Vascular endothelial cells are thought to be the main source of plasma tissue-type plasminogen activator (t-PA) and von Willebrand factor (VWF). Previous studies have suggested that both t-PA and VWF are acutely released in response to the same stimuli, both in cultured endothelial cells and in vivo. However, the subcellular storage compartment in endothelial cells has not been definitively established. We tested the hypothesis that t-PA is localized in Weibel-Palade (WP) bodies, the specialized endothelial storage granules for VWF. In cultured human umbilical vein endothelial cells (HUVECs), t-PA was expressed in a minority of cells and found in WP bodies by immunofluorescence. After up-regulation of t-PA synthesis either by vascular endothelial growth factor (VEGF) and retinoic acid or by sodium butyrate, there was a large increase in t-PA-positive cells. t-PA was exclusively located to WP bodies, an observation confirmed by immunoelectron microscopy. Incubation with histamine, forskolin, and epinephrine induced the rapid, coordinate release of both t-PA and VWF, consistent with a single storage compartment. In native human skeletal muscle, t-PA was expressed in endothelial cells from arterioles and venules, along with VWF. The 2 proteins were found to be colocalized in WP bodies by immunoelectron microscopy. These data indicate that t-PA and VWF are colocalized in WP bodies, both in HUVECs and in vivo. Release of both t-PA and VWF from the same storage pool likely accounts for the coordinate increase in the plasma level of the 2 proteins in response to numerous stimuli, such as physical activity, β-adrenergic agents, and 1-deamino-8d-arginine vasopressin (DDAVP) among others. (Blood. 2002;99:3637-3645)
rapid release kinetics. In their experiments, t-PA expression was up-regulated by cell pretreatment with sodium butyrate. In vivo, t-PA has also been mainly located to endothelial cells, but again the subcellular t-PA storage compartment has not clearly been identified. To clarify these issues, we have reinvestigated the localization of t-PA both in HUVECs after up-regulation of endogenous t-PA expression, and in endothelial cells in vivo.

Materials and methods

RPMI 1640 and M199 media were from Gibco BRL (Gaithesburg, MD), fetal calf serum (FCS) and collagenase were from Seromed (Berlin, Germany). Endothelial cell growth supplement (ECGS) was from Upstate Biotechnology (Lake Placid, NY). Anti-VWF antibodies were from Dako (Glostrup, Denmark). Anti- t-PA antibodies (monoclonals ESPI, ESP4, ESP5, PAM3, and goat polyclonal) were from American Diagnostica (Greenwich, CT). Fluorescein isothiocyanate (FITC)–conjugated goat antimouse, Texas Red–conjugated goat antirabbit and Texas Red–conjugated donkey antigoat antibodies were from Jackson laboratories (West Grove, PA). FITC–conjugated sheep antirabbit antibodies were from Roche (Rotkreuz, Switzerland). Recombinant t-PA (Actilyse) was provided by Dr J. Krause (Dr K. Thomae GmbH, Biberach an der Riss, Germany); dilutions of t-PA were made in 0.2 M L-arginine, 0.11 M phosphate, 0.01% Tween 80, pH 7.2. Histamine, human thrombin, IBMX (3-isobutyl-1-methyl-xanthine), epinephrine, and forskolin were from Sigma (St Louis, MO). VEGF, all-trans retinoic acid (ATRA), sodium butyrate, and cycloheximide were also from Sigma.

Cell culture

Primary cultures of HUVECs were obtained from individual human umbilical veins by collagenase digestion as described previously. They were grown in RPMI 1640 or M199 media supplemented with 10% FCS, 90 μg/mL heparin, and 15 μg/mL ECGS. Cells were used during passages 1 or 2. Tissue culture dishes as well as the 24-well plates (Costar, Cambridge, MA) and the glass coverslips were coated with 0.1% gelatin.

Secretion studies

Confluent monolayers of HUVECs grown in 24-well dishes were pretreated with VEGF, ATRA, or sodium butyrate in complete medium for 20 hours. They were then washed 3 times and preincubated in 1 mL Krebs Ringer–bicarbonate buffer (120 M NaCl, 4.75 M KCl, 1.2 M KH2PO4, 0.6 M MgSO4, 1.2 M CaCl2, 25 M NaHCO3, 25 M Hepes, pH 7.4 [KRBH], supplemented with 0.1% bovine serum albumin [BSA]) for 5 minutes at 37°C. After a fourth wash, cells were incubated in 0.3 mL to 0.5 mL KRBH with the different agents for 30 minutes. The supernatants were precleared 37°C until the assays. All pharmacologic agents were either directly dissolved in incubation medium or dissolved in dimethyl sulfoxide (DMSO). The 96-well plates were coated with monoclonal anti- t-PA antibodies ESP1 and ESP5, 1 μg/mL each in carbonate buffer (NaCO3 50 mM, pH 9.6), 100 μL/well. After 4 washes with TBS-T (Tris 20 mM, NaCl 140 mM, Tween-20 0.1%, pH 7.4), the remaining protein binding sites were blocked with 100 μL/well blocking solution (TBS-T plus 3% BSA) added for 2 hours at 37°C. Samples and standards diluted in blocking solution (0.1 mL/well) were then added and incubated overnight at 4°C. After 4 washes with TBS-T, a goat anti–human t-PA antibody (American Diagnostica, no. 381) diluted 1:2000, was added for 2 hours at 37°C. A third incubation with a rabbit anti–goat antibody conjugated to horseradish peroxidase (HRP) (Dako P0449, diluted 1:10 000) was performed for 2 hours at 37°C. Finally, bound HRP activity was detected using o-phenylene diamine as a substrate and measuring optical density at 490 nm. A standard curve was constructed using purified recombinant t-PA. t-PA was reliably detected at concentrations of 0.025 ng/mL to 3.2 ng/mL. The assay recognizes t-PA both in free form and bound to PAI-1. Indeed, the optical signal obtained with recombinant t-PA was not modified by adding a 50-fold excess of purified PAI-1. The excess PAI-1 caused a quantitative shift of t-PA to a PAI-1-bound, high-molecular-weight complex as determined by Western blot (not shown).

VWF was measured by ELISA as described previously. A standard curve was constructed from serial dilutions of normal pooled plasma, assuming a plasma concentration of 10 pg/mL. Results are expressed in ng/well per unit time. Unless indicated otherwise, results are shown as the mean ± SEM. Statistical analysis was done using the 2-tailed, paired Student t test.

Cell immunofluorescence

HUVECs grown on glass coverslips were fixed for 30 minutes in 3.7% formaldehyde and permeabilized for 15 minutes with 0.5% Triton X-100 in phosphate buffered saline (PBS). The coverslips were then incubated with the anti-t-PA monoclonal antibody ESP-4 diluted at 1:100 and a rabbit anti–human VWF antibody diluted at 1:30 000, and subsequently with FITC–conjugated goat antimouse and Texas Red–conjugated goat antirabbit antibodies, both diluted at 1:200. The slides were mounted with ProLong Antifade (Molecular Probes, Eugene, OR) and examined using a confocal microscope (Zeiss LSM 410, Germany).

Cell transduction with recombinant adeno virus

t-PA recombinant adeno virus (AdCMV-t-PA) was propagated on a monoc- layer of 293 cells and titrated by plaque assay, as previously described. HUVECs grown to 50% confluency were transduced for 1 hour at 37°C in serum-free media. For transduction, cells were washed and incubated for 48 hours at 37°C before fixation.

Tissue immunofluorescence

Samples of abdominal skin, gastrocnemius, and serratus anterior muscles were obtained at plastic surgery from unaffected patients who had given informed consent, in accordance with the guidelines of our institutional committee for clinical investigation. All samples were rapidly frozen in liquid nitrogen and cryosectioned. Sections were fixed in 4% paraformaldehyde containing 0.1% Triton X-100 and processed as previously described. 21 Briefly, sections were rinsed, incubated for 2 hours at room temperature with either the rabbit polyclonal serum against von Willebrand factor (diluted 1:20 000) or the goat polyclonal serum against t-PA (diluted 1:50), rinsed again and incubated for one hour at room temperature with either FITC–conjugated sheep anti–rabbit antibodies (diluted 1:500) to detect VWF or Texas Red–conjugated donkey anti–goat antibodies (diluted 1:200) to detect t-PA. After rinsing, sections were viewed with an Axioskop microscope (Zeiss, Oberkochen, West Germany). In all experiments, negative controls included exposure of sections during the first incubation to either normal rabbit or goat serum, fluorescein-conjugated anti–rabbit antibodies or Texas Red-conjugated anti–goat antibodies. None of these incubations resulted in a specific staining of the tissues examined (not shown).

Immunoelectron microscopy

HUVECs or tissue fragments were fixed in 1.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Cryosections were made using an ultracytomic- rotome (Reichert Ultracut S) and ultrathin sections mounted on Formvar-coated gold grids were prepared. During incubations at room temperature the grids were floated on the surface of droplets as previously described. Briefly, the sections were incubated for 15 minutes with PBS 15% glycine; for 5 minutes with PBS 15% glycine, 0.1% BSA; and for 20 minutes with PBS 15% glycine, 0.1% BSA, 10% normal goat serum followed by 1 hour incubation with a mixture of the mouse monoclonal anti-t-PA antibodies ESP-4 and PAM 3 and/or with a rabbit polyclonal antibody to VWF. The
primary antibodies were diluted 1:50 in PBS 15% glycine, 0.1% BSA, 4% normal goat serum. After extensive rinsing in PBS 15% glycine, 0.1% BSA, sections were incubated for 30 minutes with gold-labeled secondary goat antimouse or goat antirabbit antibody or both in case of double-labeling, with a gold particle size of 10 nm (GAM 10) and/or 5 nm (GAR5), respectively (British Biocell, Cardiff, Wales). Sections were then washed for 30 minutes with PBS 15% glycine, stained with 2% uranyl acetate for 10 minutes, and air dried. Examination was performed in a Philips CM 10 electron microscope.

Results
Up-regulation of t-PA synthesis from HUVECs

The localization of t-PA in HUVECs, as well as the regulation of t-PA secretion are difficult to study given the low levels of t-PA expression in these cells. To circumvent this problem, we set up an ultrasensitive ELISA for t-PA. This ELISA allowed the detection of free or complexed t-PA at a concentration of 0.025 ng/mL (see “Materials and methods”). This high sensitivity permits studies on the regulation of t-PA synthesis and release. Basal t-PA release from confluent HUVECs was 0.71 ± 0.04 ng/well over 20 hours (mean ± SD). Incubation for 20 hours with VEGF (40 ng/mL), ATRA (10⁻⁶ M), or sodium butyrate (3 mM), caused a 3.3-, 1.9-, and 13-fold increase in basal t-PA release, respectively. VEGF and ATRA added together had an additive effect, with a 5.1-fold increase in t-PA release. Estradiol (100 nM) had no effect. Thus, ATRA, VEGF, and sodium butyrate all cause an increase in synthesis and constitutive release of t-PA, in agreement with previous reports.

Acute agonist-induced t-PA release from HUVECs

Acute VWF release from WP bodies occurs in response to a variety of agonists, that act either via an increase in intracellular free calcium ([Ca²⁺]), or via activation of adenylate cyclase and an increase in cellular cAMP. Histamine is a potent activator of the [Ca²⁺]-dependent pathway. Forskolin, a direct activator of adenylate cyclase, induces VWF secretion when added together with IBMX (an inhibitor of the phosphodiesterases that degrade cAMP). Epinephrine, which acts via a receptor-mediated, G-protein-coupled pathway, also raises cAMP and induces VWF secretion, although its effect is weaker than that of forskolin (Figure 1B, and Vischer and Wollheim). We tested whether these agonists also induce acute t-PA release.

Confluent HUVEC monolayers were incubated with histamine (10⁻⁵ M), forskolin (10⁻⁵ M), and epinephrine (10⁻⁶ M) for 30 minutes at 37°C. The latter 2 agents were added together with IBMX (10⁻⁴ M) (Figure 1A). Histamine, forskolin, and epinephrine induced a 2.0-, 2.4-, and 1.5-fold increase in t-PA release, respectively (from 0.016 ± 0.002 to 0.031 ± 0.005, 0.040 ± 0.008, and 0.024 ± 0.003 ng/well per 30 minutes, respectively; mean ± SEM, n = 4). These values were close to the assay detection level and the results of borderline statistical significance (P = .05-.1).

The acute release experiments were also performed after up-regulation of t-PA synthesis. After a combined VEGF and ATRA pretreatment, acute t-PA release increased 3.1-, 3.2-, and 1.5-fold in response to histamine, forskolin, and epinephrine, respectively (from 0.05 ± 0.002 to 0.17 ± 0.03, 0.17 ± 0.03, and 0.08 ± 0.01 ng/well per 30 minutes; n = 4; P < .003 for all 3 comparisons). After sodium butyrate pretreatment, the corresponding increases were 2.6-, 1.6-, and 1.3-fold, respectively (from 0.44 ± 0.1 to 1.14 ± 0.22, 0.74 ± 0.1, and 0.60 ± 0.13 ng/well per 30 minutes; n = 4; P < .004 for all 3 comparisons). IBMX added alone had no effect on t-PA release (data not shown). Thus, the up-regulation of t-PA synthesis by pretreatment with either VEGF and ATRA or sodium butyrate is associated with the induction of a rapidly releasable t-PA pool. In these experiments we verified that histamine, forskolin, and epinephrine induce acute VWF secretion as previously reported (Figure 1B). The rapidly releasable t-PA pool is thus responsive to the agonists that induce acute VWF release.

To confirm that t-PA is released from a preformed store, the secretion experiments were repeated in the presence of cycloheximide (CHX), an inhibitor of protein synthesis. HUVECs were incubated with CHX (5 μg/mL) or medium alone for 4 hours and then subjected to acute secretion experiments (Figure 2A). Basal t-PA release was strongly decreased by CHX pretreatment, indicating an inhibition of t-PA synthesis and constitutive release. Increases in t-PA release were still observed in response to histamine, forskolin, and epinephrine. After subtraction of the
corresponding control value, there was no decrease in t-PA release in response to these 3 agonists. Similar results were obtained when the cells were pretreated with sodium butyrate, added to increase t-PA synthesis (Figure 2B). CHX did not affect basal or agonist-induced VWF release (not shown). These data confirm the presence of an agonist-sensitive, preformed t-PA storage compartment in HUVECs.

t-PA localization in HUVECs

We studied the subcellular localization of t-PA in HUVECs by immunofluorescence. HUVECs grown on glass coverslips were preincubated with either VEGF and ATRA or sodium butyrate, to increase t-PA synthesis and storage. The cells were then fixed, permeabilized, and stained by double-labeling immunofluorescence for t-PA and VWF (Figure 3). In untreated HUVECs, only occasional cells were positive for t-PA. In these cells, t-PA was localized to WP bodies, identified as rod-shaped granules that were stained with anti-VWF antibodies (Figure 3A,B). No other structures were stained by anti-t-PA antibodies. After pretreatment with VEGF and ATRA, the number of cells positive for t-PA was markedly increased, and t-PA was again colocalized with VWF in WP bodies (Figure 3C,D). After pretreatment with sodium butyrate, the majority of cells were positive for t-PA, and the t-PA stain was again entirely localized to WP bodies (Figure 3E,F). t-PA was occasionally seen in the perinuclear area, but no particulate structure other than WP bodies was revealed by anti-t-PA antibodies. Thus, increased t-PA synthesis is associated with increased t-PA storage in WP bodies.

t-PA localization to WP bodies in HUVECs was further documented by immunoelectron microscopy (Figure 4). To increase t-PA expression, HUVECs were infected with t-PA-expressing adenovirus, as previously described. After 48 hours, the cells were trypsinized, pelleted, and fixed in 1.5% glutaraldehyde (Figure 4). After immunostaining, t-PA was seen as 10-nm gold particles over WP bodies, identified as elongated electron-dense organelles with parallel tubular structures. Specific labeling was observed in no other intracellular structures (Figure 4A). t-PA was not found in untreated HUVECs, in accordance with the low levels of t-PA expression observed by ELISA and by immunofluorescence (Figure 4B). In double-label experiments, the elongated electron-dense structures that bound t-PA antibodies (seen as 10-nm beads) also bound anti-VWF antibodies (seen as 5-nm beads), confirming their identity as WP bodies (Figure 4C). We also

Figure 2. t-PA is released from a preformed, cycloheximide-resistant cellular pool. Confluent HUVECs were pretreated for 20 hours with control medium (panel A) or sodium butyrate (3 mM) (panel B). The cells were then incubated for 4 hours in the absence (black bars) or presence (gray bars) of cycloheximide (5 μg/mL) to block protein synthesis, followed by stimulation for 30 minutes with histamine, forskolin, and epinephrine as in Figure 1. The supernatants were assayed for t-PA by ELISA. Sodium butyrate pretreatment caused a large increase in basal and stimulated t-PA release (compare scales in panels A and B). Cycloheximide decreased t-PA release from unstimulated cells but did not inhibit agonist-induced t-PA release. Results are the mean ± SEM of 4 independent experiments; *P < .05 by the paired Student t-test, compared to CHX alone.

Figure 3. t-PA colocalizes with VWF in WP bodies. Preconfluent HUVECs were pretreated with control medium (panels A and B), VEGF (40 ng/mL) and ATRA (10⁻⁶ M) (panels C and D), and sodium butyrate (NaBut; 3 mM) (panels E and F). The cells were then processed for double-label indirect immunofluorescence. Identical fields are shown for t-PA (FITC channel; panels A, C, and E) and VWF (Texas Red channel; panels B, D, and F). WP bodies are identified as rod-shaped granules that stain with anti-VWF antibodies. The proportion of t-PA-positive cells was markedly increased in response to VEGF/ATRA and NaBut. t-PA was always colocalized with VWF in WP bodies, with no evidence for an alternative storage pool. The bar represents 20 μm.
studied cells pretreated with sodium butyrate (Figure 5). WP bodies were again identified as electron-dense, elongated, and striated structures stained by anti-VWF antibodies (Figure 5A). Similar structures were also labeled by anti–t-PA antibodies (Figure 5B,C). These results confirm that both heterologous and endogenous t-PA are localized to WP bodies in HUVECs.

**The subcellular localization of t-PA in human tissues**

To evaluate whether t-PA is localized to WP bodies also in native tissues, we studied human skeletal muscle, since acute t-PA release has been demonstrated in human forearm perfusion studies. Muscle specimens obtained at surgery were quickly frozen, sectioned, and...
fixed with paraformaldehyde. t-PA and VWF were then visualized by double-label immunofluorescence (Figure 6). VWF antibodies stained the intimal layer of endothelial cells of arterioles and venules, whereas capillaries were usually not stained. t-PA antibodies identified only some of the VWF-positive arterioles and venules. In these cases, t-PA was localized in the intimal layer of these vessels, but again not in capillaries (Figure 6). Double labeling of the same sections with different fluorochromes showed that t-PA and VWF were expressed by the same set of cells (Figure 6). A similar distribution of both t-PA and VWF was observed in human skin specimens (not shown).

The subcellular localization of t-PA in these vessel segments was studied by immunoelectron microscopy (Figure 7). Tissue fragments of skeletal muscle were fixed in 1.5% glutaraldehyde, and thin sections were sequentially incubated with anti-t-PA antibodies and anti-mouse Ig antibodies coupled to gold particles. In arterioles, the gold particles indicating the presence of t-PA were found concentrated over WP bodies, identified as elongated, striated, electron-dense structures. WP body labeling was highly specific; in particular, no gold particle concentration was seen over other dense structures such as mitochondria. These results indicate the presence of t-PA in WP bodies in vascular endothelial cells in vivo.

Discussion

Our results provide direct evidence that t-PA is localized to WP bodies, both in cultured HUVECs and in vivo. In previous work, we have found that t-PA is expressed at low levels in HUVECs. However, after heterologous expression via an adenoviral system, higher amounts of t-PA were also found in WP bodies. The present study extends these results and shows that endogenous t-PA is localized to WP bodies. In untreated HUVECs or after up-regulation of t-PA synthesis with VEGF, ATRA, or sodium butyrate, t-PA was found in WP bodies by immunofluorescence. By immunoelectron microscopy, t-PA was found in WP bodies after heterologous t-PA overexpression and after up-regulation of endogenous t-PA by sodium butyrate. We found no evidence for any other storage compartment. We also demonstrate agonist-induced t-PA release from HUVECs in less than 30 minutes, including after pretreatment with CHX, an inhibitor of protein synthesis. The rapid
time course and the lack of inhibition by CHX indicate that agonist-induced t-PA release is due to release from a preformed store rather than to increased synthesis. Acute t-PA release occurred in response to histamine, forskolin, and epinephrine, which are all well-characterized agonists for VWF release from WP bodies. These morphologic and functional data indicate that both t-PA and VWF are stored in and released from WP bodies. Importantly, we demonstrate by immunoelectron microscopy that t-PA is localized to WP bodies of endothelial cells of skeletal muscle vessels, also in vivo.

The existence of a t-PA storage compartment other than that represented by WP bodies has been proposed by Emeis et al. In cell fractionation experiments of rat lung, these authors found t-PA at a different density than VWF on sucrose gradients, although t-PA and VWF migrated at the same density on Nycodenz gradients. In HUVECs, they identified t-PA in small, round vesicles by immunofluorescence and immunoelectron microscopy. Although our approach was quite similar, we found no evidence for such a distinct vesicle type. We have no obvious explanation for these discrepancies. Like these authors, we performed t-PA immunolocalization after pretreating HUVECs with sodium butyrate, and we used culture conditions that are nominally quite similar. Datta et al reported t-PA localization to WP bodies in most HUVECs, even without pretreatment, again without obvious differences in cells or culture conditions used. These divergent results are likely due to subtle, unaccounted differences in culture conditions. The relevance of
our results is strongly supported by our finding that t-PA is localized to WP bodies also in vivo, at least in the arterioles of native skeletal muscle.

The conclusion that t-PA and VWF colocalize in WP bodies implies that the 2 proteins are coordinately released in response to secretion agonists. Indeed, our secretion studies in HUVECs indicate a coordinate t-PA and VWF release in response to calcium-mobilizing agents such as histamine, and to cAMP-raising agents such as forskolin and epinephrine. In vivo, physical activity, β-adrenergic agents, and intravenous injection of DDAVP induce an increase in the plasma levels of both t-PA and VWF. In human forearm perfusion studies, t-PA release was induced by DDAVP, bradykinin, and purine nucleotides among other agonists. However, contrary to our prediction, DDAVP failed to induce coordinate VWF release. Thus, DDAVP appears to induce VWF secretion after intravenous injection but not after perfusion into the brachial artery. We have previously shown a direct effect of DDAVP on VWF release from WP bodies in endothelial cells. We believe it is extremely unlikely that DDAVP induces t-PA release not only from WP bodies, but also from an additional t-PA storage compartment that escaped detection in our study. This hypothesis would imply that DDAVP results in exocytosis from such a compartment but not from WP bodies. The discrepancy in t-PA and VWF secretion could also be explained by analytical considerations. In forearm perfusion studies, net t-PA release is calculated as the product of the arterio-venous difference in t-PA concentration and blood flow. Since DDAVP also increases blood flow, significant increases in t-PA release may translate into fairly small increases in the arterio-venous difference in t-PA concentration. VWF has a much longer half-life than t-PA (6-12 hours vs 5-10 minutes), accounting in part for a higher basal circulating level (≈10 μg/ml vs 5-10 ng/mL). It is therefore quite likely that after an acute stimulus, a smaller relative increase in the arterio-venous difference in VWF levels would have escaped detection. It is also...
worth noting that the relative level of expression of t-PA and VWF has not been studied in detail in different human organs and vessel types. In mice, VWF expression in skeletal muscle is very low. If confirmed in humans, this observation would suggest that the t-PA/VWF ratio may be high in skeletal muscle, contributing to the apparent increase in t-PA but not in VWF release after DDAVP in forearm perfusion studies.

In conclusion, we have shown that t-PA is colocalized in Weibel-Palade bodies, along with VWF, both in HUVECs and in endothelial cells in native skeletal muscle vessels. Release of both t-PA and VWF from the same storage pool likely accounts for the coordinate increase in the plasma level of the 2 proteins in response to numerous stimuli such as physical activity, β-adrenergic agents, and DDAVP.

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References

Tissue-type plasminogen activator (t-PA) is stored in Weibel-Palade bodies in human endothelial cells both in vitro and in vivo

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