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RESEARCH ARTICLE

UROKINASE ENZYME; RECENT ADVANCES IN UNDERSTANDING OF ITS RECEPTOR, STRUCTUREAND PHYSIOLOGICAL ASPECTS

***Manoj G Tyagi, RohitKodagali, PichumaniBalagurumoorthy, VishakhaTyagi and VikramS Gota**

Department of Biotechnology, Vellore Institute of Technology, Vellore, Tamilnadu, India

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ABSTRACT

The urokinase enzyme is a serine protease released by the kidney and is aimportant member of the Pulmonary renal cascade. The uPA system consists of the serine proteinases uPA and plasmin, two serpin plasminogen activator inhibitors (PAI; PAI-1 and PAI-2), the serpin plasmin inhibitor α2-anti-plasmin, and the cell surface uPA receptor (uPAR), a member of the glycosylphosphatidylinositol (GPI)-anchored protein family. There are two types of plasminogen activators (PAs): tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA), both of which can catalyze the conversion of the inactive plasminogen zymogen to plasmin, as well as cause the activation of additional protease zymogens and other latent growth factors. Urokinaseis structurally unique and does contribute to multiple physiological roles and its effects are also prominent on the pulmonary system whereby along with the erythropoietin and heparin binding growth factors it plays a critical role in the pulmonary renal cascade.

INTRODUCTION

Urokinase is an important enzyme released by the kidney and is a critical member of the pulmonary renal cascade an important neuro-humoral and physiological cascade (Tyagi *et al*., 2004). A soluble-cleaved form of the urokinasereceptor (scuPAR), arising from lipolytic cleavage of the glycosylphosphatidylinositol (GPI) anchor by factors including phospholipase D (Ryu *et al*., 2014; Thuno *et al*., 2009), also exists i.e asscuPARwhich is structurally identical to the membrane-bound receptor but is able to interact with a number of cell factors, for example integrins and G protein-coupled receptors. scuPAR is thought to havefunctions beyond acting as a putative receptor for the main uPAR ligands, for example uPA (Selman *et al*., 2004; Bradford *et al*., 2014).Urokinase plasminogen activator (uPA) also has fibrogenic actions, although its pathological role in idiopathic pulmonary fibrosis (IPF) is not properly understood. uPA-generated plasmin can cause concomitant collagen proteolysis and transactivation of protease activated receptor-1 (PAR-1), integrins and transforming growth factor-β receptor (TGFβ R), indirectly implicating this system in IPF pathology (Richeldi, 2014; Imokawa *et al*., 1997; Lardot *et al*., 1999).

Corresponding author: **Manoj G Tyagi**
Department of Biotechnology, Vellore Institute of Technology, Vellore, Tamilnadu, India

However, plasmin-mediated fibrinolysis is attenuated in IPF, contributing to the accumulation of airspace fibrin. Suppressed fibrinolysis corresponds with lower levels of uPA detected in the bronchoalveolar lavage fluid of IPF patients, conversely with higher levels of plasminogen activator inhibitor-1 (PAI-1). Consequently, uPA has been considered to be protective, rather than being detrimental in IPF. However, fibrinolysis is the physiological role of tissue-type plasminogen activator (tPA), which has fibrin specificity, unlike u PA (Schuliga *et al*., 2015; Chambers, 2012). Nevertheless, intranasal administration of higher than physiological concentrations of uPA or the overexpression of uPA in bronchial Clara cells of the epithelium enhance airspace fibrinolysis, has been shown to be protective in experimental lung injury. This article reviews the current status of the research in urokinase and its receptor and recent insights into the modulation of its structure for therapeutic purpose.

Soluble urokinase receptor

The Cell surface uPAR can be shed by several proteases,leaving it devoid of the GPI anchor, to generate a soluble form of uPAR (suPAR) (Jo *et al*., 2003; Blasi *et al*., 2012). suPAR is a stable threedomain (D1, D2, and D3) protein that retains most of uPAR activities; both uPAR and suPAR are involved in the cell attachment, motility, and migration through their interaction with the integrins

(Ranadheer, 2017). Further cleavage through the linker connecting D1 and D2 domains generates a soluble D1 fragment and the residual D2-D3 fragment, which may remain membrane-bound or detach from the membrane (Eugen-Olsen, 2010). suPAR circulates in blood and other body fluids and has been identified in various pathological conditions: elevated plasma suPAR levels are predictive of cancer (Donadello *et al*., 2014), cardiovascular disease (CVD) (Persson *et al*., 2012).

Crystal structure of the murine urokinase enzyme

X-ray crystollaography is an established technique to study the chemical configurations in a crystallised structure. Many X-ray crystal structures of trypsin-like serine proteases are available and reveal surface-exposed loopstrapped in different conformations (Jacobsen *et al*., 2008). On the other hand, techniques such as NMR and hydrogen-deuterium exchange mass spectrometry (HDXMS) suggest the existence of substantial internal dynamics in serine proteases that are not observed by X-ray crystallography (Llinas *et al*., 2005; Kjaergaard *et al*., 2008; Montuori *et al*., 2002). In general Urokinase is a 411-residue protein, consisting of three domains: the serine protease domain, the kringle domain, and the growth factor domain. Urokinase is synthesized as a zymogen form (prourokinase or single-chain urokinase), and is activated by proteolytic cleavage between Lys158 and Ile159. The two resulting chains are kept together by a disulfide bond.It has previously been shown that the N-terminal Ile16 in the murine version of uPA (muPA) is susceptible to carbamylation indicating that Ile16 in solution is not stably incorporated into the activation pocket as it would be if the protease adopts an active conformation (Huai *et al*., 2001; Thuno *et al*., 2009). Upon binding a stimulatory monoclonal antibody to an allosteric site in the 37s and 70s loops in the N-terminal β-barrel, N-terminal Ile16 carbamylation was reduced. In contrast, the binding of an inhibitory monoclonal antibody to the same region resulted in even higher levels of carbamylation (Rand *et al*., 2008; Kromann-Hansen *et al*., 2013). These studies suggested the existence of a conformational equilibrium in muPA and that allosteric molecules and/or substrate binding modulates the conformational equilibrium of muPA to affect distal functional regions. However, the structural determinants for the different muPA conformations have remained elusive till far.

Urokinase and lung function

It is now evident from several research reports that Urokinase and erythropoietin work in tandem for the 'Pulmonary renal cascade' and regulate multiple patho-physiological functions in the body. It does appear that these two and the heparin binding growth factors also released from the kidney play an important role in maintaining physiological homeostasis. The G protein-coupled receptor, PAR-1, is activated by proteases and implicated in many lung diseases. PAR-1 expression is increased in macrophages and fibroblasts in fibrotic lesions in IPF. Inhibition of PAR-1 activation reduces lung inflammation and fibrosis in bleomycin-induced lung injury. Furthermore, PAR-1 activation in lung fibroblasts elicits increased cytokine production, collagen expression and proliferation (Liu *et al*., 2012; Sidenius *et al*., 2000; Kotani *et al*., 1995). In a recent study, plasmin stimulated IL-6 production by lung fibroblasts was attenuated by directly inhibiting PAR-1 using genetic (RNA*i),* immunological (neutralizing IgG) or pharmacological (SCH79797) inhibition of PAR-1, or indirectly by selective inhibition of MAPK kinases known to signal downstream of PAR-1. Interestingly reducing PAR-1 expression also attenuated plasminogen activation (Refer Fig.1). Thus, PAR-1 is likely to have a role in the increased uPA production that occurs with plasminogen and/or plasmin exposure. A decrease in uPA levels in IPF is possibly explained by a decrease in uPA and increase in PAI-1 expression in the alveolar epithelium in the disease state (Hattori *et al*., 2004; Stewart *et al*., 2009). Meanwhile, basic research indicated that PAI-1 regulated lipopolysaccharide-induced inflammation via targeting on the TLR4/NF-κB signaling pathway in alveolar macrophages, and elevated PAI-1 expression promoted alveolar epithelial cell apoptosis and exacerbated lung inflammation induced by influenza A virus (de Torres *et al*., 2006; Ren *et al*., 2015).On the other hand, Chen *et al* found that C reactive protein (CRP) could increase the expression of PAI-1 by mediating oxidative stress and MAPK signal pathway in the human coronary endothelial cells (Chen *et al*., 2008).

Urokinase and asthma and targeting the uPAR:

It has been shown that the same single nucleotide polymorphisms (SNPs) were associated with soluble PLAUR levels in blood, airway hyper-responsiveness (AHR) and accelerated lung function decline in asthma; a clinical feature linked to airway wall remodeling (Ierodiakonou *et al*., 2016). Therefore, it was hypothesised that PLAUR may contribute to structural changes in asthma via increased levels of the membrane bound or soluble receptor. Studies subsequently showed that PLAUR levels were elevated in the airway epithelium of asthma patients and that PLAUR has a role in epithelial repair responses (Barton *et al*., 2009). Through the world the mortality from asthma have reached more than 180,000 annually. Asthma is considered a complex genetic disorder. It has been previously shown that genetic polymorphisms spanning the urokinase plasminogen receptor (uPAR) gene are associated with the risk of developing asthma. uPAR and is a serine protease receptor that interacts with several ligands and has proteolytic via plasmin activation functions including ECM degradation as well as non-proteolytic functions, e.g. cell migration and proliferation. In particular, a polymorphism that results in a Lys220Arg substitution in Domain 3 of uPAR was associated with asthma risk, lung function decline and airway structural changes in multiple asthma/respiratory cohorts (Stewart, 2012; Hogg *et al*., 2004; McEwanm, 2011).

Similarly, through sequencing it was that identified novel coding region variants only found in the asthma population. The molecular mechanisms underlying these observations remains to be resolved and may be important when targeting this receptor for therapeutic benefit. PLAUR is a serine protease receptor with many downstream effects including ECM degradation as well as non-plasmin functions, e.g. cell migration and proliferation (Woessner, 1991). These recent studies provide further indications that PLAUR may be involved in airway remodelling in asthma through alterations in epithelial function, which is, at least in part, driven by genetic polymorphisms. Further investigation into the role of PLAUR in asthma may provide therapeutic opportunities to target airway remodelling in asthma.

Urokinase plasminogen activator system and Serpin 2

Synthetic formulations of the urokinase are used in cardiovascular and other manifestations from treating thromboembolic disorders to its role in myocardial infarction and empyema. Several studies have implicated the serineprotease urokinase-type plasminogen activator(uPA) and its receptor (uPAR) to be of special importance in cancer proliferation and metastasis (Dass *et al*., 2008). uPAR is a receptor consisting of 3 domains (DI, DII, DIII) boundtothe cell surface via a glycolipid-anchor. uPA binds with high affinity to uPAR and consequently converts plasminogen to active plasmin, which activates several proteases related to the proteolytic degradation of extracellular matrix, thereby facilitating cancer cell invasion. In addition uPA/uPAR directly influencesmultiple other aspects of tumor progression and development by eliciting tumor-associated processes, such as cell proliferation, cell adhesion and migration, chemotaxis, and cell survival through interactions with co-receptors to affect intracellular downstream signaling (Ulisse *et al*., 2009; Wahlberg, 1998). In clinical studies, uPAR has significant prognostic information in cancers, such as breast, lung, colorectal, and prostate, in which patients with high levels of cleaved uPARthat form in the blood experience a shorter overall survival (Langkilde *et al*., 2011; Kwaaan *et al*., 2013) (Refer Fig.2). On the other hand,The SERPIN protease nexin-1 has important role in the metabolism of urokinase. Firstly, as a serine protease inhibitor, serpinE2/protease nexin-1 is found in many organs (Candia *et al*., 2006), and it can be secreted into the extracellular space, and next expresses in cytosol and plasma membrane, according to the subcellular localization database (compartments). SerpinE2 can bind to the extracellular matrix on the surface of fibroblasts and several other cultured cells6. SerpinE2 forms complexes with certain serine proteases, like urokinase-type plasminogen activator (uPA), tissue-type plasminogen activator (t-PA), plasmin and trypsin14 in the extracellular environment. Since uPA-PN-1 forms a complex with uPAR (uPA-uPAR-PN-1), which then binds to the cells and are rapidly internalized and degraded by the low density lipoprotein-related receptor protein (LRP) (Francois *et al*.,). uPA play an important role in promoting extracellular matrix (ECM) deposition. Intriguingly, serpinE2 requires to internalizeuPAR-bound uPA to form the complex, then further inhibits the uPA that plays a pivotal role by mediating the degradation of extracellular matrix proteins (Conese *et al*., 1994). Actually, serpinE2 is the phylogenetically closest relative of Plasminogen activator inhibitor type 1 (PAI-1) that is implicated in the pathology of fibrosis in multiple organs including the heart, lung, kidney, liver and skin.

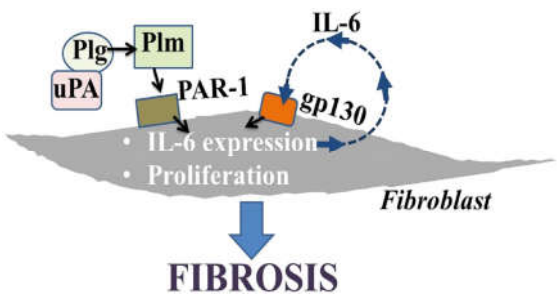

Fig.1. uPA signaling in pulmonary fibroblast cells (Couresy; Michael Schuliga*et al*, Scientific Reports, 2017)

High altitude sickness and urokinase

High altitude related sickness is a problem for both the mountaineers and military personnel and workforce not acclimatized to the harsh atmosperhic conditions for e.g Himalayan borders of South Asia. High altitude related diseases such as high altitude pulmonary edema (HAPE) and acute mountain sickness (AMS) play an important role for an increasing number of mountaineers and workforce operating at high altitude. Preventive efforts include limiting the rate of ascent to 300–600 m/day (Matthias *et al*., 2016) and administering medication before ascent such as nifedipine or tadalafil to prevent HAPE oracetazolamide to prevent AMS (Basnyat *et al*.; Luks *et al*., 2014; Bärtsch *et al*., 1991) in persons at risk. However, identification of risk factors is not easy. Measurements taken during hypoxic exposure such as low hypoxic ventilatory response and accentuated hypoxic pulmonary vasoconstriction (Basnyat *et al*., 2006) are only loosely associated with HAPE risk.

The difference in suPAR plasma concentration at sea level found in this study supports that HAPE susceptibles are a group of persons with a low grade inflammation that may encourage the development of HAPE once exposed to the stimulus of hypoxia. It is thus implied that HAPE susceptibility may be predicted without previous high altitude exposure by determining suPAR plasma concentration as a measure of such low grade inflammation, though with a limited sensitivity and specificity. This finding further suggests that low grade leukocyte activation enhances lung capillary vulnerability to hydrostatically induced leakage (Schirlo *et al*., 2012). Such a low grade inflammatory condition may be a component of HAPE susceptibility, which is in agreement with observations of higher HAPE incidence related to pre-existing inflammatory conditions (Eldridge *et al*., 1996; -54). Thus, the mechanisms underlying HAPE seem to be modulated, rather than caused, by inflammatory processes. At high altitude such a difference is not discernible anymore, possibly being inundated by the acute hypoxic stimulus. Despite an increase during hypoxic exposure suPAR plasma concentrations before ascent were not different between the groups later developing AMS or not. This suggests that cellular based inflammation does not play a role on the central form of high altitude disease, comprising AMS and high altitude cerebral edema.

Technique for uPAR Western blotting (WB)

Canine lung lysates are prepared in an extraction buffer (150 mMNaCl, 20 mMTris/HCl [pH 7.3], 0.1% sodium-dodecyl sulfate [SDS], 1% sodium deoxycholate, 1% Triton X-114 with proteinase inhibitory mixture [5 µg/mL aprotinin, 1 µg/mL leupeptin, 1 µg/mL trypsin inhibitor, and 1 mMphenylmethylsulfonyl fluoride] and incubated for 5 minutes at 37°C. Extracts are then centrifuged at 2500 × *g* for 5 minutes to separate a detergent phase that contains mainly hydrophobic membrane proteins including GPI-anchored uPAR. Human HL-60 leukemia cell extracts prepared in SDS–polyacrylamide gel electrophoresis (PAGE) buffer (Enzo, Farmingdale, NY, USA) are used as a WB-positive control. The total protein concentration is obtained using a bicinchoninic assay kit (BCA-1; Sigma–Aldrich, St Louis, MO, USA), and 20 µg of protein from these samples was separated under nonreducing conditions by 15% SDS–PAGE and transferred to nitrocellulose membranes (Bio-Rad,

Hercules, CA, USA). After blocking with phosphate-buffered saline–Tween and 5% nonmilk fat at room temperature for 2 hours, the membrane was blotted with a monoclonal murine anti-uPAR antibody (1:500 dilution; Sigma, St Louis, MO, USA) for 1 hour at room temperature. According to the manufacturer, this antibody is cross-reactive with human and canine uPAR. The membrane was rinsed and incubated with goat anti-mouse horseradish peroxidase secondary antibody (1:1,000 dilution; BD Bioscience, San Jose, CA, USA) for 1 hour at room temperature and developed using an enhanced chemiluminescence detection kit (GE Healthcare Life Sciences, Piscataway, NJ, USA) to allow for relative quantification of uPAR. A rabbit anti-β-actin polyclonal antibody (Abcam, Cambridge, MA, USA) is used as for protein loading. Quantitative analyses are performed using a high-resolution digital imaging (Amersham 600; GE Healthcare) and smart chemiluminescent software system (Melanie, 2D Gel Package; GE Healthcare) (ValentinaPirazzoli, 2013).

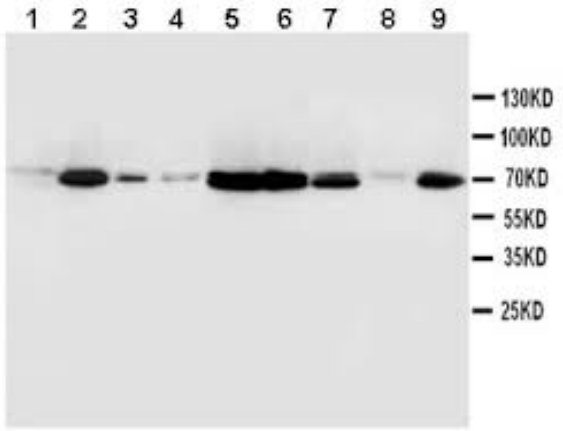

Fig. 2 .Western blotting of uPA receptor using an antibody (2 µg/ml), Image courtesy; Abcam (Cambridge, MA, USA)

Conclusion

The urokinase like serine proteases play pivotal roles in blood coagulation, the innate immune system and tissue remodeling, the recent research reports may be important for the development of new drugs for the treatment of various diseases. The crystal structures of serine protease like the uPAgave the researchers five different snapshots of urokinase and revealed that the urokinase peptide chain undergoes surprisingly large movements. Eminent structural biologist Tobias Kromann-Hansen explains: "With these results, we have found that urokinase can exist in equilibrium between an active and an inactive state (56). We now know the form of the inactive state. By developing molecules that specifically recognize and stabilize the inactive state, we may shift the equilibrium towards the inactive state, thereby unmasking disease-promoting activity of urokinase. Urokinase in tandem with erythropoietin seems to contribute to the homeostatic regulation of multiple physiological activities. Thus urokinase is a unique enzyme which plays an important role in physiological homeostasis and is an critical component of the 'pulmonary renal cascade'.

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