Tissue-type plasminogen activator (tPA) is a fibrinolytic enzyme used in the treatment of arterial and venous thromboembolic diseases. The beneficial effects of tPA in managing acute coronary syndromes are clear. In contrast, the beneficial effects of tPA in the treatment of stroke are less striking, and its effectiveness in the management of pulmonary embolism is uncertain (2, 19, 20). Indeed, results of a recent meta-analysis questioned the utility of tPA in reducing mortality from pulmonary embolism in hemodynamically stable patients or in preventing fatal recurrence compared with heparin alone (18).

Why a potent fibrinolytic agent like tPA has variable utility in disorders caused by thrombotic vascular occlusion is not entirely evident and may be related to its extrinsic fibrinolytic signal-transducing activities (11). tPA is a multifunctional protein (Fig. 1) composed of an aminoterminal growth factor-like domain, a finger-like domain, two kringle domains, and the carboxyterminal light chain that contains both the catalytic site and the plasminogen activator inhibitor type 1 (PAI-1) docking site (9), with each domain exerting potentially deleterious off-target, i.e., nonfibrinolytic, activities.

We reported that tPA regulates the contractility of smooth muscle cells in isolated blood vessels (11) and cerebrovascular contractility and cerebral blood flow (3, 11). tPA also increases the permeability of the blood-brain barrier (BBB) (1, 17, 21). Prior studies also indicate that the effect of tPA on cerebrovascular contractility and permeability contribute to its hemorrhagic and neurotoxic side effects and that neutralizing either or both of these extrinsic fibrinolytic effects improves neurological outcome after thrombolytic therapy for acute thromboembolic stroke (5).

This strategy is based on the finding that tPA is an “allosteric” enzyme that is regulated through its PAI-1 docking site (11). Occupancy of this regulatory site by specific PAI-1-derived peptides abolishes its signal-transduction effects in the vasculature and the attendant side effects without attenuating its beneficial fibrinolytic effects (5). Similar effects may occur in the lung.

tPA exerts its deleterious effects in the brain in part by activating receptors for N-methyl-D-aspartate (NMDARs) (14). Activation of NMDARs by endogenous glutamate also contributes to local vasodilation and opening of the BBB during hypoxia/reoxygenation (8). NMDARs have also been identified in the lungs, where activation by the agonist glutamate induces pulmonary edema (15). However, it is not known whether tPA activates NMDARs in the pulmonary vasculature and whether this may affect pulmonary vascular contractility or permeability so as to counteract its beneficial fibrinolytic activity in the setting of pulmonary embolism.

The data shown here demonstrate that therapeutic concentrations of tPA impair pulmonary artery contractility and promote vascular permeability in the lung through an interaction between its docking site and NMDA-R1 in a murine model of pulmonary embolism. Blocking the interaction of tPA with NMDA-R1 improves vascular contractility, inhibits the development of lung permeability induced by tPA, and facilitates resolution of pulmonary emboli.
with wild-type (WT) tPA provided by American Diagnostica and tPA purchased from Genentech (South San Francisco, CA) and with Activase from Boehringer (Ingelheim, Germany).

**tPA variants.** tPA variants (South San Francisco, CA) were synthesized and characterized as previously described (13). cDNAs encoding mature human tPAs were cloned into the pMT/BiP/V5-HisA plasmid (Invitrogen, Carlsbad, CA). To generate catalytically inactive tPA (S184A) variant and the PAI-1 docking site (DS) variant (K296A/H297A/R298A/R299A), mutations were introduced in WT tPA by PCR using the QuickChange Mutagenesis kit (Stratagene, La Jolla, CA), and the complete sequences were verified. The protein contains two extra amino acids, R5, at the NH2 terminus, resulting from introduction of the Bgl II cloning site. Proteins were expressed in S2 Drosophila Expression System (Invitrogen) according to the manufacturer’s protocol and purified by antibody affinity chromatography using anti-tPA coupled to CN-Br activated Sepharose. The final products migrated as single bands on SDS-PAGE at the expected sizes, and the plasminogen activator activity was assessed using the plasmin chromogenic substrate Spectrazyme PL (gift of American Diagnostica), as described (7). Proteins were stored at −70°C or lyophilized before use.

**Contractile response of isolated pulmonary rings.** All experimental protocols involving the use of vertebrate animals were approved by the Israeli Board for Animal Experiments. Sprague-Dawley rats (Harlan Laboratories, Jerusalem, Israel), average weight 250–275 g, were anesthetized with an intraperitoneal injection of ketamine (85 mg/kg) and xylazine (10 mg/kg). Mice were anesthetized as described above. tPA (1 mg/kg) was injected into the vein tail of the anesthetized mice (estimated plasma concentration 20 nM). No effect on mean arterial blood pressure was observed. Thirty minutes later, Evans blue dye (20 mg/kg) in 250 μl of 0.9% saline was infused into the left internal jugular vein of the anesthetized mice and allowed to circulate for 60 min. A tracheotomy was performed. The trachea was cannulated with a 20-gauge intravenous catheter, which was sutured in place. Bronchoalveolar lavage (BAL) was performed using 1.5 ml of warmed sterile Hanks balanced salt buffer (30°C). BAL fluid was collected, centrifuged at 14,000 revolution/min for 20 min at 4°C, the supernatant was removed, and the optical density at 620 nm was measured. The animals were euthanized by exsanguination under anesthesia.

**Permeability in vitro.** Human pulmonary microvascular endothelial cells (PMVECs), passages 4 to 12, were plated onto culture inserts (3-μm pore size; Falcon/BD, Heidelberg, Germany) (1.0 × 105 cells/ml) within 24-well plates and grown to confluence at 37°C under 5% CO2. Permeability to FITC-dextran was measured as described (10). Briefly, PMVECs were incubated without or with tPA, or tPA PAI-1-derived peptide in endothelial basal medium-2 containing 50 mg/ml FITC-dextran (40 kDa) for 2 to 120 min at 37°C, and permeability was assessed by measuring fluorescence emission in aliquots taken from the bottom chamber. Immunoprecipitation. Pulmonary arterial rings isolated from tPA−/− mice (Jackson Laboratories) were incubated in an oxygenated (95% O2-5% CO2) Krebs-Ringer bicarbonate solution (composition in mM): 118 NaCl, 4.7 KCl, 1.3 CaCl2, 1.2 KH2PO4, 1.2 MgSO4, 25 NaHCO3, 11 glucose, and 0.05 Na2-EDTA. Where indicated, WT tPA, WT tPA with 18-aa PAI-1-derived peptide, or the tPA variant that lacks a functional DS (DS tPA) were added. The rings were homogenized in five volumes of cold RIPA buffer composed of 1% NP40, 0.5% sodium deoxycholate, 10% sodium lauryl sulfate, and 0.5 mM PMSF in PBS in the absence of protease inhibitors at 4°C for 30 min to prevent possible interference by endogenous tPA. The lysates were cleared by centrifugation; the supernatant fractions were then precleared with protein A-agarose beads that had been preblocked with 1% BSA. The supernatants were then incubated for 2 h with beads containing anti-tPA, anti-NMDA-R1 IgG, or irrelevant IgG. The beads were washed five times with PBS, the proteins were eluted by three additions of 0.1 glycine buffer for 5 min each and centrifuged, and the supernatants were analyzed by Western blotting. Samples were applied to nitrocellulose membranes. The membranes were blocked with horse serum and incubated initially with anti-tPA or anti-NMDA-R1 antibodies (as indicated) and then with a species-specific secondary antibody conjugated to horseradish peroxide. All experiments were performed in triplicate and were repeated a minimum of three times. Irrelevant IgG was added in lieu of specific primary antibodies as a control. Western blotting to identify the NR1 subunit of the NMDA-R1 was performed as described (14). Briefly, immunoprecipitates were electrophoresed using an 8–10% SDS glycine polyacrylamide gel. Separated proteins were electroblotted onto
nitrocellulose membranes. The membranes were blocked with low-fat dry milk in 10 mM Tris HCl (pH 7.4), 150 mM NaCl, and 0.05% Tween 20, and the NMDA-R1 subunit was detected with the same antibodies. The membranes were incubated with secondary antibodies conjugated with peroxidase and developed with the appropriate colloidal substrates.

Pulmonary arterial diameter and flow. Echocardiography was performed before and after intraperitoneal injection of tPA using a 13-MHz probe (Vivid 7; GE Medical Systems, Milwaukee, WI). Rats were lightly sedated with intraperitoneal zolazepam (25 mg/kg) and xylazine (50 mg/kg). After shaving the left hemithorax, two-dimen-
sional echocardiographic images and pulsed-wave Doppler-derived recordings were acquired from the short-axis view at the level of the large arteries at a frame rate of 250–300/s. Pulmonary artery diameter (d) and the time-velocity integral (TVI) as a surrogate for stroke volume (SV) were measured. The cross-sectional area (CSA) of the pulmonary artery and cardiac SV were calculated using the following formulas: CSA = 0.785 × d² and SV = CSA × TVI. All parameters were evaluated during an average of three consecutive beats. A single echocardiographer, blinded to intervention, performed all data acquisition.

Pulmonary microembolism model. The murine model of pulmonary microembolism (ME) was performed as previously described (4). Briefly, 125I-fibrin microparticles were injected into the jugular vein of WT mice. Ten minutes later, mice were injected intravenously with tPA ± 18-aa PAI-1 peptide (0.5 and 1 mg/kg, respectively) in saline or saline alone as a control. Fifty minutes later, lungs were harvested, washed, and counted for radioactivity.

Statistical analysis. In vitro and ex vivo experiments were performed in triplicate and were repeated three to six times using independent samples or tissues. All data are presented as means ± SD. Differences were analyzed using Student’s t-test, one- or two-way ANOVA with Newman-Keuls post hoc test, or with Mann-Whitney or Kruskal-Wallis rank tests for the comparisons of nonparametric variables, as indicated. Comparison among the treatment groups of animals exposed to ME was determined using the two-tailed t-test with GraphPad Prism (version 4.0 for Windows; GraphPad Software, San Diego, CA). Statistical significance was set at P < 0.05.

RESULTS

Biphasic effect of tPA on pulmonary arterial contractility. Our initial goal was to examine the effect of tPA on the contractility of the pulmonary arterial vasculature. To do so, we first examined the effect of WT tPA on isolated pulmonary arterial rings. Physiological concentrations of tPA (1 nM) stimulated the contraction of isolated pulmonary artery rings induced by PE (P < 0.005, by both Mann-Whitney rank test and 2-way ANOVA with the Newman-Keuls post hoc test) (Fig. 2A). Addition of IP (1 nM) decreased the EC50 of the arterial rings from 22.3 ± 4.7 to 4.3 ± 1.6 nM (P < 0.004, 1-way ANOVA) (Fig. 2B). However, the effect of tPA on pulmonary arterial contractility was dose dependent. At higher concentrations, tPA (20 nM) inhibited the contractility of pulmonary arterial rings (P < 0.006, by both Mann-Whitney rank test and 2-way ANOVA with the Newman-Keuls post hoc test) (Fig. 2A). Addition of tPA (20 nM) increased the EC50 of the arterial rings from 22.3 ± 4.7 to 147 ± 11.8 nM (P < 0.007, 1-way ANOVA) (Fig. 2B).

Role of catalytic activity in tPA-mediated vasoactivity. We then studied the relationship between the catalytic activity of tPA and its effect on pulmonary arterial contractility. To do so, we examined the effect of a catalytically inactive variant (tPA-S481A). Catalytically inactive tPA maintained its capacity to stimulate the contractility of the pulmonary arterial rings at a 1 nM concentration. Addition of tPA-S481A (1 nM) decreased the EC50 of the arterial rings from 22.3 ± 4.7 to 5.4 ± 1.7 nM (P < 0.004, 1-way ANOVA) (Fig. 2B). However, higher concentrations of tPA-S481A (20–50 nM) failed to inhibit the contractility of the pulmonary arterial rings, yielding an EC50 almost identical to that attained at the 1 nM concentration (Fig. 2B).

Fig. 2. Effect of tPA on the contraction of pulmonary artery rings. A: effect of tPA on the contraction pulmonary arterial rings is concentration dependent. Contraction of isolated pulmonary arterial rings was induced by phenylephrine (PE) at the indicated concentrations in the absence (■) or presence of 1 nM wild type (WT) tPA (□) or 20 nM WT tPA (▲). B: effect of tPA on the contraction of isolated pulmonary arterial rings is partially independent of its catalytic activity. Contraction of isolated pulmonary arterial rings was induced by PE in the absence (control) or presence of 1 nM WT tPA or 20 nM WT tPA (△, ||) or 1, 20, or 50 nM catalytically inactive tPA-S481A. The mean ± SD of at least 3 experiments is shown. Significant differences are noted as follows: *Control vs. tPA-S481 1 nM; ‡Control vs. WT tPA 20 nM; #Control vs. WT tPA 20 nM; §Control vs. WT tPA 20 nM. C: involvement of N-methyl-D-aspartate receptors (NMDARs) and lipoprotein-related receptor (LRP) in regulating contractility of isolated pulmonary arterial rings by tPA. Contraction of isolated pulmonary arterial rings was induced by PE in the absence (control) or presence of 1 nM or 20 nM WT tPA alone or together with MK-801 (100 nM) (MK), glutamate (150 μM) (Glut), recombinant receptor-associated protein (rRAP) (20 nM), anti-LRP antibodies (20 nM) (Anti LRP), or MK-801, glutamate, or anti-LRP antibodies alone. Significant differences are noted as follows: *Control vs. WT tPA 1 nM ± MK and Glut + MK; #Control vs. WT tPA 20 nM; §Glut vs. Glut + MK. The mean ± SD of at least 3 experiments is shown in each panel.

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Involvement of NMDA-R1 in tPA-mediated inhibition of pulmonary arterial contractility. In view of the requirement for catalytic activity for its inhibitory effect on the contractility of the pulmonary arterial rings, we next turned our attention to a substrate/receptor of tPA, NMDA-R1, as a potential mediator of contractility. Occupancy of NMDA-R1 by tPA in the brain is followed by receptor cleavage, which promotes glutamate-induced intracellular signal transduction and accelerates neuronal apoptosis (14). To examine this possibility, we tested the effect of the NMDA-R antagonist MK-801 on tPA-mediated pulmonary arterial contraction. The NMDA-R1 antagonist blocked the inhibition of contractility induced by 20 nM tPA (P < 0.004, 1-way ANOVA) (Fig. 2C) but had no effect on the procontractile effect of 1 nM tPA (Fig. 2C). The NMDA-R1 antagonist by itself stimulated the contraction of the pulmonary rings (P < 0.01, 1-way ANOVA) (Fig. 2C), suggesting that the receptor exerts constitutive procontractility in this tissue. We then examined the effect of the NMDA-R agonist glutamate on pulmonary artery contractility. As would be deduced from the procontractile effect of the NMDAR antagonist, the NMDA-R1 agonist glutamate inhibited the contraction induced by phenylephrine (P < 0.01, 1-way ANOVA) (Fig. 2C). This supports the concept that NMDA-R1 constitutively inhibits vascular tone in the pulmonary vasculature, consistent with its described role in causing pulmonary edema (15).

LRP mediates tPA-induced stimulation of pulmonary arterial contractility. We previously reported that tPA stimulates the contraction of isolated aortic rings induced by phenylephrine through LRP (11). Therefore, we tested the effect of the LRP antagonists rRAP and anti-LRP antibodies on phenylephrine-induced contraction of pulmonary artery rings in the presence of 1 nM tPA. Both LRP antagonists abolished the stimulatory effect of tPA on PE-induced vasoconstriction (P < 0.009 “for rRAP” and P < 0.01 “for anti-LRP”, 1-way ANOVA) (Fig. 2C). This indicates that the procontractile and anticontractile vascular responses are mediated through different receptors and that vascular contractility in vivo reflects the balance between these opposing systems.

Effect of tPA on pulmonary arterial contractility in vivo. Several experiments were performed to determine whether tPA similarly affects pulmonary arterial contractility in vivo. First, we examined the effect of tPA on pulmonary arterial diameter and SV in rats by Doppler. A single intravenous injection of tPA into the tail vein (1 mg/kg; estimated initial plasma concentration 20 nM) increased the diameter of the pulmonary artery by ~6.5% (P < 0.001; Table 1). Second, we observed that a PAI-1-derived 18-aa peptide (Ac-RMAPPFP-NH2-amide) inhibited the vasoactivity of tPA (5). The effect of tPA on pulmonary arterial dilation was almost totally inhibited by the 18-aa PAI-1-derived peptide and by the NMDAR antagonist MK-801 (Table 1). tPA also increased the calculated pulmonary arterial CSA by 13.4% and increased the SV by 25.4%. In contrast, injection of lower doses of tPA (0.05 mg/kg, estimated initial plasma concentration 1 nM) decreased the diameter of the pulmonary artery by ~3% and reduced the TVI by 6.9% (Table 1). tPA (0.05 mg/kg) decreased significantly (P < 0.05) the calculated pulmonary arterial CSA by 12.5% and increased the SV by 18.4%. The reduction in pulmonary artery diameter induced by tPA and TVI was almost totally inhibited by the PAI-1-derived peptide and the LRP receptor antagonist rRAP, but not by MK801, consistent with their ex vivo effects on vascular contractility of isolated rings.

Effect of tPA on pulmonary vascular permeability. Activation of NMDARs in rat lungs by glutamate triggers an acute increase in permeability leading to pulmonary edema (15). Therefore, we asked whether activation of NMDA-R1 by tPA exerts a similar effect. To do so, we studied lung permeability after sequential intravenous injections of tPA and Evans Blue (6). Injection of tPA (1 mg/kg) increased the extravasation of intravenous Evans Blue into the lung (P < 0.01, 1-way ANOVA) (Fig. 3A). The NMDA-R1 antagonist MK-801 abolished the effect of tPA and glutamate on lung permeability (P < 0.009, 1-way ANOVA) (Fig. 3A). Activation of NMDARs in the lungs by tPA induces pulmonary edema by activating nitric oxide synthase (NOS) (15). Therefore, we examined the effect of the NOS antagonist on tPA-induced lung permeability. Nitro-L-arginine methyl ester (L-NAME) (5 mg/kg) inhibited tPA-induced endothelial cell permeability in vivo (Fig. 3A).

Role of tPA catalytic activity in pulmonary vascular permeability. The data in Fig. 2B indicate that the inhibition of pulmonary artery contractility by 20 nM tPA requires intact catalytic activity, whereas we previously observed that the induction of BBB permeability by tPA does not (5). Therefore, we next asked whether induction of pulmonary vascular permeability requires catalytic activity. tPA-S481A, which lacks catalytic activity, whereas we previously observed that the inhibition of pulmonary vascular contractility by 20 nM tPA requires intact catalytic activity, whereas we previously observed that the induction of BBB permeability by tPA does not (5).

Table 1. Pulmonary arterial diameter and flow

<table>
<thead>
<tr>
<th></th>
<th>TVI, cm</th>
<th>SD</th>
<th>PA d, cm</th>
<th>SD</th>
<th>CSA, cm²</th>
<th>SV, ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.56</td>
<td>1.32</td>
<td>0.31</td>
<td>0.064</td>
<td>0.0754</td>
<td>0.57</td>
</tr>
<tr>
<td>tPA 1 nM</td>
<td>7.04</td>
<td>0.13</td>
<td>0.29</td>
<td>0.039</td>
<td>0.066</td>
<td>0.465</td>
</tr>
<tr>
<td>tPA 20 nM</td>
<td>8.36</td>
<td>1</td>
<td>0.33</td>
<td>0.044</td>
<td>0.0855</td>
<td>0.715</td>
</tr>
<tr>
<td>tPA 20 nM + Peptide</td>
<td>8.03</td>
<td>1.2</td>
<td>0.31</td>
<td>0.061</td>
<td>0.0754</td>
<td>0.606</td>
</tr>
<tr>
<td>tPA 20 nM + MK801</td>
<td>7.97</td>
<td>1.7</td>
<td>0.3</td>
<td>0.054</td>
<td>0.07</td>
<td>0.56</td>
</tr>
</tbody>
</table>

Echocardiography was performed in 5 different Sprague-Dawley rats (Haran Laboratories, Jerusalem, Israel) before and after intraperitoneal injection of tissue-type plasminogen activator (tPA) as described in MATERIALS AND METHODS. Pulmonary artery (PA) diameter (d) and the time-velocity integral (TVI) as a surrogate for stroke volume (SV) were measured. The cross-sectional area (CSA) of the PA and cardiac SV were calculated using the following formulas: CSA = 0.785 x d² and SV = CSA x TVI. All parameters were evaluated during an average of 3 consecutive beats. A single echocardiographer, blinded to intervention, performed all data acquisition. SD, standard deviation.
mutated so that it retained catalytic activity (data not shown). We also asked whether tPA induced permeability through a direct effect on the endothelium as opposed to an indirect effect on hemodynamic activity leading to increased intravascular volume and hydrodynamic pressure. To do so, we examined the effect of tPA on the permeability of monolayers human PMVECs in vitro. WT tPA increased the permeability of PMVEC monolayers to FITC-dextran in a dose-dependent manner (Fig. 5C). The effect of tPA on endothelial cell permeability was inhibited by the 18-aa PAI peptide and was independent of tPA catalytic activity (Fig. 5D). MK-801 failed to inhibit the effect of tPA on endothelial cell permeability (Fig. 5D) in contrast to its effect on tPA-mediated vasoactivity and permeability in vivo. This outcome is consistent with the expression of NMDA-R1 on human pulmonary SMC but not on endothelial cells (T. C. Allen, unpublished observations). Activation of NMDARs in the lungs by NMDA induces pulmonary edema by activating NOS (15), and the NOS antagonist L-NAME but was unable to bind to NMDA-R1 (13). This docking site variant of tPA neither inhibited pulmonary arterial contractility (Fig. 5A) nor induced lung permeability (Fig. 5B). These results are consistent with the catalytic activity not being required for tPA to increase lung permeability. They also indicate that inhibition of vascular contractility and increased lung permeability both require an intact DS.

**tPA induces endothelial cell permeability.** We also asked whether tPA induced permeability through a direct effect on the endothelium as opposed to an indirect effect on hemodynamic activity leading to increased intravascular volume and hydrodynamic pressure. To do so, we examined the effect of tPA on the permeability of monolayers human PMVECs in vitro. WT tPA increased the permeability of PMVEC monolayers to FITC-dextran in a dose-dependent manner (Fig. 5C). The effect of tPA on endothelial cell permeability was inhibited by the 18-aa PAI peptide and was independent of tPA catalytic activity (Fig. 5D). MK-801 failed to inhibit the effect of tPA on endothelial cell permeability (Fig. 5D) in contrast to its effect on tPA-mediated vasoactivity and permeability in vivo. This outcome is consistent with the expression of NMDA-R1 on human pulmonary SMC but not on endothelial cells (T. C. Allen, unpublished observations). Activation of NMDARs in the lungs by NMDA induces pulmonary edema by activating NOS (15), and the NOS antagonist L-NAME.
inhibited tPA-induced lung permeability in vivo (Fig. 3A). Consistent with that observation, "NAME (200 μM) also inhibited tPA-induced endothelial cell permeability in vitro (Fig. 5D).

Role of NMDA-R1 cleavage in tPA-mediated vascular permeability. We then asked whether NMDAR was activated and/or cleaved by other proteases to help understand the biological effect of catalytically inactive tPA on vascular permeability. To do so, we examined the effect of the two tPA variants on NMDA-R1 cleavage. WT tPA and tPA-S481A bind to NMDA-R1 (Fig. 6A) and activate signal transduction pathways that stimulate lung permeability (Fig. 3B). However, whereas WT tPA leaves the receptor, the catalytically inactive variant does not (Fig. 6B), affirming that the tPA signal can be transmitted without cleavage of NMDA-R1 (16). The 18-aa PAI-1 peptide that abolished the signal-transduction effect of both variants on lung permeability (Fig. 4B) also inhibited the binding of WT tPA and tPA-S481A to NMDA-R1 (Fig. 6C), further supporting the contention that both variants exert their effects on the lung vasculature through this receptor.

Contribution of vascular contractility and permeability to thrombolysis. We previously reported that the effects of tPA on cerebrovascular contractility and BBB permeability diminished its beneficial effects in models of thrombotic stroke and that these neurological outcomes were improved when these nonfibrinolytic activities were inhibited (5). Therefore, we asked whether the effect of tPA on pulmonary vascular contractility and permeability might limit or even mask its beneficial effects in the treatment of pulmonary embolism. To examine this possibility, we studied the effect of the 18-aa PAI-1 peptide that inhibits tPA-mediated vasorelaxation and permeability without affecting its fibrinolytic activity (5) in a model of pulmonary embolism. Pulmonary vascular occlusion by radiolabeled microemboli was produced as previously described (4). tPA (0.5 mg/kg) enhanced clearance of the labeled microemboli from the lungs, and the effect was enhanced significantly by coinjection of the 18-aa PAI-1 peptide (1 mg/kg) (Fig. 7). This outcome supports the inference that the effects of WT tPA on vascular contractility and permeability limit its effectiveness in the resolution of pulmonary emboli.

DISCUSSION

The results in this paper show that tPA is involved in two essential functions, vascular contractility and vascular permeability, that play important roles in lung physiology and pathophysiology. The mechanism by which tPA modifies pulmonary vascular function involves an interplay between several of its domains and their respective vascular receptors.

Our data indicate that tPA regulates the contractility of isolated pulmonary arterial rings by acting on two receptors. At physiological concentrations (1 nM), tPA stimulates the vaso-
tPA regulates pulmonary vasoactivity and permeability

Contraction induced by phenylephrine through LRP, consistent with previous observations in isolated aortic rings (11); at higher concentrations that are well within the range attained during thrombolytic therapy (20 nM) tPA overrides this signal by acting through NMDA-R1 to inhibit pulmonary arterial contractility. Inhibition of NMDA-R1-mediated vascular contractility by tPA requires intact catalytic activity, whereas vasoconstriction at lower concentrations does not. However, both functions are ablated by mutations in the DS of tPA and by peptides that block the site.

tPA also increases pulmonary vascular permeability at concentrations that impede arterial contractility. Increased pulmonary vascular permeability in vivo is in part mediated through NMDA-R1, consistent with previous studies demonstrating activation of this receptor by tPA (13, 14), and data implicating this receptor in the development of pulmonary edema induced by glutamate (15). However, induction of pulmonary permeability by tPA, in contrast to its effect on contractility, does not require catalytic activity, which is in line with previous observations on the mechanism of BBB disruption in the central nervous system (5, 16). The capacity of the PAI-1-derived peptide that binds to the DS (5, 11), but does not affect fibrinolysis (11), to inhibit the capacity of WT tPA and tPA-S481A to enhance pulmonary vascular permeability strongly suggests that binding of tPA to the NMDAR through this site is essential. This hypothesis is supported by the inability of a tPA variant lacking a functional DS, but that retains its catalytic activity, to increase lung permeability. The finding that catalytically inactive tPA retains the capacity to signal through the NMDA-R1 and the inhibitory effect of PAI-1 peptides that do not block catalytic activity (11) strongly suggest that cleavage of the receptor is not required for this signaling pathway.

tPA induced permeability of PMVEC monolayers in vitro. MK-801 failed to inhibit this in vitro effect although it inhibited tPA-mediated permeability in vivo. This strongly suggests that paracrine interactions between smooth muscle cells and endothelial cells are induced by tPA in vivo. This contention is further supported by the fact that the tPA variant that stimulates the vasoconstriction in an NMDAR-independent manner increases lung permeability in vivo that is inhibited by MK-801. This interpretation is consistent with our observation that NMDA-R1 is expressed by pulmonary vascular smooth muscle cell but not by PMVEC. We hypothesize that this crosstalk involves amplification of endothelial NOS activation in PMVEC through activation of pulmonary vascular smooth muscle cell NMDA-R1. The mechanism by which this occurs is presently under investigation.

Fig. 6. Interaction between tPA and NMDA-R1 in the pulmonary artery. A: WT tPA and catalytically inactive tPA form a complex with NMDAR from pulmonary arteries. Homogenates of pulmonary arterial rings isolated from tPA−/− mice were preincubated with WT tPA (lane 1) or a catalytically inactive mutant tPA (Mut tPA; lane 2) (40 nM each) at 4°C, precipitated with an antibody against the NR1 subunit of NMDA-R1, followed by immunoblotting with an antibody against tPA or irrelevant Ig (lanes 4 and 5); lane 3 (c) is the control WT tPA. B: same experiment was performed as in A except that the immunoprecipitation was performed with anti-tPA antibodies followed by immunoblotting with an antibody against the NR1 subunit of NMDA-R1 or irrelevant Ig (lanes 3 and 4). The results of an experiment representative of 3 performed experiments are shown. C: WT tPA but not catalytically inactive tPA cleaves the NR1 subunit of NMDA-R1. Pulmonary arterial rings from tPA−/− mice were incubated in buffer alone or buffer containing 20 nM WT tPA or catalytically inactive tPA (Mut) for 120 min at 37°C or with WT tPA (WT) for 30 min at 4°C (control). Tissue homogenates were analyzed by SDS-PAGE and Western blotting using an antibody against the NR1 subunit of NMDA-R1. The results of an experiment representative of 3 performed experiments are shown. D: interaction between NMDA-R1 and tPA is inhibited by the 18-aa PAI-1-derived peptide. Pulmonary arterial rings from tPA−/− mice were incubated in buffer alone or buffer containing 20 nM WT tPA or catalytically inactive tPA ± the 18-aa PAI-1-derived peptide (18 aa P: 1 μM). Tissue homogenates were analyzed by SDS-PAGE and Western blotting using an antibody against the NR1 subunit of NMDA-R1. The results of an experiment representative of 3 so performed are shown.

Fig. 7. Clearance of 125I-microemboli (M. E.) from the lungs by tPA ± 18-aa PAI-derived peptide. 125I-fibrin microparticles were injected into the jugular vein of WT mice; 10 min later mice were injected intravenously with tPA ± 18-aa peptide (0.5 and 1 mg/kg, respectively) in saline or saline alone as a control. 50 min later, lungs were harvested, washed, and counted for radioactivity. The data are expressed relative to the total injected dose of radioactivity. *Significantly different. The data show residual radioactivity in the lungs as the mean ± SD of 4–6 mice in each treatment group (*P < 0.05).
We hypothesized that effects of tPA on pulmonary vascular contractility and permeability may impair its beneficial effects of fibrinolysis, as we observed in the central nervous system. In support of this hypothesis, neutralizing tPA-induced vasoactivity and permeability using the 18-aa PAI-1-derived peptide accelerated the clearance of pulmonary emboli after treatment with tPA. We suggest that further refinements to the DS of tPA or the use of DS inhibitors may improve the beneficial effects of tPA in the treatment of pulmonary embolism and other disorders caused by thrombotic vascular occlusion. Additional studies are also required to study the thrombolytic effect of tPA at higher clot burdens, including those that may cause morbidity and/or lethality, to verify the applicability of our findings to the clinical setting.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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