Studies on the Acute Release of Tissue-Type Plasminogen Activator From Human Endothelial Cells In Vitro and in Rats In Vivo: Evidence for a Dynamic Storage Pool

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The process of acute release of tissue-type plasminogen activator (tPA) is important in locally speeding up fibrinolysis. Using a sensitive enzyme-linked immunosorbent assay for tPA, we investigated the acute release of tPA from cultured human umbilical vein endothelial cells. The addition of thrombin (0.003 to 3 NIH U/mL) caused the dose-dependent release of noncomplexed, enzymatically active tPA into the medium. The amount of tPA released into the medium by thrombin was similar to the difference in the amounts of tPA present in extracts from thrombin-treated cells and control cells. The process of acute release of tPA was complete in 1 minute, whereas the concomitant release of von Willebrand factor into the medium was slightly slower (maximum after 3 minutes). By increasing (c.q. decreasing) tPA synthesis, it was found that the amount of tPA constitutively secreted, the amount acutely released, and the amount in cell extracts were increased (c.q. decreased) to the same extent. The same relation was found in vivo. When rats were pretreated with cholerotoxin or retinoic acid to increase tPA synthesis, plasma levels of tPA were increased, whereas acute release of tPA, as induced by bradykinin, was increased to the same extent. Acutely released tPA and constitutively secreted tPA were liberated from different pathways in human umbilical vein endothelial cells; tPA had, relative to the in vivo situation, a shorter residence time in the acutely releasable pathway.

**MATERIALS AND METHODS**

**Materials.** Cell culture reagents were from Flow Laboratories (Irvine, Scotland). Sterile, pyrogen-free, human serum albumin (HSA; 20% wt/vol) was from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). L-methionine was from Life Technologies (Breda, The Netherlands). S-Methionine, labeling grade, was from Amersham (’s-Hertogenbosch, The Netherlands). Human α-thrombin, calcium ionophore-free acid (A23187), sodium butyrate, choletoxin, bradykinin, and all-trans retinoic acid (RA) were from Sigma (St Louis, MO). Materials used in the human and rat tPA ELISA and in the vWF ELISA have been described. Casein was from Merck (Darmstadt, Germany), and sodium pentobarbital (Nembutal) from Sanofi (Paris, France). Mouse monoclonal antibody against fluorescein isothiocyanate (FITC) was kindly provided by Dr. R. Bos of our institute.

**Definitions.** Constitutive secretion of tPA (or vWF) is defined as the amount of tPA antigen (or vWF antigen) secreted during the 30-minute preincubation period in M199 (M199/HSAs. The tPA (vWF) present in the cell extract is defined as the amount of tPA (vWF) found in control extracts at t = 3 minutes. The thrombin-induced acute released tPA (vWF) is defined as the amount of tPA (vWF) in medium of thrombin-treated cells minus the amount of tPA in medium of control cells at t = 3 minutes.

**Cell culture.** Human umbilical vein endothelial cells (HUVECs) were isolated using the method of Jaffe et al. and were cultured as previously described in M199, containing 10% (vol/vol) human serum albumin (HSA; 20% wt/vol), 10% (vol/vol) heat-inactivated newborn calf serum, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 2 μg/mL L-glutamine (henceforth called total M199 culture medium) under 5% CO2 at 37°C. Cells were passaged once by trypsin/EDTA treatment at a split ratio of 1:3 and were cultured to confluency in 2-cm² wells (final density 2 × 10⁴ cells/well).

**Induction of the acute release of tPA in vitro.** To exclude any interference of HS, ECGF, or heparin with the acute release mechanism, HUVECs were cultured in M199 medium, containing 20% (vol/vol) newborn calf serum, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 2 μg/mL L-glutamine (“deficient” medium) for 2 hours before an experiment. The 2-hour conditioned media were collected. The cells were then washed twice with sterile phosphate-buffered saline (PBS; pH 7.4) and 0.3 mL M199/HSA medium.
(M199, containing 0.03% (wt/vol) HSA, 100 IU/mL penicillin, 100 
µg/mL streptomycin, and 2 mM L-glutamine) was added to the 
wells. Thirty minutes later, at t = 0, 15 µL human α-thrombin in 
M199/HSA was added to the indicated final concentration (generally 
1 NIH U/mL). The same volume of M199/HSA medium was added 
to control cells. The media were then collected after 3 minutes, 
unless otherwise stated. The cells were washed once with ice-cold 
PBS and immediately put on ice. Cell lysates were made by scraping 
the cells with a rubber policeman into 0.3 mL PBS containing 0.05% 
(vol/vol) Tween 20, 0.01 mol/L EDTA, and 0.5% (vol/vol) Triton 
X-100. All incubations were performed at 37°C.

Decreasing the constitutive secretion of tPA. To decrease the 
constitutive tPA secretion, HUVECs were cultured for 0, 2, 4, 8, 
and 24 hours in the deficient medium described above.

Increasing the constitutive secretion of tPA. HUVECs were in-
cubated for 24 hours with 0, 0.3, 1, or 3 mM sodium butyrate or 
with 1 µm/L RA in total M199 medium to increase the consti-
tutive secretion of tPA.

Methionine-labeling of the constitutive and regulated tPA path-
ways. HUVECs were cultured for 24 hours in total M199 medium, 
containing 3 mM/L sodium butyrate. The cells were then incubated 
in total M199 medium that had been made free of methionine by 
night dialysis against methionine-free M199 and that contained 
radiolabeled 35S-methionine (20 µCi/mL) and 3 mM sodium butyrate. A 100-fold excess of unlabeled methionine was added after 
0, 1, 2, 3, or 4 hours. After a total incubation time of 4 hours, release 
was induced; media and cell extracts were prepared, and casein 
and EDTA were added as described. The collected media and cell 
extracts were incubated overnight at 4°C in microtiter plate wells, 
which had been coated with antibodies against tPA as described 
for the ELISA procedure. For controls, we used wells that had been 
coated with the same concentration of a monoclonal mouse antibody 
against FITC. After washing, the bound protein was dissolved with 
0.3 mol/L NaOH, neutralized with 1.5 mol/L HCl, and counted.

Inhibition of the constitutive and regulated tPA pathways by inhib-
iting protein synthesis. To inhibit protein synthesis, HUVECs were 
icubated with 5 µg/mL cycloheximide in total M199 for 0, 0.5, or 
1 hour. The acute release was then induced in the presence of 5 µg/ 
ml cycloheximide (without culturing the cells for 2 hours in defi-
cient medium). In this experiment, 2 µmol/L calcium ionophore 
A23187 was used to induce acute release of tPA, because calcium 
ionophore A23187 is a receptor-independent stimulant of tPA and 
vWF release, which is not dependent on protein synthesis. Also, 
A23187 has been shown to induce the acute release of tPA.

Measuring overall protein synthesis. Overall protein synthesis 
was determined by incorporating 35S-methionine into endothelial 
cell proteins and measuring the amount of 35S-methionine in the 
trichloroacetic acid-precipitable fraction, as described.

Induction of the acute release of tPA in vivo. A control group 
of eight male Wistar rats (Ifa-Credo, Someren, the Netherlands), 
weighing 200 to 300 g, were fed a standard laboratory diet. To a 
second group of eight animals, RA (8 mg/kg body weight) was 
orally administered daily for 5 days to increase tPA synthesis. A third 
group was intravenously injected with cholesterolic toxin (500 µg/ 
kg body weight) 48 hours before an experiment. Rats were anesthe-
thesized with Nembutal (60 mg/kg intraperitoneally) and were cannu-
lated, and bradykinin (30 µg/kg body weight) was injected as a bolus 
into the vein of the penis. Blood was collected through a carotid 
artery cannula, and citrated plasma was prepared. Rat tPA antigen 
concentrations were determined in citrated plasma by ELISA. Animal 
experiments had been approved by the Animal Experiments 
Committee of the Netherlands Organization for Applied Scientific 
Research TNO and were in accordance with the guidelines on animal 
experimentation presented to the International Committee of Throm-
busis and Haemostasis.

Assays. Human tPA antigen was measured by ELISA as de-
scribed. Recombinant human one-chain tPA (Activase; Genetech, 
San Francisco, CA) was used for making calibration curves in a 
range of 30 to 500 pg/mL. The detection limit in this assay is 10 
pg/mL. Both tPA and tPA-PAL1 complexes are detected with equal 
efficiency by this ELISA. Rat tPA antigen was measured by ELISA 
using rabbit-antirat tPA IgG, as described. vWF antigen was mea-
sured by ELISA, essentially as described, with the following minor 
modifications: the rabbit antihuman vWF IgG was diluted 1:2,000, 
and 0.1% (wt/vol) of casein was used in all buffers instead of 0.05% 
(wt/vol) bovine serum albumin. Human pooled plasma in a range 
of 0.078% to 1.25% was used for calibration. Lactate dehydrogenase 
activity was determined using a kit, according to the manufacturer’s 
(Sigma) instructions.

Fibrin zymography. Samples were put immediately in sample 
buffer containing 2% sodium dodecyl sulfate (SDS) and were frozen 
at −20°C. SDS/9% polyacrylamide slab gels were prepared ac-
cording to Laemmli. Gels were then washed, placed on top of a 
plasminogen-rich fibrin layer, and incubated for 36 hours at 37°C, 
as we described in detail elsewhere.

Data presentation and units used. The data are presented as 
mean ± SD of three measurements. Human tPA antigen is given 
as picograms or nanograms per milliliter, using Activase as standard. 
Rat tPA antigen is given as nanograms per milliliter, using rat L2 
tPA as standard. vWF antigen is given as units per milliliter, 100 
U being defined as the amount of vWF antigen present in 1 mL of 
pooled human plasma.

RESULTS

The regulated (acute) release of tPA in vitro. The constitu-
tive and regulated secretion of tPA was studied in vitro 
using first-passage human endothelial cells. In a representa-
tive experiment (Fig 1A), control cells constitutively se-
creted 13 pg of tPA per 2 × 10⁶ cells into the medium over 
10 minutes (1.3 pg tPA/min/2 × 10⁶ cells). This rate of 
constitutive tPA secretion resembled the rate of constitutive 
tPA secretion during the 30-minute preincubation period (1.5 
pg tPA/min/2 × 10⁶ cells) and during the 2 hours preceding 
the experimental period (2.2 pg tPA/min/2 × 10⁶ cells). 
These data suggested that no major changes in constitutive 
secretion occurred because of manipulation of the cells dur-
ing an experiment. On addition of human thrombin (1 NIH 
U/mL), the cells very rapidly released tPA into the medium. 
The highest increase of tPA occurred during the first minute, 
resulting in an additional 25 pg tPA/min/2 × 10⁶ cells in the 
medium. The tPA content of the extracts from thrombin-
treated cells was always lower than the content of the extracts 
from control cells (see Fig 1A). This difference in tPA con-
tent between extracts from thrombin-treated cells and control 
cells was of the same magnitude as the amount released 
into the medium. In five experimental series, the difference 
averaged 94% ± 30% (mean ± SD) of the amount of tPA 
acutely released by thrombin into the medium at 1 minute.

Because, in vivo, acute release of vWF and tPA are often 
observed together, the secretion of vWF was measured in this 
experiment as well. vWF was, similar to tPA, constitutively 
secreted by control cells, 0.033 U/10 min/2 × 10⁶ cells (see 
Fig 1B). On addition of thrombin (1 NIH U/mL), vWF was 
released slightly slower into the medium than tPA. The in-
crease in vWF was maximal at 3 minutes, at which time an 
additional 0.22 U/mL of vWF had been released into the 
medium. Figure 1B also shows that the increase of vWF in
The acute release of tPA was induced by 1 NIH U/mL of human thrombin for 3 minutes. The plasminogen activator activity in the conditioned media and cell extracts was visualized by fibrin zymography after SDS/polyacrylamide-gel electrophoresis. Lane 1, recombinant tPA (Activase); lane 2, conditioned medium of control cells; lane 3, conditioned medium of thrombin-treated cells; lane 4, cell extract of control cells (diluted 1:7); and lane 5, cell extract of thrombin-treated cells (diluted 1:4). The lower bands in lanes 2 to 5 correspond with the band of free, uncomplexed recombinant tPA (lane 1), which is denoted by the arrow (→). The upper bands in lanes 2 to 5 represent tPA complexed to PAI-1, which is denoted by the arrowhead (▲). All PA-activity could be quenched by antibodies against human tPA (data not shown).

To study whether free tPA or tPA-plasminogen activator inhibitor (PAI) complex was acutely released, samples of media and cell extracts were analyzed by fibrin zymography. Despite the presence of an approximately fivefold excess of PAI-1 in these media, complex formation is prevented by a 1:1 dilution in an SDS-containing buffer of the media, immediately after a 3-minute stimulation. Figure 2 shows that only free tPA, but not tPA:inhibitor complex, was acutely released into the medium on addition of thrombin (Fig 2, lanes 2 and 3), and that free tPA had disappeared from the cell extracts (Fig 2, lanes 4 and 5). Please note that tPA in cell extracts has the same mobility (ie, the same M,) as constitutively secreted and acutely released tPA in media (see Fig 2, lanes 2 through 5).

Dose-dependence of tPA release by thrombin. Figure 3 shows that thrombin increased the amount of tPA (and vWF) released in a dose-dependent manner. In this experiment, the constitutive secretion of tPA was 60 pg/2 × 10⁵ cells in 30 minutes. In the presence of 1 NIH U/mL of thrombin, maximal acute release of 40 pg/2 × 10⁵ cells was observed at 3 minutes. From 0.01 NIH U/mL upwards, the acute release exceeded the constitutive secretion by at least twice the standard deviation. Similar data were obtained for vWF (Fig 3).
STUDIES ON THE ACUTE RELEASE OF TPA

**Fig 3.** Thrombin dose-dependently induces the acute release of tPA and vWF. Concentrations of 0 to 3 NIH U/mL of thrombin were used to induce acute release of tPA and vWF. The amount of tPA (f; pg/2 x 10^6 cells) that was acutely released into the medium by the indicated concentration of thrombin and the amount of acutely released vWF (A; 10^-3 U/2 x 10^6 cells) are shown.

stimulation after maximal thrombin stimulation, cells treated with thrombin (1 NIH U/mL for 3 minutes) were subsequently incubated with calcium ionophore A23187 (1 µmol/L) for another 7 minutes. Cells treated with thrombin followed by ionophore did not release more tPA than did cells treated only with thrombin. The doubly stimulated cells released 114% ± 44% (n = 6; n.s.) of tPA, similar to the amount released by cells treated only with thrombin. Doubly stimulated cells released in 10 minutes 148% ± 73% (n = 6) of vWF, as compared with cells treated for 10 minutes with thrombin only.

**Constitutive tPA secretion and acute release of tPA in various cell isolates.** To investigate whether constitutive tPA secretion (representing tPA synthesis) influenced acute tPA release in HUVECs, a correlation study was performed with data from 17 isolates of HUVECs. These 17 isolates provided naturally occurring variable levels of constitutive tPA secretion (from 2 to 10 ng/mL/24 h). By linear regression analysis, a correlation between constitutive tPA secretion and acute release of tPA of 0.68 was found (Fig 4). The percentage of tPA in the cell extract that could be released averaged 33% with an SD of 17%.

**Constitutive tPA secretion and acute release of tPA: Decreased constitutive secretion of tPA.** When HUVECs were cultured in deficient medium, the levels of the three tPA parameters constitutive secretion, acute release, and cellular concentration (expressed as percentages of the level at t = 0) significantly decreased in parallel in time to about 20% after 24 hours (Fig 5; the absolute values of these parameters are given in the legend to the figure). These parallel changes suggest that constitutive secretion, acute release, and cellular content of tPA are closely linked by some, as yet undetermined, physiological process(es). At 24 hours, the rate of overall protein synthesis did not differ from that in control cells (127% ± 18%, n = 6), showing that the viability of cells cultured in deficient medium had not diminished. Furthermore, vWF release did not differ between cells cultured in deficient medium and cells cultured in total medium (data not shown). These data suggest that cells in deficient medium were as sensitive to thrombin stimulation as were cells cultured in total medium.

**Constitutive tPA secretion and acute release of tPA: Increased constitutive secretion of tPA.** HUVECs were treated with 0 to 3 mmol/L sodium butyrate or with 1 µmol/L RA to increase tPA synthesis. After 24 hours, acute release of tPA was induced. The constitutive secretion of tPA was enhanced dose-dependently by sodium butyrate, showing a 20-fold stimulation at 3 mmol/L (Fig 6). These

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**Fig 4.** tPA synthesis and thrombin-induced acute release of tPA in 17 isolates of HUVECs. In 17 isolates of HUVECs, the acute release of tPA was induced by 1 NIH U/mL of human α-thrombin, as described in Materials and Methods. The mean constitutive tPA secretion (during 30 minutes) and the mean tPA release (3 minutes after thrombin-addition) are plotted; the horizontal and vertical bars represent the corresponding SDs (n = 3). After linear regression, a correlation of 0.68 was found (n = 17).
increases in constitutive tPA secretion were paralleled by increased concentrations of tPA in the cell extracts and by increased acute release of tPA (Fig 6), again suggesting that the processes involved are closely linked. RA enhanced the constitutive secretion twofold, which resulted in a 1.1-fold increase of tPA in the cell extracts and in a 1.6-fold increase of acute release of tPA. Increasing the constitutive tPA secretion did not influence the time profile of acute release, whereas, in most experiments using thrombin stimulation followed by ionophore, all available tPA was released by thrombin (data not shown). Sodium butyrate and RA did not change constitutive vWF secretion, the amount of vWF in cell extracts, or the acute release of vWF.

An acutely releasable tPA pool in HUVECs. The close correlation between constitutive tPA secretion and acutely releasable tPA, described above, might suggest that acutely releasable tPA is derived from the same pathway as constitutively secreted tPA. If so, the induction of acute release of tPA should cause a decrease in constitutive secretion over the subsequent time period. This was studied as follows: at t = 0, acute release was induced with thrombin; after 3 minutes, hirudin (0.67 μg/mL) was added to the cells to inactivate thrombin; and the media were collected at 0, 3, 10, 60, and 120 minutes after the addition of thrombin. Figure 7 shows that thrombin-treated cells had released 17 pg tPA/10^5 cells at t = 3 minutes, and that this difference persisted for the next 2 hours. These data suggested that the releasable pool was not part of the constitutive tPA pathway. In a first attempt to discriminate between tPA following the constitu-

Fig 5. The effects of a decreased tPA synthesis on acute release of tPA. First-passage HUVECs were cultured for 0, 2, 4, 8, and 24 hours in deficient medium (without ECGF, HS, or heparin). Acute release was induced with 1 NIH U/mL of human thrombin. The absolute amounts of tPA constitutively secreted, acutely released, and present in cell extracts at t = 0, shown as mean ± SD (n = 3), were 47 ± 4, 59 ± 9, and 240 ± 0 (pg/2 × 10^5 cells), respectively. The constitutive tPA secretion (m), the acute release of tPA (A), and the amount of tPA in the cell extracts (e) are expressed as percentages of the concentrations at t = 0 hours.

Fig 6. Sodium butyrate increases tPA synthesis and tPA release. First-passage HUVECs were cultured for 24 hours with various concentrations of sodium butyrate. Acute release of tPA was then induced as described in Materials and Methods. The constitutive tPA secretion (A), the acute release of tPA (e), and the amount of tPA in the cell extracts (m) are shown as mean ± SD (n = 3).

Fig 7. The secretion of tPA over a longer period. At t = 0, acute release was induced as described above, and the reaction was stopped at t = 3 minutes with hirudin. The concentration of tPA was measured after 0, 3, 10, 60, and 120 minutes. tPA is continuously secreted into the medium (A), and, on addition of thrombin, additional tPA is secreted into the medium (e), which is still found in the medium after 60 and 120 minutes. Data shown are mean ± SD (n = 3).
In this study, we addressed the questions of how acute release of tPA from human endothelial cells proceeds and how this release is related to the constitutive secretion of tPA. Using first-passage HUVECs, we observed, as have many others, constitutive secretion of tPA into the medium at a steady rate over many hours. On the addition of thrombin, acute release occurred, as evidenced by the rapid appearance of uncomplexed, enzymatically-active tPA in the medium. The acute release of tPA was very rapid, showing a maximal increase of tPA within 1 minute. In both these respects, the acute release reaction in vitro resembled acute release of tPA as observed in humans in vivo. Release induced by thrombin was dose-dependent. At maximal stimulation by thrombin (≥ 1 NIH U/mL), most if not all endothelial tPA was released into the medium, because a second

tative or regulated pathway, we analyzed the retention time of tPA. To this end, HUVECs that had been cultured for 24 hours with butyrate were metabolically labeled with 35S-methionine, chased for 0 to 4 hours with an excess of unlabeled methionine, and then induced to release tPA. Constitutive secretion and acute release of labeled tPA were determined by overnight binding of radiolabeled material in the media to anti-tPA-coated microtiter wells. These data (Fig 8) are expressed as percentages of the amount of radiolabeled tPA at t = 0 (ie, unchased). Background binding to anti-FITC coated wells was about 6% for unchased media. Binding of radiolabeled tPA could not be determined in cell extracts, because background binding on anti-FITC-coated plates was too high to permit reliable determination of specific binding (data not shown). As shown in Fig 8, after a 1-hour chase with unlabeled methionine, the amount of radioactivity bound from media of unstimulated cells was less than 50% relative to the amount bound from unchased medium of unstimulated cells. In media from thrombin-stimulated cells, the amount of radiolabel additionally released by thrombin was still 100% relative to the amount additionally released from unchased cells. After 2 hours, both pathways contained about 25% of the amount of label present in unchased media. Therefore, these data suggest that the releasable tPA pool is separate from the constitutive secretion pathway. The inset of Fig 8 shows that comparable data are obtained when protein synthesis is inhibited with cycloheximide (overall protein synthesis, as measured by incorporation of radiolabeled methionine, was blocked for 90% after treatment of the cells for 1 hour with cycloheximide). The constitutive secretion of tPA antigen was decreased by 57% after 1 hour. In contrast, the acutely released amount of tPA antigen was still 114%, supporting the evidence for a separate intracellular source for tPA. Blocking of protein synthesis for more than 1 hour severely reduced the cellular adenosine triphosphate content (data not shown); thus, experiments were not continued beyond 1 hour.

Acute release of tPA in vivo. To study the relation between constitutive secretion and acute release of tPA in vivo, rats were pretreated for 2 days with cholera toxin or for 5 days with RA to increase tPA synthesis. After 2 days of cholera toxin treatment, tPA mRNA levels in heart and lung were significantly increased as were the tPA antigen concentrations, showing increased tPA synthesis in these animals (data not shown). Treatment of rats with RA also resulted in increased levels of tPA activity and tPA antigen in several tissues. Treatment with cholera toxin increased the steady-state plasma levels of tPA 5.4-fold as compared with that for control animals (ie, t = 0 in Fig 9); treatment with RA increased tPA plasma levels 1.4-fold (Fig 9). The acute release of tPA was then induced in these animals by bradykinin. As shown in Fig 9, the acute release of tPA had, at 1 minute, increased 1.9-fold in RA-treated rats and 3.7-fold in cholera toxin-treated animals, as compared with that for control animals. These data indicate that an increased tPA synthesis gives rise to an increased acute release of tPA in vivo as well.

**DISCUSSION**

In this study, we addressed the questions of how acute release of tPA from human endothelial cells proceeds and how this release is related to the constitutive secretion of tPA. Using first-passage HUVECs, we observed, as have many others, constitutive secretion of tPA into the medium at a steady rate over many hours. On the addition of thrombin, acute release occurred, as evidenced by the rapid appearance of uncomplexed, enzymatically-active tPA in the medium. The acute release of tPA was very rapid, showing a maximal increase of tPA within 1 minute. In both these respects, the acute release reaction in vitro resembled acute release of tPA as observed in humans in vivo. Release induced by thrombin was dose-dependent. At maximal stimulation by thrombin (≥ 1 NIH U/mL), most if not all endothelial tPA was released into the medium, because a second
The amount of thrombin-releasable tPA strongly correlated with its rate of constitutive secretion (ie, tPA synthesis) in HUVECs (Figs 4 through 6). A similar correlation was found in vivo in rats (Fig 9). Because the constitutive secretion of tPA (tPA synthesis) was coupled, at least quantitatively, so closely with the release of tPA, the question arose how closely the two pathways are coupled functionally. The following evidence suggests that both pathways are not identical. Thrombin-induced release of tPA does not diminish the subsequent constitutive secretion (Fig 7), indicating that the former is not a precursor of the latter. After metabolic labeling (Fig 8), released tPA retains label about 1 hour longer than constitutively secreted tPA. Also, after inhibition of protein synthesis by cycloheximide, release of tPA is maintained for at least 60 minutes at control level, whereas the level of constitutively secreted tPA decreases strongly. These observations suggest that releasable tPA was delayed in an additional compartment. This conclusion is supported by the observation that acutely released tPA is not complexed to an inhibitor (Fig 2). Because tPA in the subcellular matrix and tPA on the plasma membrane are complexed to PAI-1, the tPA released must be derived from an intracellular compartment (see also Fig 2, lane 4), in line with previously published data, which showed that plasma membrane-bound tPA is not released. Moreover, on subcellular fractionation, the tPA content of an intracellular high-density fraction, free of plasma membrane markers, is diminished after stimulation with thrombin (Schrauwen et al and Emeis and van den Eijnden-Schrauwen, manuscript in preparation). Therefore, we propose that, after synthesis, tPA is temporarily stored in an intracellular compartment, presumably consisting of high-density granules. In all these respects, the pathway followed by tPA destined for acute secretion resembles that of other secretory proteins, although the storage pool is less permanent than pools of other stored proteins.

We observed a close correlation between the rate of synthesis of tPA and the amount of acutely releasable tPA. This suggests that a fixed percentage of newly synthesized tPA enters the tPA storage compartment, regardless of the rate of synthesis. This has also been described for vWF storage.

tPA that is not acutely released from this pool into the medium still disappears from the pool, because the time tPA stays in the pool is only about 1 hour. What happens to this tPA is not known. We call this tPA that has disappeared "lost tPA," because it is not secreted in the constitutive pathway (Fig 7) or in the acute release pathway. One possibility is that this lost tPA is deposited in the subcellular matrix. However, we have been unable to detect the required amounts of tPA in the matrices of our cell cultures. Another possibility is that the lost tPA is degraded intracellularly, possibly in a lysosomal compartment, because label leaves the storage compartment but is not recovered in the constitutive pathway (Fig 8; compare also Fig 7). In an attempt to find further evidence for this hypothesis, we incubated cells for 4 hours in conditions that diminish lysosomal enzyme activity (50 μmol/L chloroquine). Under these conditions, the constitutive secretion did not change, whereas the amount of tPA in cell extracts did indeed increase. However, this increase could account for only some 10% of the lost tPA (our unpublished observations).

The storage pool in HUVECs (residence time about 1 hour) was less stable than the tPA storage pool in vivo in rats, the latter pool being hardly diminished by inhibiting protein synthesis for 5 hours. This might explain why endothelial cells in vitro possess a relatively small storage pool for tPA as compared with that for rats. In cell extracts from HUVECs about 0.1 ng of tPA/cm² is present, whereas 0.5 to 2 ng of tPA/cm² has been estimated to be present in vivo (calculations in Emeis).
or growing thrombi. Indeed, in vivo in primates, thrombin formation has been shown to induce acute release of large amounts of tPA. In quantitative terms, acute secretion of 50 pg tPA/cm² (as in Fig 6) will result, in a capillary with a diameter of 6 μm, in a local concentration of 340 ng tPA/mL. If tPA synthesis is increased (Fig 6), up to 900 pg tPA/cm² can be released, resulting in a local concentration of 6 μg tPA/mL in that same capillary (compare with Schrauwen et al 1). This concentration is even higher than the plasma tPA concentration reached during thrombolytic therapy. Moreover, tPA present before or during coagulation will be much more potent in dissolving a thrombus than tPA present only after thrombus formation has occurred, and is the case during thrombolytic therapy.

The results of this study show that in human endothelial cells tPA can be acutely released from an intracellular pool, and that it should be possible to enhance this capacity for acute tPA release by increasing tPA synthesis. Therefore, in combination with the possibility to induce the release of tPA without inducing the release of vWF, it should be possible to increase local tPA concentrations specifically and to speed up fibrinolysis/thrombolysis when required.

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Studies on the acute release of tissue-type plasminogen activator from human endothelial cells in vitro and in rats in vivo: evidence for a dynamic storage pool

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