunon) for 30 minutes. Sections were incubated with biotinylated secondary antibody for 30 minutes and with streptavidin-peroxidase reagent for 30 minutes. After incubation with fresh chromogen solution for 30 minutes, sections were counterstained with hematoxylin for 2 minutes. After dehydration, sections were mounted with resin-based medium. Matched stimulation and control vessel preparations were examined pairwise with light microscopy (Olympus BX-60; Olym-
**Oligonucleotide Primers and Probes Used for TaqMan Real-Time Quantitative PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligonucleotide</th>
<th>Sequence</th>
<th>Position</th>
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<tbody>
<tr>
<td>tPA</td>
<td>Sense primer</td>
<td>5'-GGC CTT GTC TCC TTT CTA TTC G-3'</td>
<td>31561-31565, 32429-32444</td>
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<tr>
<td></td>
<td>Antisense primer</td>
<td>5'-AGC GGC TGT ATG GGT ACA C-3'</td>
<td>32472-32490</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>5'- (FAM) TGA CAT GAG CCT CTC TCA GCC GCT (TAMRA) -3'</td>
<td>32446-32469</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Sense primer</td>
<td>5'-CCG CAT GCC TCA GAC ACC AT-3'</td>
<td>1435-1454</td>
</tr>
<tr>
<td></td>
<td>Antisense primer</td>
<td>5'-CCG GGC CAA TAC G-3'</td>
<td>3123-3138</td>
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<tr>
<td></td>
<td>Probe</td>
<td>5'- (FAM) AAG GTG AGT GTC GGA GTG AAC GGA TTT G (TAMRA) p-3'</td>
<td>1459-1481, 3116-3120</td>
</tr>
</tbody>
</table>

**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' nuclease activity 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-tetramethylrhodamine) 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 end via a linker arm nucleotide. During the extension phase, the reporter dye is released and the increase in dye emission is monitored.

The average optical density for each vessel preparation was compared pairwise.

**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 260g, and the cell pellet was resuspended in denaturing solution (4 mol/L guanidine 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 guanidine thiocyanate–phenol-chloroform method according to Chomczynski and Sacchi. Total RNA concentration was determined with a spectrophotometer (A260 and A280 (A260/A280 = 1.7 to 1.9) spectrophotometric measure - matic). The selected area was spectrally inverse correlation was observed between CT values and the slope of standard curve line (n) were calculated for each of the 2 target genes. The input amount (CTg) was determined by the formula TGI = ((cell containing C_T value) – b)/m. The IPA-to-GAPDH cDNA ratio was used to determine the initial amount of normalized tPA cDNA.

**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 M33197)(1) (Table). Each primer pair was selected so the amplicron 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 IPA 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). 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. 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. Transcrip sequencing 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 C_T 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/cm\(^2\) 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±1% (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.

**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/cm\(^2\)) 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.
tPA Secretion Into the Perfusion Medium

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 x 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 = 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 thrombo-protective 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.12–15 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.3 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,13 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.16 Acute regulated release is important for rapid elevation of the local tPA concentration in response to the activation of platelets or plasma coagulation.1 Under basal conditions in vivo, the
steady state plasma concentration of tPA is mainly maintained through constitutive secretion, although a low-degree activation of the stimulated pathway (eg, by catecholamines or other humoral agonists) may contribute to the basal secretion rate. It is not known which pathway mediates tPA secretion during ex 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. However, this stimulatory effect has a slow onset with a lag phase of 8 to 10 hours, 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. 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.

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.

Previous studies have used various stretch devices to induce mechanical deformation on cultured cell monolayers. The few studies that investigated the effect of stretch on tPA production have shown inconsistent results. Carosi and McIntire 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 observed a significant increase in both expression and release of tPA in third-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 vitro studies. In cultured endothelial cells, subcultivation induces phenotypic changes in the expression of tPA, 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, we recently showed that shear stress increases tPA expression. 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. 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.

Acknowledgments

This work was supported by grants from the Swedish Medical Research Council (project 09046), the Bank of Sweden Tercentenary Foundation, the Swedish Heart-Lung Foundation, the Swedish Hypertension Society, the Åke Wiberg Foundation, and the Gothenburg Medical Society. We wish to thank laboratory technicians Hannele Korhonen and Camilla Ejdestig for excellent technical assistance. The cooperation of the midwives at the Department of Obstetrics, Sahlgrenska University Hospital/Ostra, Göteborg, in collecting umbilical cords is gratefully acknowledged.

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_Hypertension_. 2000;35:1002-1008
doi: 10.1161/01.HYP.35.4.1002

_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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