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A Role for Low Density Lipoprotein Receptor-related Protein 1 in the Cellular Uptake of Tissue Plasminogen Activator in the Lungs

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Abstract

Purpose—To gain knowledge of lung clearance mechanisms of inhaled tissue plasminogen activator (tPA).

Methods—Using an *in vivo* mouse model and *ex vivo* murine whole organ cell suspensions, we examined the capability of the lungs to utilize LRP1 receptor-mediated endocytosis (RME) for the uptake of exogenous tPA with and without an LRP1 inhibitor, receptor associated protein (RAP), and quantitatively compared it to the liver. We also used a novel imaging technique to assess the amount LRP1 in sections of mouse liver and lung.

Results—Following intratracheal administration, tPA concentrations in the BALF declined over time following two-compartment pharmacokinetics suggestive of RME clearance mechanism. *Ex vivo* studies showed that lung and liver cells are similarly capable of tPA uptake via LRP1 RME which was reduced by ~50% by RAP. The comparable lung and liver uptake of tPA is likely due to equivalent amounts of LRP1 of which there was an abundance of LRP1 in alveolar epithelium.

Conclusions—Our findings indicate that LRP1 RME is a candidate clearance mechanism for inhaled tPA which has implications for the development of safe and effective dosing regimens of inhaled tPA for the treatment of plastic bronchitis and other fibrin-inflammatory airway diseases in which inhaled tPA may have utility.

Keywords

receptor mediated endocytosis; plastic bronchitis; therapeutic proteins

Introduction

Plastic bronchitis is a rare, primarily pediatric disease that is characterized by the formation of obstructive, exudative, inflammatory fibrin casts in the tracheobronchial tree (1–4). Inhaled nebulized tPA (alteplase, Genentech, South San Francisco, CA, USA) is often clinically used off-label to treat acute exacerbations of PB (5–9). tPA is a serine protease that degrades fibrin via the activation of plasminogen which yields plasmin and is used therapeutically to dissolve thrombus associated with MI, pulmonary embolism, and AIS (10–12). For PB treatment, inhalation of tPA permits targeted delivery to the site of drug action which requires repeated administration into the airways (5).

The dosing regimen of inhaled tPA for PB treatment was derived from the weight-based adult intravenous regimen used in the treatment of MI or AIS. Systemic administration and clearance of tPA in adults has been well characterized (10–12). The drug has a reported initial (α) half-life of 5 minutes that is followed by a more prolonged terminal (β) phase which is characteristic of a two-compartment model (13, 14) and follows non-linear pharmacokinetics (15, 16). Its clearance from the systemic circulation occurs by hepatic RME via LRP1 and mannose receptors (12, 16–19) and RME likely contributes to the non-linearity of tPA clearance (15, 16). LRP1 is ubiquitous and is expressed on hepatocytes and many other cell types (20), including lung fibroblasts and macrophages (21, 22) and it has been detected in pneumocytes and bronchial epithelium (23). To date, most of the pulmonary LRP1 related research has focused on the receptor's role in inflammatory lung disease (24), pulmonary function (25) and lung cancer (26).

To study the fate of tPA in the lungs, animal studies have been performed following IT administration of tPA. Following IT dosing, tPA is detectable in BALF of healthy mice up to several hours after dosing and fatal pulmonary hemorrhage occurred in a group of mice that received high, repeated doses of tPA ($> 1\text{ mg/kg/d}$), suggesting that tPA may accumulate in the airways (27, 28). Since LRP1 plays an essential role in the elimination of tPA from the systemic circulation, we hypothesized that pulmonary LRP1 will bind and transport inhaled tPA from the airway lumen into the epithelial cell lining, and potentially into the circulation. We previously reported that lower levels of LRP1 in the airways compared with the liver, may explain slower elimination of inhaled tPA in the lungs (29). Here, we have expanded on the details of these findings and now show that lung LRP1 is abundant in type II alveolar epithelium through a combined approach of *ex vivo* studies and state-of-the-science imaging techniques. In addition, whole organ cell studies show that the lung has a similar capability as the liver for LRP1-mediated uptake of tPA. This knowledge has significant implications for improving understanding of the fate of therapeutic macromolecules in the airways as well as for the development of safe and effective dosing regimens of inhaled tPA for the treatment of PB and other fibrin-inflammatory airway diseases (30–32) in which inhaled tPA may have utility.

Materials and Methods

Animals

Male mice (C57BL/6; 8 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and housed for at least one week prior to experimentation. The animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Michigan in accordance with the principles of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Isolation of Mouse Alveolar Epithelial Cells

Murine type II alveolar epithelial cells were isolated using the method developed by Corti et al. (33) and as previously described (34). Briefly, following CO₂ euthanasia, the pulmonary vasculature was perfused, and the lungs were first filled with dispase (1mL; Worthington, Lakewood, NJ) and then low melting point agarose (1mL) and placed in ice-cold PBS to harden. The lungs were then submerged in dispase for 45 min before being minced and incubated in DMEM with 0.01% DNase for 10 min. A single-cell suspension was obtained by passing the lung mince over a series of nylon filters. Myeloid cells were removed by first incubating cells with biotinylated antibodies against CD32 and CD45 (BD Pharmingen, San Diego, CA) and then streptavidin-coated microbeads (Promega, Madison, WI), followed by negative selection using a magnetic tube separator. Mesenchymal cells were removed by overnight adherence in a Petri dish. Non-adherent AEC were plated on fibronectin-coated plates and cultured for 72 h in complete DMEM. At the time of harvest, media was removed, lysis buffer was added to the plate and samples were frozen (−80°C). The method is known to result in a sample that consists of approximately 93% and 95% type II cells by Papanicolaou staining, which detects the lamellar bodies of type II cells (35), and ultrastructural analysis, respectively.

Administration of Tissue Plasminogen Activator (tPA)

Human recombinant tissue plasminogen activator (htPA; alteplase, Activase®, Genentech, South San Francisco, CA, USA) was formulated and nebulized for pulmonary delivery as previously described (36). The collected aerosol was assayed by UV spectroscopy to determine protein concentration and the extent of protein aggregation (28, 36). Using the protein concentration derived from this method, mice received a single IT dose (via the oral route) of 0.3mg/kg. For this, mice were anesthetized under isoflurane (Fluriso; Vet One, Boise, ID), placed on a rodent intubation table (Hallowell EMC, Pittsfield, MA, USA), and the vocal cords were visualized using an otoscope (Welch-Allyn Medical Products, Skaneateles Falls, NY, USA) (27, 28). The tip of a gavage needle (22G, Hallowell EMC) attached to a syringe containing a dose of either pf-mtPA or an equivalent volume of sterile saline was gently moved past the vocal cords and into the trachea; the dose was delivered by depression of the syringe's plunger. Following drug or saline administration, the mouse was held upright for approximately 5 s, after which it was returned to a cage and allowed to recover from anesthesia.

Collection of BALF and Blood Samples and Measurement of htPA

At each respective time point, mice were anesthetized with ketamine (50mg/kg) and xylazine (5 mg/kg) administered by intraperitoneal injection. A blood sample was collected via cardiac puncture into a heparinized tube which was centrifuged ($1000 \times g$, 4°C for 5 min) to generate plasma. These samples were stored (-80°C) until the time of assay. After the collection of the blood sample, a tracheotomy was performed. The BALF was collected by flushing $2 \times 400\mu\text{L}$ of PBS into the lung via the tracheal cannula. The BALF was centrifuged ($1000 \times g$, 4°C for 5 min) and the supernatant was collected and frozen (-80°C) until the time of assay. At the time of assay, samples were thawed on ice and the concentration of htPA was measured using an enzyme-linked immunosorbent assay (ELISA). For this, the wells of a 96-well microtiter plate were coated with $100\mu\text{L}/\text{well}$ of capture antibody ($1\mu\text{g}/\text{mL}$; rabbit anti-human tPA, Molecular Innovations, Novi, MI) and incubated for 1 h (37°C). The plate was washed with wash buffer (0.05% Tween-20 in 1X PBS, pH 7.2) using a 96-well plate washer (BioTek ELx405, Winooski, VT) three times after which blocking reagent (5% milk in 1X PBS 0.05% Tween-20 buffer) was added to each well. The plate was covered and incubated (1 h) at room temperature (RT) and the wash step was repeated. Upon completion of the second wash, standards (htPA, 0–1000 ng/mL) and diluted samples ($100\mu\text{L}/\text{well}$) in duplicate were added to respective wells and the plate was covered and incubated (1 h, RT). The plate was washed again and $100\mu\text{L}$ of detection antibody ($1\mu\text{g}/\text{mL}$; mouse anti-human tPA, Calbiochem, Gibbstown, NJ) was added to each well and the plate was covered and incubated (1 h, RT). Following this incubation, the plate was washed and $100\mu\text{L}$ of a horseradish peroxidase (HRP) conjugated antibody ($1\mu\text{g}/\text{mL}$; goat anti-mouse IgG HRP, Millipore, Billerica, MA) was added to each well followed by incubation (20 min, RT) and the addition of TMB substrate solution ($100\mu\text{L}/\text{well}$). The reaction was stopped by the addition of sulfuric acid ($100\mu\text{L}$ of 2N). The assay was previously validated to show no cross reactivity with rat or mouse tPA and the lower limit of detection was determined to be $10\text{ ng}/\text{mL}$. The absorbance data were acquired using a ThermoMax microplate reader (Molecular Devices, Sunnyvale, CA). The total tPA concentration of each sample was derived from the linear phase of the standard curve as analyzed by reader's software (Softmax PRO 4.1, Molecular Devices).

The resulting mean BALF htPA concentration data were plotted as $\ln$ concentration versus time which revealed a pharmacokinetic profile with a biphasic elimination pattern indicative of a two-compartment model (14). Best line fitting of the first five, initial (α) phase, $\ln$ concentration measurements and the last three, terminal (β) phase, $\ln$ concentration measurements were used to calculate the elimination rate constants (K_d) of each phase. The elimination half-life ($t_{1/2}$) of each phase was calculated by dividing 0.693 by the respective K_d .

Generation of Primary Cell Suspensions

Whole lung and liver organs were harvested from mice for generation of primary lung and liver cell suspensions. Mice were euthanized under CO_2 in a contained chamber and following thoracotomy and appendectomy, the organs were perfused blood free with PBS (Life Technologies, Carlsbad, CA, USA). Blood-cleared lungs and livers were excised *en bloc*, placed into DMEM high glucose media (Life Technologies) and were manually

dispersed and passed through cell strainers to remove connective tissues. The resulting whole organ cell suspensions were then centrifuged ($800 \times g$, 4°C , for 5 min) to acquire primary cells. The pelleted cells were resuspended in PBS and residual red blood cells (RBC) were lysed with the addition of RBC lysis buffer (Sigma-Aldrich, St. Louis, MO, USA). The cell suspension was washed with PBS and centrifuged an additional three times after which the final cell suspensions were maintained in DMEM on ice. Primary lung and liver cells were stained with trypan blue and counted using an automated cell counter (Countess, C10227, Life Technologies). For the experiments, 20,000 cells were plated per well into 24-well plates in DMEM media and maintained at 37°C (5% CO_2).

Mouse Tissue Plasminogen Activator (mtPA) Treatment

In advance of tPA treatment, 400 nM of receptor associated protein (RAP, P30533, Molecular Innovations), an inhibitor of LRP1, or an equivalent volume of PBS was added to some cells and they were incubated (30 min, 5% CO_2 , 37°C). Recombinant mtPA protein (P11214, Molecular Innovations, Novi, MI, USA) in doses of 25, 50, or 100 ng or an equivalent volume of PBS was added to whole lung and liver primary cells and the plates were incubated (5% CO_2 , 37°C) for 30 min. Following incubation, the contents of each well were transferred to a microcentrifuge tube and centrifuged ($800 \times g$, 4°C , for 5 min). The supernatant was removed and stored (-80°C). Cell pellets were washed with PBS and centrifuged again, after which wash buffer supernatant was removed and stored (-80°C). The final cell pellet was resuspended in radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich) and frozen (-80°C). Time course studies (0–30 min) of mtPA uptake by whole lung and liver cell suspensions in the presence and absence of RAP (400 nM) were done as described above following the addition of mtPA (50 ng).

Western Blotting

Frozen samples containing 10,000 whole cell lysates were eluted with Laemmle 2X sample buffer (Sigma-Aldrich) with 5% β -mercaptoethanol and separated by 10% SDS-PAGE (Bio-Rad, Hercules, CA). Proteins were transferred to polyvinylidene fluoride (PVDF) membranes and blocked (5% milk in PBS with Tween-20 (Sigma-Aldrich)). Membranes were immunoblotted overnight (4°C) with a anti-mouse tPA (ASMTA-GF, Molecular Innovations) and LRP1 (ab92544, Abcam, Cambridge, MA, USA) in 1:5000 and 1:15,000 dilutions (5% milk in PBS with Tween 20), respectively. Post immunoblotting, membranes were washed with PBST (PBS with Tween 20, three times for 5 min each). Then membranes were incubated with a horseradish peroxidaseconjugated secondary antibody (goat anti-rabbit, AQ132P, Millipore, Billerica, MA, USA) in 1:10,000 dilution (2 h at RT). Signal detection was achieved using enhanced chemiluminescence (Pierce ECL Western Blotting Substrate, Thermo Scientific, 32106, Rockford, IL) and exposure to radiographic film (BioMax Light Film, Z370371, Kodak, Rochester, NY). The image was acquired using a Medical Film Processor Model SRX-101A (Konica Minolta) and Image J (ImageJ 1.44b, NIH, Bethesda, MD, USA) was used to quantify protein band density.

Measurement of mtPA Uptake by Lung and Liver Cell Suspensions

The concentration of mtPA in lung and liver cell lysates (intracellular fraction) and supernatants (extracellular fraction) was detected using mtPA total antigen assay ELISA kit (MTPAKT-TOT, Molecular Innovations). The mtPA concentrations of the samples were derived from the standard curve.

Immunohistochemistry

Existing C57BL/6 mouse and 13N159 macaque monkey lung and liver formalin fixed, paraffin embedded (FFPE) samples were sectioned at the University of Michigan Unit for Laboratory Animal Medicine and analyzed by immunohistochemical staining for LRP1. Heat-induced antigen retrieval of the sample sections was performed using Rodent Decloaker (RD913, Biocare Medical LLC, Concord, CA) and EDTA Decloaker (CB917, Biocare Medical). Tissue sections were then blocked with Peroxidized 1 (PX968M, Biocare Medical) and Rodent Block M (RBM961, Biocare Medical). Immunohistochemical staining was assessed using a titer titration of the primary antibody (1:500, 1:1000, and 1:5000) and the pathologist determined that the 1:1000 dilution was optimal. Samples were incubated in monoclonal LRP1 antibody diluted 1:1000 (rabbit α -mouse ab92544, Abcam). Secondary and tertiary incubations were carried out with Rabbit-on-Rodent HRP-polymer (RMR622, Biocare Medical) and intelliPATH Universal HRP Tertiary Reagent (IPT5002, Biocare Medical), respectively. LRP1 was detected and visualized using 3'3'-diaminobenzidine (DAB) (intelliPATH FLX™ DAB Chromogen Kit, IPK5010, Biocare Medical) followed by DAB post-enhancing solution (DAB sparkle, DS830G, Biocare Medical). All sections were counterstained with hematoxylin (CAT Hematoxylin, CATHE-M, Biocare Medical). Negative control slides were also generated by omitting the DAB staining step.

Image Acquisition and Analysis

Digital images of stained and unstained lung and liver sections at 40 $\times$ magnification were acquired on a Ti Eclipse inverted microscope (Nikon Instruments, Inc., Melville, NY, USA) using bright field optics under Kohler illumination. To acquire and quantify DAB stained images, a red filter (655 ± 30 nm), which corresponds to the absorbance peak of the background staining, and a blue filter (480 ± 30 nm), which corresponds to the absorbance peak of DAB, were used. The density of DAB staining is indicative of LRP1 receptor density. To analyze quantify DAB staining density all images were analyzed with ImageJ. A detailed outline of the image acquisition, processing and analysis workflow is in the online supplement.

Statistical Analysis

Statistical analyses were performed using PRISM 6 software (GraphPad, La Jolla, CA, USA) and differences between groups and treatments were estimated by unpaired Student's t-test or ANOVA with post-hoc analyses, as appropriate. Dose-response was assessed by linear regression. A p-value of ≤ 0.05 was considered statistically significant.

Results

BALF Levels of Lung Delivered htPA Declined Over Time

Instillation of htPA into the mouse lung resulted in detectable levels of htPA that declined over time (Figure 1). At no time point was htPA detected in the blood. Pharmacokinetic fitting of the htPA log concentration-time data revealed that the change in htPA concentration was most consistent with a two-compartment model.

Antagonism of the LRP1 Receptor Reduced mtPA Uptake in Whole Lung Cell Suspensions

To determine the role of LRP1 on lung clearance of tPA, we proceeded to establish that the uptake of mtPA into primary murine lung and liver whole cell suspensions was affected by the presence of a known LRP1 inhibitor, receptor associated protein (RAP). Quantitation and representative Western blots of both lung and liver cell uptake of mtPA in the presence and absence of RAP are shown in Figure 2. The amount of mtPA uptake into lung, but not liver cells, was dose dependent ($r^2=0.823$, $p<0.0001$ and $r^2=0.192$, $p=0.155$, respectively). In both lung and liver, mtPA uptake was reduced by 20–67% in the presence of RAP.

Timecourse of mtPA Uptake in the Presence and Absence of RAP is Similar in the Lung and Liver

To further substantiate the role of LRP1 in the uptake of mtPA, we assessed the time course of mtPA uptake in lung and liver whole cell suspensions. Pre-treatment with RAP reduced LRP1-mediated uptake of mtPA in both lung and liver cells as measured by Western blot (Figure 3A and B). This finding was corroborated by a corresponding increase in intracellular fraction mtPA levels as measured by ELISA (Figure 3C and D). Although initial (time 0) intracellular fraction concentrations were lower in liver than in lung cells (Student's t-test $p=0.036$), RAP inhibited mtPA uptake in both liver and lung cells (Figure 3E and F). Using the extracellular fraction mtPA concentrations that are less prone to degradation than intracellular fraction mtPA, the average percent RAP-mediated reduction in LRP1 uptake of mtPA over the entire 30 min time course was similar for the lung and liver cell suspensions, 58% and 52%, respectively.

Lung and Liver Cell Suspensions Have Similar Amounts of LRP1 Protein

The relative amounts of LRP1 in murine lungs and murine liver samples were quantified and displayed similar amounts of the receptor. Based on a linear relationship between the measured LRP1 protein density (Figure 4A and B) and cell number, densitometry data show that the LRP1 protein density per cell is similar (mean $\pm$ S.D.: 1.2 ± 1.1 vs. 1.0 ± 0.78 , $p=0.81$) in liver and lung cells, respectively. LRP1 was also detected in isolated type II alveolar epithelial cells (Figure 4C).

The Lung and Liver Have Similar Ratios of LRP1 Staining

As an independent measurement of the relative amounts of LRP1 protein in liver and lung cells, FFPE liver and lung tissue sections were immunohistochemically stained for LRP1. Brightfield images were acquired using a transmitted light microscopy set up (see online supplement) with two different wavelengths of light (655nm and 480nm) corresponding to

the peak absorbance signal of the LRP1-specific probe (brown DAB staining), and the peak absorbance of the nonspecific, background tissue signal (also see Figure E1 in the online supplement). In the liver (Figure 5A), brown DAB staining corresponding to the distribution of LRP1 was ubiquitous across the section (also see Figure E3A and B in the online supplement). In the lung (Figure 5B), brown DAB staining corresponding to the distribution of LRP1 was localized along the epithelial lining of the alveoli and in macrophages (also see Figure E3C and D in the online supplement). Based on quantitative image analysis, the density of LRP1 staining over the cellular regions of liver and lung sections was not different between the two organs (Figure 5C). Image analysis of LRP1 stained 13N159 macaque lung and liver sections produced similar staining results (Figure E4 in the online supplement).

Discussion

Inhalation of nebulized tPA has therapeutic potential as a treatment for the rare, pediatric illness, PB (5–9). However, very little is known about the transport of tPA following delivery into the airways. Here, we show that the concentration of IT administered tPA declined over time in the BALF and that the time-dependent tPA concentration change followed two-compartment pharmacokinetics with an estimated α -phase elimination half-life of 0.7h and an estimated β -phase elimination half-life of 5.8h. We cannot confirm non-linear kinetics because we only tested one dose of tPA. However, similar to what has previously been reported (15), the BALF tPA concentration versus time curve showed an upward trend suggesting a zero-order component. This is consistent with RME (16, 17) and suggests that tPA is either degraded or removed from the airway space. Potentially contributing to the clearance of tPA in the airways, is the significant expression of pulmonary LRP1, which was primarily detected in the alveolar epithelium, at levels comparable to the liver. In addition, our data show that, in both lung and liver cell suspensions, the peak measured intracellular fraction tPA concentration occurred within 1 min of the application of tPA to the cells and this concentration was reduced by half within 10 min. This observation implies that LRP1-mediated uptake into cells occurs very quickly (16, 17) and likely contributes to the $\alpha_{t_{1/2}}$ of tPA in the airways. The longer $\beta_{t_{1/2}}$ may be attributable to the diffuse distribution of tPA in the lungs that is known to occur after IT administration (36, 37) and involves other clearance mechanisms such as mucociliary action which is categorized as slow (Figure 6) because it can take several hours for macromolecules to be cleared from the airway by this process. Transcellular absorptive transcytosis is also reported to occur over hours (38) but, to date, there is no evidence that tPA is removed from the airway via this mechanism. Thus, we infer that LRP1-mediated binding and uptake contributes, at least in part, to the early clearance of tPA from the airways. This conclusion is supported by the rapid time course of the α phase of BALF clearance and by two other pieces of evidence: First, using a whole lung cell suspension model, that lung cells are capable of taking up tPA from the surrounding media via LRP1 to the same extent as liver cells; and second, state-of-the-science image analysis techniques, which permitted measurements of a cell-associated LRP1 signal following immunohistochemical staining. This revealed that the expression of LRP1 protein by cells from the lung was comparable to that of cells from the liver in both mice and macaque

monkeys. Western blotting also showed an abundance of LRP1 in type II alveolar epithelial cells.

Our findings are important and relevant because they point to a potential mechanism for removal of inhaled tPA from the airways. The clearance mechanisms affecting the half-life of pulmonary delivered therapeutic macromolecules from the airways, via absorption, metabolism or other routes have not been fully elucidated (38–40). However, there is evidence that these mechanisms are distinct from those that regulate small molecule and ion transport in the lungs (41, 42). Therapeutic proteins have been successfully delivered to the lungs via inhalation but issues related to utility and safety have been raised (38, 43). Nevertheless, technological advances in pulmonary drug formulation and devices for the delivery of macromolecules, including siRNA and DNA, are being made (44, 45). This active area of drug development highlights the need for more mechanistic knowledge about the fate of therapeutic proteins in the lungs.

Endocytosis of tPA by LRP1 in the liver has been well studied and is known to result in the degradation of this protein (12, 16–18). This process is essential for the regulation of tPA plasma concentrations, contributes to the short elimination for the systemically administered drug, and is a saturable process (15, 16). In our study, the rate of intracellular processing of tPA in liver cells appeared to occur at a faster rate than that in lung cells. This was evidenced by longer α and β elimination half-lives compared with those of systemically administered tPA (13, 14) and the lower intracellular fraction concentrations of mtPA present in liver cells compared to lung cells, with similar extracellular fraction concentrations across both cell types (see Figure 3). Given the functional differences of these organs, it is reasonable to expect that the pharmacokinetics of tPA endocytosis in the lungs will be somewhat different from that of the liver. However, since LRP1 appears to function in lung epithelial cells in the same manner as it does in hepatocytes, it is reasonable to hypothesize that RME followed by degradation of tPA (12, 16–18) is also active in the lung. Collectively, details of this process remain to be elucidated as RME of tPA in alveolar epithelium is not yet fully understood (Figure 6).

The importance of improving understanding of the mechanisms of the fate of lung delivered proteins is further illustrated by the broad functions of the receptors that bind tPA protein and/or that participate in RME (Figure 6). While delivery of therapeutic proteins to the lungs can result in cellular uptake via endocytic receptors and ultimately lead to degradation in lysosomes, this process can also initiate subsequent cell signaling pathways. In the context of LRP1, which is a transmembrane receptor responsible for the endocytosis and degradation of a number of ligands including metalloproteinases (MMP) and apolipoproteins (22, 24), it can function as a signaling receptor (Figure 6). The expression of LRP1 in the airway epithelium suggests an important but not yet fully understood physiological role in the lungs. Its ability to mediate MMP endocytosis and other extracellular matrix proteins may be particularly important in inflammatory conditions, like acute respiratory distress syndrome, in which the extracellular domain is shed and results in dysregulation of MMP homeostasis (24). It is also worth noting that tPA-mediated activation of LRP1 can lead to activation of the ERK1/2 pathway (46, 47) with consequent effects on cell physiology (48). In fact, depending on the cell type, the tPA-LRP1 interaction can

trigger a range of different responses (12). Activation of downstream signaling can also occur via other tPA receptor proteins such as annexin A2 and mannose receptors both of which have been detected on alveolar macrophages (23). Accordingly, as the use of inhaled tPA for PB or other fibrin-inflammatory lung diseases advances, it will be important to assess whether LRP1 binding leads to downstream effects on cell function.

We acknowledge that there are limitations of our study. Experimentally, it is possible that the intracellular fraction may contain contamination by extracellular tPA that occurred during the separation process. However, the purpose of our studies was to evaluate the role of LRP1-mediated uptake in the lung rather than the specific mass action of tPA. Since any contamination would likely occur to similar extent across all samples it has little impact on the interpretation of our results. Next, as previously mentioned, additional mechanisms likely contribute to the clearance of tPA in the lungs. This includes the mannose receptor which may contribute to α -phase elimination and is prevalent on alveolar macrophages (18, 49). The LRP1 receptor is also expressed by macrophages (50) and we did not separately account for macrophage-mediated uptake of tPA in our model. We also elected to conduct the bulk of our study in an *ex vivo* murine model using a small dose of tPA that was directly applied to the cells. This did not permit assessment of mucociliary clearance which likely contributes to the β -phase of airway elimination. While we generated evidence of LRP1 in the airway epithelium of Macaque monkeys, we recognize that LRP1-RME of tPA in the airways will need to be confirmed using *in vivo* models including the human model so that we can confirm this mechanism.

In conclusion, the abundance of LRP1 in the alveolar epithelium suggests that there is a site accessible mechanism of elimination for inhaled tPA. Furthermore, our study found that exposure of whole lung cell suspensions to exogenous tPA resulted in LRP1-mediated uptake of tPA that followed a similar extent and time course to that which occurred in whole liver cell suspensions. Therefore, LRP1-mediated cellular uptake is a candidate mechanism responsible for the fate of tPA in the airways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PB plastic bronchitis

tPA	tissue plasminogen activator
RME	receptor mediated endocytosis
LRP1	Low density lipoprotein receptor-related protein 1
MI	myocardial infarction
AIS	acute ischemic stroke
IT	intratracheal
BALF	bronchoalveolar lavage fluid

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Figure 1.

Log₁₀ concentration of human tissue plasminogen activator (htPA) versus time in bronchoalveolar lavage fluid (BALF) from mice that received a single intratracheal dose of nebulized htPA (0.3mg/kg). BALF was acquired from individual animals at each time point and at the same time an associated blood sample was obtained. Mice that received an equivalent volume of phosphate buffered saline (PBS) did not have detectable levels of htPA in the BALF and there was no detectable htPA in any of the blood samples from either htPA- or PBS-treated mice. The initial (α) elimination half-life (t_{1/2}) was followed by a more prolonged, terminal (β) t_{1/2}, which is indicative of a two-compartment model. Data are the mean (±SEM) of 3-4 mice at each time point. htPA concentrations were determined by ELISA.

Figure 2.

Receptor associated protein (RAP) reduced LRP1-mediated uptake of mtPA in primary murine whole lung and liver cell suspensions. A total of 20,000 lung or liver cells in the presence or absence of the LRP1 inhibitor, RAP (400 nM), were treated with mtPA (25–100ng). A representative Western blot of intracellular fraction mtPA and quantitated data from (A) primary liver cell suspension lysates and (B) primary lung cell suspension lysates show that RAP reduced the amount of intracellular fraction mtPA by an average of 67, 53 and 39% for 25, 50 and 100ng of added mtPA, respectively, in liver cells and by an average of 20, 43, and 52% for 25, 50 and 100ng of added mtPA, respectively, in lung cells. In the presence of RAP, the mean amount of mtPA (100ng) was significantly lower in liver cells compared to the absence of RAP (* $p=0.014$ by ANOVA post-hoc Holm-Sidak test). In the lung, but not the liver, the mtPA dose response was significant by linear regression ($r^2=0.82$; $p<0.0001$ and $r^2=0.19$; $p=0.19$, respectively). Data are the mean ($\pm$ S.E.M.) of 3–4 samples/group.

Figure 3.

The concentration of intracellular fraction mtPA was lower over time in the presence of LRP1 inhibitor, receptor associated protein (RAP) in primary liver and lung cell suspensions (20,000 cells each). Cells were exposed to mtPA (50ng) at time 0 in the presence or absence of RAP (400nM). Representative Western blots of intracellular fraction mtPA over time in (A) primary whole liver cell lysates and (B) primary whole lung cell lysates. These findings were corroborated by the measurement of intracellular fraction mtPA concentrations measured by ELISA in (C) liver cell (* $p=0.0132$ at 1 min) and (D) lung cell (* $p=0.009$; + $p<0.001$; # $p=0.066$) lysates as well as in extracellular fraction (supernatant) media of (E) liver (* $p=0.005$; + $p=0.0005$) and (F) lung (* $p=0.024$; + $p=0.001$) cell suspensions. Data are the mean ($\pm$ S.E.M.) of 3-4 samples/group and p values were generated from post ANOVA Holm-Sidak tests.

Figure 4.

Protein band density of LRP1 increases with increasing cell number in both murine whole liver and lung cells. Representative Western blot and quantitation of LRP1 in primary whole (A) liver cell and (B) lung cell lysates at cell densities from 20,000 to 500,000 cells. The correlation coefficient (r^2) for liver and lung LRP1 and cell number was 0.74 and 0.92, with p values of 0.13 and 0.04, respectively. (C) Representative Western blot of LRP1 in mouse type II alveolar epithelial cell (AEC) lysates with actin as a loading control. Lanes 1, 2: Mouse AEC (30 µg protein); Lanes 3, 4: Mouse AEC (20 µg protein); Lane 5: Mouse primary whole lung cell lysate (20,000 cells).

Figure 5.

The percent (%) tissue area with LRP1 staining in mouse liver and lung sections was similar. Representative color light micrographs of (A) liver and (B) lung sections (40X) stained for LRP1 using 3'3'-diaminobenzidine (DAB) as a chromogen lead to brown staining; see text for details. (C) Quantitative image analysis revealed that the % tissue area with LRP1 stain in liver and lung sections was not different ($p=0.483$ by unpaired Student's t-test). Data are mean ($\pm$ S.E.M.) of 7 sections/group.

Figure 6.

A number of transport mechanisms for macromolecules exist in the airways. **Panel A:** Transcellular transport can occur as either absorptive transcytosis or via transporter proteins (e.g., OAT P2B1) (42) and often involve movement of molecules through the cell, which can be a slow (hours) process (38). Paracellular transport, in which macromolecules move through the airway epithelial barrier between cells, is thought to be a primary transport mechanism for small (< 40kDa) proteins and can occur within minutes (39). Mucociliary action is pivotal for maintaining the integrity of the airway environment and also serves as a mechanism for slow airway clearance of macromolecules (42). **Panel B:** At the receptor level, membrane-associated tissue plasminogen activator (tPA) binding proteins include annexin A2 and p11 binding complex, LRP1 and the mannose receptor. The LRP1 and mannose receptors are transmembrane proteins which are the only known endocytic receptors for tPA (18, 49), the former of which can be blocked by receptor associated protein (RAP). Annexin A2 serves as the site where tPA assembles with plasminogen (PLG) for localized generation of plasmin (51). Most abundant on endothelial cells, annexin A2 has been detected on macrophages and pneumocytes (23) and tPA binding to annexin A2 has been reported to lead to activation of downstream signaling pathways (47, 52). The binding of tPA to LRP1, which is ubiquitous, and mannose receptors, which are prevalent on macrophages, is independent of tPA protease activity and can occur in the presence or absence of its primary physiological inhibitor, plasminogen activator inhibitor. Following tPA binding, the protein is endocytosed and packaged into endosomes. In the liver, this process leads to lysosomal degradation of tPA. Given this function and the abundance of LRP1 in the alveolar epithelium, it is reasonable to expect that the same occurs in the lung. In addition to the processing of macromolecules, activation of LRP1 and mannose receptors can trigger a cadre of downstream signaling events including the activation of ERK and MAP kinases (46, 53). “Lungs diagram simple” by Patrick J. Lynch, medical illustrator - Patrick J. Lynch, medical illustrator. Licensed under CC BY 2.5 via Wikimedia Commons - http://commons.wikimedia.org/wiki/File:Lungs_diagram_simple.svg#mediaviewer/File:Lungs_diagram_simple.svg