Depletion of tissue plasminogen activator attenuates lung ischemia-reperfusion injury via inhibition of neutrophil extravasation

Yunge Zhao, Ashish K. Sharma, Damien J. LaPar, Irving L. Kron, Gorav Ailawadi, Yuan Liu, David R. Jones, Victor E. Laubach, and Christine L. Lau

Division of Thoracic and Cardiovascular Surgery, Department of Surgery, University of Virginia Health System, Charlottesville, Virginia

Submitted 7 July 2010; accepted in final form 23 February 2011

Zhao Y, Sharma AK, LaPar DJ, Kron IL, Ailawadi G, Liu Y, Jones DR, Laubach VE, Lau CL. Depletion of tissue plasminogen activator attenuates lung ischemia-reperfusion injury via inhibition of neutrophil extravasation. Am J Physiol Lung Cell Mol Physiol 300: L718–L729, 2011. First published March 4, 2011; doi:10.1152/ajplung.00227.2010.—Ischemia-reperfusion (IR) injury following lung transplantation remains a major source of early morbidity and mortality. Histologically, this inflammatory process is characterized by neutrophil infiltration and activation. We previously reported that lung IR injury was significantly attenuated in plasminogen activator inhibitor-1-deficient mice. In this study, we explored the potential role of tissue plasminogen activator (tPA) in a mouse lung IR injury model. As a result, tPA knockout (KO) mice were significantly protected from lung IR injury through several mechanisms. At the cellular level, tPA KO specifically blocked neutrophil extravasation into the interstitium, and abundant homotypic neutrophil aggregation (HNA) was detected in the lung microvasculature of tPA KO mice after IR. At the molecular level, inhibition of neutrophil extravasation was associated with reduced expression of platelet endothelial cell adhesion molecule-1 mediated through the tPA/LDL receptor-related protein/NF-κB signaling pathway, whereas increased P-selectin triggered HNA. At the functional level, tPA KO mice incurred significantly decreased vascular permeability and improved lung function following IR. Protection from lung IR injury in tPA KO mice occurs through a fibrinolysis-independent mechanism. These results suggest that tPA could serve as an important therapeutic target for the prevention and treatment of acute IR injury after lung transplantation.

P-selectin; PECAM-1; tPA knockout; NF-κB

LUNG ISCHEMIA-REPERFUSION (IR) injury is one of the most important complications of lung transplantation, affecting 20–30% of all lung transplant recipients (6, 17). This event represents the most frequent cause of early mortality and prolonged ICU stay following lung transplantation. Mortality rates are significantly higher in recipients with IR injury, and those who do survive have significantly impaired physical function and an increased risk of chronic rejection or bronchiolitis obliterans syndrome (8). IR injury is an inflammatory response mediated by multiple cellular and molecular mediators (5, 33). During IR injury, leukocytes, especially neutrophils, infiltrate into the lung and release superoxides/oxidants and proteases that damage lung tissues (11). Furthermore, activated neutrophils release inflammatory mediators, such as cytokines/chemokines and enzymes, which promote the recruitment and activation of greater numbers of leukocytes into the injured tissues and amplify the severity of tissue damage.

Since neutrophils are intimately involved in the pathogenesis of the acute phase of IR injury, the neutrophil-mediated inflammatory cascade during reperfusion represents an important therapeutic target for lung IR injury (5, 33).

Tissue plasminogen activator (tPA), a member of the serine proteinase family, is highly expressed in both adult and fetal tissues (26). tPA is expressed by vascular endothelial cells and functions to convert the zymogen plasminogen to the active protease plasmin (29), thus initiating a potent fibrinolytic process. On the basis of the interplay between the fibrinolytic cascade and the inflammatory process in acute lung injury models, we previously investigated the relationship between these two systems in lung IR injury. We demonstrated that increased fibrinolysis through depletion of plasminogen activator inhibitor-1 (PAI-1) attenuated lung IR injury (14). Accordingly, we believed that alternative mechanisms involved in impaired fibrin breakdown would result in an increased inflammatory response. In this study, we hypothesized that increased fibrin levels due to impaired fibrinolysis in tPA knockout (KO) mice would exacerbate acute lung inflammation following IR. We further examined the underlying mechanisms involved in the tPA-mediated lung response at the molecular, cellular, and functional levels.

METHODS

Mice and experimental protocol. In our experimental model, we utilized tPA KO mice (on C57BL/6 background) and C57BL/6 mice [wild-type (WT) mice] (23–28 g), purchased from Jackson Laboratory (Bar Harbor, ME). The Animal Care and Use Committee at the University of Virginia reviewed and approved all aspects of our experimental protocol before experimentation. All experimental mice received humane care in accordance with “Principles of Laboratory Animal Care” according to the National Society for Medical Research and “The Guide for the Care and Use of Laboratory Animals,” prepared by the National Academy of Science and published by the National Institutes of Health (NIH).

Mouse lung IR injury model. Three experimental groups (WT sham, WT IR, and tPA KO IR) were evaluated by using an in vivo left hilar clamp model of lung IR as previously described (14). Briefly, WT IR and tPA KO IR groups underwent 1 h of left hilar clamping followed by 2 h of reperfusion. The tPA KO sham and WT sham groups underwent left thoracotomy but no hilar clamping. Experimental groups included six to nine mice per group.

Pulmonary function assessment. Lung function (pulmonary compliance, pulmonary artery pressure, and airway resistance) was measured following 2 h of reperfusion by using an in situ buffer-perfused mouse lung system (14, 25). Briefly, the animal was anesthetized and a tracheotomy performed. Positive-pressure (2 cmH2O) ventilation (120 breaths/min, tidal volume = 15 ml/kg) was initiated before the thorax was opened. Fifty units of heparin were injected via the jugular vein. The lungs were perfused with Krebs-Henseleit buffer, containing 2% albumin, 0.1% glucose, and 0.3% HEPES. The buffered
perfusate was prepared to mimic right ventricular, mixed venous blood by using a gas bubbling stone with titrated gases generating a pH of 7.40–7.45. Po2 60–70 mmHg and PcO2 50–60 mmHg. The buffered perfusate and isolated lung apparatus were maintained at 37°C. Lungs were allowed to equilibrate for a 10-min stabilization period. Pulmonary compliance, pulmonary artery pressure, and airway resistance were then measured and recorded by use of Pulmodyne data-acquisition software (Hugo Sachs Elektronik, March-Hugstetten, Germany).

**Histology and immunohistochemical staining.** Whole-lung tissue specimens were immediately fixed in 10% formalin. Following 24 h, fixed lungs were embedded in paraffin, and sections were stained with hematoxylin and eosin or stained by immunohistochemistry. Immunohistochemical staining of mouse migratory macrophage, neutrophil, CD3, P-selectin, and platelet endothelial cell adhesion molecule-1 (PECAM-1) were performed as described in our previous methods (37, 38). For macrophage and neutrophil staining, we utilized rat anti-mouse neutrophils (AbD Serotec, Raleigh, NC) and rat anti-mouse Mac-2 (Accurate Chem, Westbury, NY) primary antibodies. We then utilized alkaline phosphatase-conjugated anti-rat IgG (Sigma, St. Louis, MO) as a secondary antibody. The signals were detected using fast red (Sigma). For negative controls, we used purified normal rat IgG (eBioscience, San Diego, CA). For immunofluorescence staining, the cell nucleus was stained with 4,6-diamidine-2-phenylindole dihydrochloride (DAPI, Roche Diagnostics, Mannheim, Germany). The number of neutrophils and macrophages per high-power field was assessed by using immunohistochemical staining of peripheral lung sections, and six fields were counted per lung. To ensure the validity of all cell counts, counting of positively stained cells was performed in the same fashion by three separate investigators. The average cell number was used for statistical analysis. For P-selectin and PECAM-1 staining, we used rabbit anti-mouse P-selectin [a gift from Drs. John M. Sanders and Klaus Ley, The Cardiovascular Research Center (CVRC) at University of Virginia] and goat anti-mouse PECAM-1 (Santa Cruz, CA) as primary antibodies. The signal for P-selectin and PECAM-1 were semi-quantified using Imaging-Pro Plus software (14, 36). The average value of each group was utilized for statistical analysis. All the pictures were taken under an Olympus BX51 microscope equipped with an Olympus DP70 digital camera (Minneapolis, MN).

**Plasma and BAL fluid collection.** For zymography, we collected plasma and bronchoalveolar lavage (BAL) samples (14). As previously described, blood samples for each mouse were collected after euthanasia. Blood samples were then centrifuged and the plasma component separated. BAL samples were collected by two separate washings with 1 ml aliquots of Hanks’ balanced salt solution instilled through the trachea. The BAL was centrifuged and stored for zymography analysis.

**Pulmonary microvascular permeability.** Lung microvascular permeability was determined by the Evans blue dye extravasation technique, which is an index of change in protein permeability. Six additional mice per group were utilized. Evans blue dye (20 mg/kg) was injected intravenously 30 min before the animals were euthanized. The lung was then perfused for 10 min with PBS to remove intravascular dye. Lungs were then homogenized in PBS to extract the Evans blue. The absorption of Evans blue was measured in the supernatant at 620 nm and corrected for the presence of heme pigments. A620 (corrected) was determined for each group and expressed as micrograms per gram wet lung weight.

**In vitro migration assay.** Cell migration was measured by a modification of the Boyden chamber assay. We filled 24-well plates (BD Biosciences, Bedford, MA) with RPMI 1640 medium (Invitrogen, Carlsbad, CA) that contained 50 nM of formyl-methionyl-leucyl-phenylalanine (fMLP, Sigma), recombinant active murine tPA (Molecular Innovations, Southfield, MI), or medium alone. The bottom of the insert was covered with a 3.0-µm-pore-size filter on which 100 µl of mouse whole blood was applied. After incubation for 90 min at 37°C, anti-Ly6G (Milleniy Biotech, Auburn, CA) staining was performed on the bottom side of the filter, and the number of transmigrated neutrophils was counted.

**HUVEC culture assay.** Human vascular endothelial cell (HUVECs) (Invitrogen) were grown in medium 200 supplemented with low serum growth supplement kit, which contains 2% FBS, 3 ng/ml BFGF, 10 µg/ml heparin, 0.2 µg/ml BSA, 1 µg/ml hydrocortisone, 10 ng/ml EGF, and 0.2% gentamicin/amphotericin B (Invitrogen) in a humidified incubator containing 5% CO2 at 37°C. When the cell density reached 80% confluence, the HUVEC was treated with 500 nM of recombinant active or inactive tPA (Molecular Innovations) and/or 500 nM of recombinant human receptor associate protein (RAP, from Enzo Life Sciences, Plymouth Meeting, PA), and/or 5 ng/ml NF-κB inhibitory peptide (23) for 24 h. The medium was then collected, and cells were lysed with RIPA buffer (50 mM Tris-HCl, PH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) for subsequent Western blot analysis.

**Western blot analysis.** The conditioned media and cell lysis were subjected to standard Western blot analysis according to previous methodology (30, 31). Equal amounts of total protein (30 µg) were loaded per lane. The primary antibodies were anti-PECAM-1 antibody (Santa Cruz, CA) and rabbit anti-mouse P-selectin (a gift from Drs. John M. Sanders and Klaus Ley. The CVRC at University of Virginia). The mouse anti-human α-tubulin antibody (Sigma) was also used as the loading control.

**Immunofluorescence staining assay.** Immunofluorescence staining of PECAM-1 was performed according to our previous description (31). Briefly, HUVECs were cultured on eight-well slides for 24 h followed by treating with active or inactive tPA for another 24 h. Cells were then fixed in 50% Acetone and 50% methanol for 15 min at room temperature and permeabilized with 0.2% Triton X-100 in Tris-buffered saline. The fixed, permeabilized cells were stained overnight at 4°C with goat anti-mouse PECAM-1 (0.4 µg/ml, cross reaction with human PECAM-1). Secondary rhodamine red-X-conjugated donkey anti-goat IgG was subsequently applied at 1:200 dilution for 1 h at room temperature. DAPI (1 µg/ml, Roche Diagnostics) was used for nuclei staining. Slow Fade mounting medium was added to the slides. Images were obtained by using an Olympus BX51 microscope equipped with an Olympus DP70 digital camera (Minneapolis, MN). Purified normal goat IgG was used as a negative control.

**Fibrinogen and gelatin zymography.** Total proteins were extracted from different organs (heart, kidney, spleen, lung, and testis) by use of proteinase extraction buffer [50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% (vol/vol) Triton X-100]. Plasma and BAL were collected as described above. Protein concentrations were determined with BCA protein assay. Equal amounts of proteins (30 µg) were loaded in each lane for matrix metalloproteinase (MMP)-2 and -9 gelatinolytic activity detection by use of gelatin zymography (36–38). Urokinase plasminogen activator and tPA activities were detected by plasminogen-fibrinogen zymography (35).

**MPO assay.** To further assess neutrophil activity as a function of lung injury, we examined myeloperoxidase (MPO) activity. After BAL fluid was collected, the lung was removed and immediately placed in 3.0 ml ice-cold homogenization buffer with protease inhibitor cocktail (Complete Roche, Penzberg, Germany) and 0.05% Triton X-100 (Sigma) in 60 ml PBS. Homogenization and sonication were followed by centrifugation (15,000×g for 15 min at 4°C). For MPO assay, 20 µl of the supernatant was added to a 96-well plate. Assay buffer (200 µl) containing 100 mM potassium phosphate, 0.834 ml o-dianisidine HCl (10 mg/ml, Sigma), and 0.083 ml 0.3% H2O2 in 50 ml deionized water was added immediately before reading. The reaction was assayed in a Biotek microplate reader (Bio-Tech Instruments) every 10 s at 465 nm. The data were expressed as the slope of change in optical density over 100 s.
P-selectin neutralizing antibody administration and its effect on neutrophil extravasation, aggregation, and lung function. Monoclonal antibody RB40.34 is a neutralizing antibody against murine P-selectin that blocks adhesion of HL-60 cells to immobilized P-selectin in vitro (3) and P-selectin-dependent rolling in vivo (12). The RB40.34 (BD Biosciences, San Jose, CA) or normal rat IgG (Sigma) were intravenously injected via the jugular vein at doses at 400 μg/kg 15 min before lung ischemia. Then the mice were then underwent 1 h of ischemia followed by 2 h reperfusion as described above. The lung function was assessed by the pulmonary function assay detailed above. The lung tissues were collected for immunohistochemical and immunofluorescence staining.

Densitometric analysis. Quantification of P-selectin and PECAM-1 expression via immunostaining was performed using Imag-Pro Plus software (36). Four photos were taken from each group with an Olympus BX51 microscope equipped with an Olympus DP70 digital camera (Minneapolis, MN). Count/size was selected within the “measure” menu. An appropriate color threshold (RGB) was chosen (for P-selectin staining, R: 29–183, G: 24–138, B: 18–100; for PECAM-1 staining, R: 0–178, G: 0–130, B: 1–89) to include the staining of signal. The whole high-power field was manually selected by irregular area of interest. Density (mean) was obtained and was equal to optical density divided by the selected area, which represents the average optical density of an object. The value of density (mean) from each group was then used for subsequent statistical analysis.

Statistics analysis. Statistical differences between groups were determined by one-way ANOVA followed by Tukey’s multiple comparison test or by Student’s t-test where appropriate. Data were reported as means ± SE from six separate experiments. P value equal or less than 0.05 was considered significant.

RESULTS

Pulmonary function is improved in tPA KO mice. The tPA KO mice were confirmed by fibrinogen/plasminogen zymography (Supplemental Fig. S1; the online version of this article contains supplemental data). Lung function of tPA KO IR mice was significantly improved compared with that of WT IR mice (Fig. 1). Mean pulmonary artery pressures were markedly higher in the WT IR (12.56 ± 1.030 cmH2O) mice compared with tPA KO IR (9.23 ± 0.536 cmH2O, P = 0.006), tPA KO sham mice (5.48 ± 0.963 cmH2O, P < 0.001), and WT sham mice (5.70 ± 0.263 cmH2O, P = 0.0009). Pulmonary artery pressures were not significantly different between tPA KO sham group and WT sham group (P = 0.33). Similarly, airway resistance among WT IR (2.24 ± 0.267 cmH2O·μl−1·s−1) mice was significantly worse compared with tPA KO IR (1.18 ± 0.114 cmH2O·μl−1·s−1, P = 0.0048), tPA KO sham (0.758 ± 0.112 cmH2O, P = 0.001), and WT sham mice (0.75 ± 0.025 cmH2O·μl−1·s−1, P = 0.0016). There were no significant differences between tPA KO sham and WT sham groups (P = 0.32). tPA KO IR mice also demonstrated higher pulmonary artery pressure and airway resistance compared with WT sham mice. Mean pulmonary compliance was similar in WT shams (5.54 ± 0.547 ml/cmH2O), tPA KO sham mice (6.79 ± 2.280 cmH2O, P = 0.001), and tPA KO IR mice (5.65 ± 0.377 ml/cmH2O, P = 0.4190), which was markedly improved compared with WT IR mice (2.54 ± 0.202 ml/ cmH2O, P < 0.001). Pulmonary compliance was not significantly different between tPA KO sham group and WT sham group (P = 0.15).

Neutrophil and migratory macrophage infiltration. Immunohistochemical staining revealed that neutrophil infiltration was significantly increased in WT IR mice compared with WT shams (P < 0.001, Fig. 2). Significantly less neutrophils infiltrated the interstitum of experimental lungs in tPA KO IR mice compared with WT IR mice (Fig. 2A, P < 0.001). However, the migratory macrophage infiltration was slightly increased in the experimental lung of tPA KO IR compared with WT IR (Supplemental Fig. S2). Importantly, neutrophils formed homotypic aggregation within the lung vasculature of tPA KO IR mice but not within WT IR mice (Fig. 2B). The
number of vessels with homotypic neutrophil aggregation (HNA) was significantly increased in experimental lungs of tPA KO IR mice (Fig. 2B, \( P < 0.001 \)). Similar aggregation patterns were not observed for macrophage and CD3+ T cells (Supplemental Fig. S3).

Pulmonary microvascular permeability was significantly decreased in tPA KO mice. To further characterize the degree of neutrophil extravasation in tPA KO IR mice, we performed pulmonary microvascular permeability studies using Evans blue dye extravasation. As expected, lungs of WT IR mice demonstrated significantly increased microvascular permeability vs. WT sham (Fig. 3, \( P = 0.0046 \)). Microvascular permeability in tPA KO IR mice was not significantly different compared with tPA KO sham mice (\( P = 0.1914 \)), but it was significantly decreased compared with WT IR mice (\( P = 0.04 \)). In addition, there was no significant difference in microvascula-
mice. Data shown are mean ± SE values for each group (n = 6).

Recombinant tPA had no chemotactic effects on neutrophil migration. To investigate whether tPA exerts a direct chemotactic effect on neutrophils, we performed an in vitro migration assay using fMLP and recombinant active tPA (a-tPA). As shown in Fig. 4, fMLP significantly increased neutrophil migration by twofold at the concentration of 50 nM for both WT and tPA KO mice (P = 0.003535). Although a-tPA (10 ng/ml) did not significantly alter neutrophil migration (Fig. 4, P > 0.05), addition of a-tPA (10 ng/ml) to the fMLP (50 nM)-treated neutrophils exhibited no synergetic stimulation of migration, suggesting that a-tPA had no significant direct chemotactic effect on neutrophil migration.

MPO activity. To assess the activation of neutrophils in the lung after IR, we assessed MPO activity. MPO activity in experimental lungs of tPA KO IR mice was significantly elevated (115.31 ± 9.37 MPO units) compared with WT mice (87.54 ± 5.62 MPO units, P = 0.042, Fig. 5). This result together with the elevation of MMP-9 plasma levels indicates that neutrophils, despite not extravasating, are activated in the blood vessels of the lung.

Effects of tPA KO on the expression of PECAM-1 and P-selectin. To further elucidate the molecular mechanisms underlying decreased neutrophil extravasation in tPA KO IR mice, we assessed two key molecules (PECAM-1 and P-selectin) known to play an important role during neutrophil extravasation. Immunohistochemistry and densitometric analysis demonstrated that the expression of PECAM-1 in experimental lungs was significantly decreased in tPA KO IR mice compared with both WT sham and WT IR mice (Fig. 6A, P < 0.001). Conversely, P-selectin expression was significantly increased within the pulmonary vasculature of tPA KO IR mice (Fig. 6B, P = 0.00129). Interestingly, the pattern of P-selectin expression was very similar to that of HNA in the pulmonary vessels, suggesting that decreased neutrophil extravasation may be a result of decreased PECAM-1 expression, whereas HNA may correlate with upregulation of P-selectin expression in the small pulmonary vessels.

Effects of anti-P-selectin functional blocking antibody on HNA, neutrophil extravasation, and lung function. To further test whether the observed HNA in tPA KO IR mice was a result of increasing expression of P-selectin in the small pulmonary vessels, functional blocking antibody (400 µg/kg) against P-selectin was injected via the jugular vein. Anti-P-selectin antibody markedly inhibited HNA in tPA KO IR mice compared with control IgG treatment at the same dosage (Fig. 7). Additionally, anti-P-selectin antibody also blocked neutrophil infiltration in WT IR mice compared with normal IgG treatment (Fig. 7).

Furthermore, we measured lung function following treatment with P-selectin neutralizing antibody and IR injury in WT and tPA KO mice (Fig. 8). Lung function of WT IR mice was significantly improved following injection of P-selectin neutralizing antibody, but no additional improvement was observed in tPA KO IR mice. Within WT IR mice, injection of anti-P-selectin antibody resulted in lower mean pulmonary artery pressures (8.73 ± 1.15 vs. 14.17 ± 0.42 cmH2O, P = 0.0033), lower airway resistance (1.30 ± 0.12 vs. 2.33 ± 0.27 cmH2O·µl−1·s−1, P = 0.0059), and improved pulmonary compliance (4.03 ± 0.47 vs. 2.78 ± 0.09 ml/cmH2O, P = 0.0120) compared with control IgG injection. Conversely, in tPA KO IR mice, anti-P-selectin antibody had no significant effect on pulmonary artery pressure (7.78 ± 0.43 vs. 8.03 ± 0.21 cmH2O)
Expression of PECAM-1, but not P-selectin, was regulated by tPA via tPA/LDL receptor-related protein (LRP)/NF-κB pathway in an in vitro cell culture model. To confirm that tPA may regulate expression of PECAM-1 or P-selectin, we tested the effects of tPA on the expression of PECAM-1 and P-selectin in HUVEC cell culture system. As a result, recombinant active and inactive tPA-stimulated expression of PECAM-1 on the HUVECs as well as in the conditioned media (Fig. 9A). The molecular...
weight of PECAM-1 was lower in conditioned media compared with cell lysis. The P-selectin level was not significantly affected with the addition of active tPA; however, it decreased with the treatment of inactive tPA. Subsequent immunofluorescence staining confirmed that both active and inactive tPA enhanced PECAM-1 expression on the surface of HUVECs (Fig. 9B). Blue signals demonstrate DAPI staining for visualizing nuclei. Furthermore, this stimulation was inhibited by RAP (500 nM), which is an endogenous antagonist of LRP, and by a recombinant NF-κB inhibitor (5 ng/ml) (Fig. 9C). These findings suggest that tPA may regulate expression of PECAM-1 through tPA/LRP/NF-κB signal pathway.

Effects of tPA KO on gelatinolytic activities of MMP-2 and MMP-9. MMP-2 and MMP-9 enzymatic activities were evaluated by gelatin zymography. We found that the gelatinolytic activity of MMP-9 was decreased in experimental lungs of tPA KO IR mice compared with WT IR mice (Fig. 10A). Sham operations induced MMP-9 gelatinolytic activity in experimental lungs of both WT and tPA KO mice, but less MMP-9 activity was expressed in experimental lungs of tPA KO compared with WT mice. MMP-2 gelatinolytic activity was not influenced in either IR or sham-operated groups. Moreover, latent MMP-9 levels were increased in the serum of tPA KO IR mice compared with WT IR and WT sham mice (Fig. 10B). However, the levels of MMP-9 and MMP-2 had

Fig. 7. Effects of P-selectin neutralizing antibody on neutrophil infiltration and homotypic neutrophil aggregation in mice after IR injury. Red indicates positive neutrophil staining. All sections were counterstained lightly with hematoxylin for viewing negatively stained cells. Purified normal rat IgG was used at the same concentration as controls. All pictures are ×40 magnification. Left: immunofluorescence imaging of fast red. Middle: 4,6-diamidine-2-phenylindole dihydrochloride (DAPI) staining for visualizing nucleus. Right: immunohistochemistry (IHC) staining of neutrophil. Pictures were taken under bright field (BF).
no significant change in the tPA KO IR mice compared with WT IR mice (Fig. 10C). Consequently, the deletion of tPA in KO mice appears to decrease MMP-9 activity within injured lungs, which may contribute to the observed attenuation of IR injury.

**DISCUSSION**

Herein, we have demonstrated the important effect of tPA depletion on the attenuation of acute lung IR injury and have further characterized the molecular, cellular, and functional mechanisms involved in this inflammatory process (Fig. 11). The present study provides important evidence that, independent of its effect on the fibrinolytic pathway, tPA, specifically, mediates neutrophil extravasation during IR and increases HNA in the postcapillary venules. These findings demonstrated that decreased neutrophil infiltration in experimental lungs of tPA KO IR mice is a result of decreased lung microvascular permeability as well a result of varying expression of key cell surface adhesion molecules, P-selectin and PECAM-1. Furthermore, we have also shown that MMP-9 expression in experimental lungs is decreased in tPA KO IR mice compared with WT IR mice and that tPA does not exert a direct chemotactic effect on migrating neutrophils following an acute ischemic insult. These results provide important evidence regarding potential molecular targets for the future treatment of acute lung IR injury following transplantation.

The interplay between fibrin deposition and pulmonary inflammation has been well established, and enhanced fibrinolysis has been demonstrated to ameliorate acute lung IR injury in both animal models and clinical practice. We have previously demonstrated that mice deficient in the tPA endogenous inhibitor, PAI-1, are protected from lung IR injury through the
enhancement of fibrinolysis (14). To further implicate the proinflammatory effects of fibrin deposition in acute lung IR injury, we sought to study the enhanced, fibrin-mediated inflammatory response expected to occur within tPA-depleted mice. Contrary to our expectations and stated hypothesis, however, our results demonstrated that tPA depletion conferred protection from IR injury. Specifically, we observed improved pulmonary artery pressures, airway resistance, and pulmonary compliance as well as decreased microvascular permeability within tPA-depleted mice. The attenuated injury we demonstrated suggests the influence of alternative, fibrinolytic-independent mechanisms through which IR injury protection occurs in the absence of tPA. Furthermore, our results revealed diminished neutrophil extravasation within experimental lungs of tPA-depleted mice. As a result, we sought to explore the underlying mechanisms involved in attenuated neutrophil migration following tPA-depleted lung IR injury. Our experimental model implicates the role of tPA modulated neutrophil

Fig. 9. tPA promotes PECAM-1 expression through tPA/LDL receptor-related protein (LRP)/NF-κB signal pathway. Human vascular endothelial (HUVEC) cells culture were cultured as described in METHODS. A: Western blot analysis of PECAM-1 and P-selectin expression in HUVECs treated with recombinant active and inactive tPA (atPA and itPA, 500 nM). A-tubulin was stained as loading controls. B: immunofluorescence staining of PECAM-1 on HUVECs in presence or absence of recombinant tPA (500 nM). Red indicates the positive staining of PECAM-1, blue is DAPI staining of cell nuclei. C: regulation of PECAM-1 expression by tPA was blocked by LRP antagonist, LDL receptor-associated protein (RAP, 500 nM), and NF-κB inhibitory peptide (5 ng/ml).
extravasation through the combined influence of specific molecular and cellular processes.

In general, IR injury describes an acute inflammatory response, which results in increased neutrophil rolling, adherence, and migration in postcapillary venules. The process of neutrophil infiltration is a highly regulated, multistep process that involves sequential events, each of which is necessary for progression to the next step. These cellular processes are governed by molecular interactions between receptors and ligands expressed on neutrophils and endothelial cells (24). Furthermore, the initial adhesion of neutrophils is a critical step for infiltration, allowing for the molecular interaction between cell surfaces of neutrophils and endothelial cells and resulting in neutrophil rolling along the vessel wall. This process likely results from accumulation of key molecular mediators, including P-selectin and PECAM-1 (5, 24, 33). P-selectin, a member of the selectin family of cell surface molecules, plays a critical role in the initial adhesion of neutrophils (15).

In this study, we demonstrated that deletion of tPA significantly increases P-selectin expression on the surface of both endothelial cells and neutrophils within the pulmonary vasculature compared with WT mice. Consequently, these results suggest that elevation of P-selectin is a key component to the attenuated migration of neutrophils from the pulmonary vasculature into the pulmonary interstitium following acute lung IR injury. Furthermore, we observed an increase in HNA in experimental lungs of tPA KO IR mice, which we believed to be partially explained by the upregulation of P-selectin activity. Such a process may be mediated by the interaction of P-selectin and its associated ligands. In fact, P-selectin-P-selectin glycoprotein ligand-1 bonds alone, in the absence of functional integrins, have been demonstrated to mediate stable platelet-neutrophil aggregation under fluid shear (12). Thus, to determine the role of P-selectin in intravascular neutrophil aggregation, we performed experiments with anti-P-selectin functional blocking antibody. Injection of anti-P-selectin antibody into tPA-depleted mice resulted in diminished HNA and decreased neutrophil extravasation. Commensurate with the increased expression of P-selectin activity and its effect on neutrophil aggregation, we detected enhanced neutrophil activation by MPO assay. The enhanced activation of neutrophils coupled with their decreased extravasation following the injection of anti-P-selectin antibody into tPA-depleted mice led us to believe that neutrophil extravasation may be a result of other key adhesion molecules in addition to P-selectin, especially PECAM-1.

PECAM-1 is an important cell surface molecule similarly expressed on pulmonary endothelial cells and neutrophils that plays a fundamental role in the process of neutrophil diapedesis (18) and extravasation from the pulmonary microvasculature. Our results demonstrate that tPA deficiency leads to a down-regulation of PECAM-1 expression on the surface and the junction of endothelial cells of small vessels, resulting in...
decreased pulmonary microvascular permeability in the ischemic lung. The importance of endothelial and neutrophil expression of PECAM-1 has been confirmed by use of in vivo murine models (7, 28). Blockage of PECAM-1 with monoclonal antibody inhibits neutrophil diapedesis (13) and reduces myocardial infarct size in both rat (9) and feline models (19) of IR injury. In the experimental lungs of tPA-depleted mice, we have further bolstered these findings by demonstrating an attenuation of neutrophil extravasation following the injection of anti-P-selectin blocking antibody. Because tPA KO mice demonstrate decreased expression of PECAM-1, blockade of upregulated P-selectin receptors with antibody expectedly resulted in no change in the degree of neutrophil migration from the pulmonary microvasculature. Moreover, the concomitant observation that lung function following IR injury was further exacerbated following injection of anti-P-selectin antibody in WT IR mice but unchanged in tPA KO IR mice further implicates PECAM-1 as an important mediator of neutrophil migration during this inflammatory process. However, the underlying mechanism of tPA regulation of PECAM-1 expression remains unknown.

On the basis of our observations and other publications to date, we postulate that tPA may act as a cytokine to regulate PECAM-1 expression through the tPA/LRP/Erk and/or tPA/LRP/NF-κB signal pathways. Zhang and colleagues (34) demonstrated that tPA mediates cerebral ischemia-induced NF-κB pathway activation through the plasminogen/plasmin-independent binding of low-density LRP. In addition, two consensus sites for NF-κB were identified at −409 (GGGGTTCTCC) and at +110/+120 (GAGGAATCCCC) in the PECAM-1 promoter (1), and at least one functional NF-κB site (+110/+120) has been identified within the PECAM-1 promoter (4). Our in vitro cell culture analysis demonstrated that both recombiant active and inactive tPA increased PECAM-1 expression in HUVECs and that this upregulation was blocked by LRP antagonist RAP and a NF-κB inhibitor. These results strongly suggest that endogenous tPA may regulate PECAM-1 expression in the pulmonary vessels via tPA/LRP/NF-κB signal pathway.

Diminished neutrophil extravasation in tPA-depleted mice was also a result of diminished MMP activity following acute IR injury. MMP-9 activity has been implicated in both brain (2, 22) and myocardial IR injury (16) as well as in myocardial infarction (27). MMP-9 plays an important role in neutrophil extravasation and migration by its ability to degrade the major components of the basement membrane (20). Our results reveal that experimental lungs of acutely injured tPA-depleted mice had decreased MMP-9 expression compared with WT IR mice. Interestingly, however, latent MMP-9 plasma levels in tPA KO IR mice were elevated compared with WT IR mice. One possible explanation for this discrepancy is that as aggregated neutrophils within the pulmonary microvasculature become activated, they release granules that contain various enzymes, including MMP-9 and MPO. This notion is further strengthened by our demonstration of increased MPO activity in the lungs of both tPA KO IR and WT IR mice compared with sham. As a result, our data suggest that tPA appears to regulate MMP-9 expression in the mouse lung IR model. The exact mechanism of tPA-mediated MMP regulation has yet to be completely elucidated. However, recent studies suggest the interaction between tPA and the LRP-activated canonical pathway of NF-κB (34). Activation of the NF-κB pathway elevates expression of MMP-9, which is associated with an increase in the permeability of the neurovascular unit during cerebral ischemia (30, 32). Moreover, in vitro studies using various cell lines indicate that the binding of tPA to LRP induces a transient tyrosine phosphorylation of the cytoplasmic domain of LRP, which leads to activation of Erk1/2 signaling pathway and subsequently increased synthesis of MMP-9 (10). In addition to its contribution to fibrinolysis, accumulating data suggest that tPA acts as an extracellular cytokine to stimulate cell proliferation (31) and upregulates the expression of MMP-9 in human cerebral microvascular endothelial cells (30) and fibroblasts (10). Hence it is conceivable that tPA binds LRP and signals the transcriptional upregulation of MMP-9 in our mouse lung IR model as well.

Finally, we investigated the potential direct chemotactic effect of tPA on neutrophil migration and extravasation following acute lung IR injury. The results of our in vitro migration assays revealed that recombinant tPA had no direct chemotactic effects on neutrophil migration compared with that observed for fMLP, a well-established chemoattractant. These results complement those of previously published reports in renal IR injury (21), which compared the chemotactic effect of r-tPA to fMLP. As a result, it appears that tPA exerts an indirect chemotactic effect on migrating neutrophils during the extravasation process following acute IR injury.

Conclusion. Our significant experimental results demonstrate that deletion of tPA confers marked protection following acute lung IR injury in a mouse model through diminished neutrophil extravasation, evident at three different levels: 1) at the cellular level, elimination of tPA results in an increase of HNA within the pulmonary microvasculature; 2) at the molecular level, deletion of tPA leads to the concomitant downregulation of PECAM-1 expression via tPA/LRP/NF-κB signaling pathway and upregulation of P-selectin expression in small pulmonary vessels as well as to decreased MMP-9 expression; and 3) at the functional level, depletion of tPA significantly decreases lung microvascular permeability, airway resistance, and pulmonary artery pressure and increases lung compliance. These results suggest that inhibition of tPA could be a novel therapeutic target for the treatment of IR injury following lung transplantation.

ACKNOWLEDGMENTS

We thank Dr. Klaus Ley and Dr. John M. Sanders for providing us with a P-selectin antibody and Dr. Klaus Ley and Dr. Joel Linden for consultation.

GRANTS

This study was supported by Award Numbers 1K08HL094704-01 (C. L. Lau), RO1 CA136705 (D. R. Jones) and T32HL007849 (D. J. LaPar) from the National Heart, Lung, and Blood Institute and by a CVRC Partner’s Grant (C. L. Lau). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Heart, Lung, and Blood Institute or the National Institutes of Health.

DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the author(s).

REFERENCES

IPAI N HIBITNS NEUTROPHIL EXTRAVASATION IN LUNG IR INJURY

L729


