Effects of a modulation of the urokinase-type plasminogen activator (u-PA) system in chronic hypoxia-induced pulmonary vascular remodeling and right ventricular hypertrophy (RVH) in mice

Inauguraldissertation
zur Erlangung des Grades eines Doktors der Humanbiologie
des Fachbereichs Medizin
der Justus-Liebig-Universität Giessen

vorgelegt von

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Giessen 2007

Aus dem Medizinischen Zentrum für Innere Medizin
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Tag der Disputation: 11. März 2008

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1 INTRODUCTION

1.1 Physiology of Pulmonary circulation

1.1.1 Functional anatomy of pulmonary circulation

During the passage of blood through the pulmonary circulation, gas exchange takes place and allows distribution of oxygen throughout the body. The right ventricle pumps desaturated blood from the body tissues and organs into the lungs via the pulmonary artery, which later branches into smaller vessels following the bronchi and bronchioles and finally, forms a huge network of capillaries. At the level of the bronchioles, the pulmonary arterioles have very thin walls. The alveoli, the terminal part of the respiratory tract, are surrounded by a diffuse network of capillaries, which provides a large surface area of approximately 30 m^2. The capillary network is the prerequisite for an efficient gas exchange. Oxygenated blood from the capillary network passes into pulmonary venules which converge into pulmonary veins. The pulmonary veins empty the oxygenated blood into the left ventricle, which pumps the blood into the systemic circulation.

The pulmonary circulation, as compared to the circulation of other organs, possesses a number of special features in order to adequately perform its job. The branching of large muscular arteries in the pulmonary circulation gives rise to small, partially muscularized vessels possessing a low perfusion resistance. Whereas the systemic arterioles have a thick layer of smooth muscle cells, the pulmonary vessels of the corresponding size lack these cells. Even in the vessels of a greater caliber, the medial smooth muscle cell layer is much thinner in the lung as compared to the extra-pulmonary vasculature. This helps to maintain low vascular resistance within the pulmonary circulation. The prevalence of low pressure and high flow in the pulmonary circulation also facilitates gas exchange by preventing fluid flux from the vessels into the interstitial space. In addition, it allows the right ventricle to operate at a low energy cost. A second anatomical feature is that arterioles and venules similar to those in the systemic circulation are not present in the pulmonary circulation. These arterioles are the site of most peripheral resistance to flow in the systemic circulation, and since they are absent in the pulmonary circulation much of the pulmonary arterial pressure is transmitted to the alveolar capillaries and probably, in a highly pulsatile flow.

Structurally, the transitional equivalents between arteries and capillaries exist in the lungs and they are often called ‘precapillary arteries’ and ‘postcapillary veins’. In contrast to the systemic arterial system, larger vessels contribute very little to the pulmonary arterial resistance. The longitudinal resistance distribution is spread relatively evenly over the precapillary, capillary, and postcapillary areas in the pulmonary circulation, where vessels are smaller than 100 μm. Besides, the lungs uniquely have two new elements: extra-alveolar vessels and corner cells. The extra-alveolar vessels are arteries and veins surrounded by connective tissue cuffs into which surrounding alveolar walls radially insert. They increase in volume and length during inflation of lungs and decrease during deflation. The corner vessels, which are single alveolar wall capillaries, are located in corners and they cannot be closed by high air pressures.

Systemic circulation also differs from pulmonary circulation regarding their regulation. The most significant difference exists in their response to hypoxia. Hypoxia causes hypoxic pulmonary vasoconstriction (HPV), whereas hypoxic vasodilation occurs in autoregulated organs of the systemic circulation. The arterial pressure is the most important controlled variable in the systemic circulation. The feedback control system, the so-called arterial baroreflex located in the brain stem, and the various associated changes in the heart, vessels and endocrine functions are well known. On the other hand, the central nervous system does not regulate the pulmonary vasotone and pulmonary pressure remains remarkably constant even during increased flow induced by physical efforts or exercise. The maintenance of relatively constant pressure in the lungs is achieved by ‘active vasodilation’. The active vasodilation is attributable either to an autonomic regulatory mechanism acting on the pulmonary vessels or to the changes caused by the arterial baroreflex. This pressure homeostasis even in the event of increased flow, however, is also maintained by the high distensibility of the pulmonary vessels and additional recruitments of perfused vessels (Passive dilatation). Vascular tone in the pulmonary circulation is very important feature as it confers lung vasculature the ability to attain maximum vasoconstriction to maximum vasodilatation. There is rich supply of the autonomic nervous system as adrenergic, cholinergic and non-adrenergic non-cholinergic (NANC) pathways in the lungs.

However, the contribution of the nervous system to pulmonary vascular tone is minor. Vessel tone rather depends on the cytoplasmic calcium concentration. The cytoplasmic calcium concentration, in turn, depends on the concentration of cyclic nucleotides as cAMP or cGMP. Besides, other factors such as catecholamine, nitric oxide, prostacycline and endothelin are also involved in modulating vascular tone. The normal vascular tone is of utmost importance for healthy vascular function as any impairment leads to pathological conditions.

1.1.2 Hypoxic pulmonary vasoconstriction (HPV)

Literature on HPV goes back to 18th century. Bradford and Dean described HPV in 1894[1] followed by J. Beyne in 1942[2]. The investigation into this phenomenon was furthered with Euler and Liljestrand's description in 1946[3]. Teleologically, HPV is an adaptive mechanism that matches local ventilation with local perfusion in the pulmonary circulation by redistributing venous blood away from poorly oxygenated alveoli to regions of the lungs that are properly ventilated. Hence, HPV may be beneficial in patients with inhomogeneous ventilation distribution such as chronic obstructive pulmonary disease. Despite the beneficial effect, HPV can become life-threatening when it is generalized, e.g. in high altitude. In such condition, it may lead to acute onset of pulmonary hypertension. The phenomenon of HPV is universal in mammals. However, considerable interspecies and inter-individual variability exist[4].

Lungs elicit vasoconstriction within a few seconds of hypoxic exposure[5] and the HPV reaction reaches its maximum after a few minutes. Interestingly, HPV is completely reversible upon returning to normal oxygen concentration. The phenomenon of HPV is present in an isolated pulmonary arterial smooth muscle cells and is elicited even in the absence of central nervous supply. This clearly implies that HPV is a local response to hypoxic environment and the mechanism is intrinsic to pulmonary artery wall. Despite a host of investigations carried out over the past years, the biochemical mechanism of hypoxic pulmonary vasoconstriction remains incompletely understood. One school of thought suggests that a decrease in partial oxygen pressure (PO_2) inhibits smooth muscle cell (SMC) voltage-dependent potassium channels leading to membrane depolarization, influx of calcium and SMCs shortening, and hence, to vasoconstriction.

Others have proposed that the O_2 sensor is linked to a NADPH oxidase, which is responsible for radical oxygen species (ROS) formation. ROS are responsible for redox modulation of transcription factors including Hypoxia-Inducible-Factor-1 (HIF-1). HIF-1α expression in lung is induced by hypoxic conditions[6]. However, a consensus on oxygen sensing and subsequent pathophysiological sequelae has yet to be arrived at.

Hypoxic vasoconstriction is inhibited by alkalosis, hypercapnia, prostaglandins, NO, calcium channel blockers, β2 agonists and endothelin antagonists, and it is enhanced by acidosis, endothelial dysfunction, NO inhibition and serotonin[7]. When only a small region of the lung is hypoxic, HPV can occur without significant effect on pulmonary arterial pressure[8]. However, when generalized hypoxia is present, as seen in many lung diseases and in high-altitude exposure, the subsequent pulmonary vasoconstriction contributes to pulmonary hypertension, right heart decompensation and, possibly, death. Persistent vasoconstriction induced by chronic hypoxia leads to structural remodeling of pulmonary vasculature and pulmonary hypertension, as will be discussed in the following section on hypoxia-induced pulmonary vascular remodeling.

1.2 Pathophysiology of pulmonary vasculature

1.2.1 Pulmonary vascular remodeling

Pulmonary vascular remodeling, characterized by structural and functional changes of the architecture of pulmonary arterial walls, can occur as a primary response to injury or to other stimuli such as hypoxia. An increased muscularization and deposition of extracellular matrix are the salient features of structural remodeling. As a result, the lumen diameter and capacity for vasodilation are decreased. The structural alteration is followed by functional consequences such as an increased pulmonary vascular resistance and sustained pulmonary hypertension. Thus, the pulmonary arterial pressure may be elevated at rest and increased further on exercise.

Understanding the morphological features of normal pulmonary arteries is important to understand the mechanism of remodeling. Proximal arteries are usually thin walled with respect to their luminal diameter.

The muscular media is composed of many elastic laminas separated by layers of smooth muscle cells. As the diameter of the arterial lumen decreases, the elastic laminas become less prominent and are replaced by smooth muscle cells. Beyond the terminal bronchioles and within the respiratory acinus, the arteries become only partially muscularized as the smooth muscle cell layer tails off in a spiral, with no smooth muscle cells found in the smaller intra-acinar arteries[9, 10]. The precapillary vessels contribute to the majority of the pulmonary vascular resistance and there is the greatest pressure drop at these precapillary segments. Hence, a small change in tone or wall structure in the precapillary vessels can lead to large elevations of pulmonary artery pressure, showing its crucial role in determining the pulmonary pressure. This area contains two smooth muscle–like cells, namely pericytes and intermediate cells. The so-called intermediate cells share the phenotypes of pericytes and smooth muscle cell. The most distal part of the precapillary arterioles consists of an endothelial layer, which is underlined by a single elastic lamina. The proximal pulmonary arteries usually differ from the distal ones in their susceptibility towards the remodeling process. The most severe alterations occur in the distal pulmonary arteries.

Previous investigations have uncovered many events of the pulmonary vascular remodeling. A common feature is the appearance of a layer of smooth muscles in small peripheral, normally non-muscular, pulmonary arteries within the respiratory acinus. However, the cellular mechanism underlying muscularization is incompletely understood. Proliferation and differentiation of intermediate cells situated inside the internal elastic lamina of precapillary vessels could be attributable to the increase in smooth muscle cells[11]. Moreover, differentiation of pericytes and recruitment of interstitial fibroblast from the surrounding lung parenchyma may contribute to the process of muscularization in the most distal vessels devoid of an elastic lamina[12]. Such vessels devoid of elastic lamina range in size of 20-30 μm diameters. The consequence of vasoconstriction and remodeling of the small distal arteries will be a higher intraluminal pressure in the more proximal muscular arteries. Subsequently, proliferation and hypertrophy of smooth muscle cells and collagen deposition occurs in the media[13, 14]. In addition, proliferation of fibroblast, deposition of collagen in adventitia and the medial thickening lead to reduced lumen size[14].

The cellular changes in the vascular layers during the process of remodeling have been summarized in table 1.

Table 1. Cellular changes in vascular layers during remodeling[15]

Endothelium	• Adhesiveness of platelets and granulocytes ↑ • Anticoagulant activity ↓ and procoagulant activity ↑ • Contribution to plexiform lesion
Intima	• Fragmentation of internal elastic layer • Myofibroblasts: -Proliferation of intermediate cells and increase in contractile filaments with transition to myofibroblast -Collagen deposition ↑ -Migration and proliferation of smooth muscle cells (?)
Media	• Smooth muscle cells - Proliferation ↑ - Elastin deposition ↑ - Distal migration (de novo muscularization of arterioles) - Migration into intima (?)
Adventitia	• Fibroblast -Proliferation ↑ -Collagen deposition ↑

Neointima is a form of vascular remodeling occurring in small and large arteries. It consists of a layer of cells and extracellular matrix between the endothelium and the internal elastic lamina[16]. Neointimal cells comprise myofibroblasts and do not express endothelial markers such as CD31, CD34 or factor VIII[16, 17]. It contributes significantly to the increased vascular resistance. Neointima formation is the hallmark of severe pulmonary hypertension. Studies with animal models have suggested that increased blood flow is important stimulus for neointima formation. The increased blood flow (induced by pneumonectomy) together with vascular injury (induced by monocrotaline) could induce the neointima formation in rat[18, 19]. It is likely that the neointimal cells arise by transdifferentiation of endothelial cells, by migration of smooth muscle-like cells from media, or by migration of adventitial fibroblast. However, the origin of neointimal cells in severe pulmonary hypertension is yet unknown.

Severe pulmonary hypertension is also characterized by the formation of plexiform lesion, another important form of vascular remodeling. The disorganized proliferation of endothelial cells gives rise to plexiform lesion.

Within the lesion the endothelial cells are supported by a stroma containing matrix proteins and α-smooth muscle actin expressing myofibroblasts[16]. The investigations of the cell types in the plexiform lesions have shown that they differ between primary and secondary pulmonary hypertension. In primary pulmonary hypertension, the cells are monoclonal in origin, whereas in secondary pulmonary hypertension they are polyclonal in origin[20.] Pulmonary hypertension patients as well as animal models were investigated in the past to elucidate the mechanisms of pulmonary vascular remodeling. In these studies many factors have been identified such as potassium channels, Transforming Growth Factor-β (TGF-β) and Bone Morphogenetic Protein (BMP), serotonin (5-HT), Platelet Derived Growth Factor (PDGF), Epidermal Growth Factor (EGF) and Fibroblast Growth Factor (FGF). However, the process of remodeling is incompletely understood.

1.2.2 Right ventricular (RV) adaptation

In the normal heart, the right atrium contracts and empties its content into right ventricle (RV). The closure of the pulmonary valve during right atrial contraction prevents blood from flowing into the pulmonary artery and hence, allows the ventricle to fill in with blood. During RV contraction the tricuspid valve closes and the pulmonary valves open. The closure of the tricuspid valve prevents blood from flowing back into the right atrium and the opening of the pulmonary valve allows the blood to flow into the pulmonary artery toward the lungs. The right ventricle is a thin walled chamber having a mass about $1/6^{th}$ of the left ventricle. It performs $1/4^{th}$ of the stroke work because the pulmonary vascular resistance is $1/10^{th}$ of the systemic vascular resistance. The RV is a compliant chamber capable of withstanding volume overload compared to pressure overload. A pressure overload, if sustained, induces RV hypertrophy as a compensatory response mechanism. The RV hypertrophy, characterized by the thicker ventricular wall and flattened inter-ventricular septum, allows RV to adapt to an increased work. However, the structural alteration during hypertrophy, accompanied by reduced contractility, is followed by a relative coronary insufficiency contributing to RV failure.

At a cellular level, cardiomyocytes undergo hypertrophy along with an enhanced protein synthesis and higher sarcomere organization.

The factors that influence cardiomyocyte hypertrophy are largely unknown.However, multiple molecular pathways involving Ca^{2+}/Calcineurin/NFAT, G-Protein-coupled receptors (adrenergic, angiotensin and endothelin receptors), Phosphoinositide 3-Kinase/Akt/Glycogen Synthase Kinase-3, Myocyte enhancer factor-2/Histone deacetylases, Na/H exchanger, Ca^{2+} cycling factors and others[21] have been proposed. A reduction in the pressure by mechanical means have been shown to result in reversal of right heart dilation and dysfunction, and in improvement of myocardial performance[22]. For example thromboendarterectomy for chronic large-vessel pulmonary embolism, correction of atrial septal defect, mitral valve replacement and, interestingly single-lung transplantation in patients with primary and secondary pulmonary hypertension. Thus, persistence of high pressure is regarded as the important factor. Overall, it seems that most of the molecular pathways leading to RV hypertrophy are provoked by the shear stress or pressure load of the right ventricle.

Although the severity of pulmonary arterial hypertension depends on the degree and distribution of the pulmonary arteriopathy, the level of pulmonary artery pressure has only modest prognostic significance. It is rather the ability of the RV to compensate for the increased afterload that determines the severity and survival[23]. Hence, RV adaptation is important in determining the fate of pulmonary hypertension and the RV function is crucial especially in patients with left heart failure and severe lung disease[23].

1.3 Pulmonary hypertension

1.3.1 Definition and classification

In simple terms, pulmonary hypertension can be referred to any increment in the pulmonary arterial pressure (PAP) above normal values (Table 2). It is a disease of the lung vasculature, where the pulmonary arteries undergo vasoconstriction and remodeling leading to an increase in right ventricular afterload and development of *cor pulmonale.* It is the third most common cardiovascular condition, after coronary heart disease and systemic arterial hypertension. The elevation in PAP may occur either as a consequence of underlying pulmonary vascular disease, which can be progressive and fatal. Moreover, the increased PAP may be simply a passive elevation in response to an elevated pressure in the left heart. An accurate diagnosis, therefore, needs a thorough and careful consideration.

Table 2. Normal values and range of pulmonary blood flow and vascular pressures

Variable	Mean	Range of normal
Q (l/min)	6.4	4.4-8.4
Heart rate (bpm)	67	41-93
PAP systolic (mmHg)	19	13-26
PAP diastolic (mmHg)	10	6-16
PAP mean (mmHg)	13	7-19
PVR (dyn s/cm^5)	55	11-99
SAP mean (mmHg)	91	71-110

Q, cardiac output; PAP, pulmonary artery pressure; PVR, pulmonary vascular resistance; SAP, systemic arterial pressure

As a disease associated with a diverse etiology, classification of pulmonary hypertension is essential in order to facilitate the diagnosis. The World Health Organization Symposium in 1973[24] coined an original classification, which classified pulmonary hypertension into groups based on the known causes. Primary Pulmonary Hypertension (PPH) was classified as a separate entity of unknown cause. Others related to diseases with identifiable causes were termed as Secondary Pulmonary Hypertension (SPH)[25]. The Second World Symposium of pulmonary hypertension, held in 1998 in Evian, France, proposed a new classification for pulmonary hypertension[26]. The second symposium focused on the pathophysiological mechanisms, clinical presentation and therapeutic options. This was a simplified classification aiming to provide a useful guide for the clinician in evaluating pulmonary hypertension patients and developing treatment plan. In addition, the New York Heart Association (NYHA) functional classification for heart diseases established a new functional classification (Table 3). The NYHA classification was useful for comparison of patients with respect to the clinical severity of the disease process. In 2003, the 3rd World Symposium on pulmonary arterial hypertension proposed some modifications to the Evian classification[27]. In the modification, the term "primary pulmonary hypertension" was replaced with "idiopathic pulmonary hypertension". In addition, the pulmonary capillary hemangiomatosis and pulmonary veno-occlusive disease were reclassified and risk factors were updated.

Moreover, the guidelines for the classification of congenital systemic-to-pulmonary shunts were also included (Table 4). An overview of the most frequent causes and triggers and different pathomechanisms underlying development of pulmonary hypertension is provided in figure 1.

Table 3. WHO functional classification of pulmonary hypertension

Class I	Patients with pulmonary hypertension but without resulting limitation of physical activity. Ordinary physical activity does not cause undue dyspnea or fatigue, chest pain or near syncope.
Class II	Patients with pulmonary hypertension resulting in sight limitation of physical activity. They are comfortable at rest. Ordinary physical activity causes undue dyspnea or fatigue, chest pain or near syncope.
Class III	Patients with pulmonary hypertension resulting in marked limitation of physical activity. They are comfortable at rest. Less than ordinary activity causes undue dyspnea or fatigue, chest pain or near syncope.
Class IV	Patients with pulmonary hypertension. They are unable to carry out any physical activity without symptoms. These patients manifest signs of right heart failure. Dyspnea and/or fatigue may even be present at rest. Discomfort is increased by any physical activity.

Table 4. Revised Clinical Classification of Pulmonary Hypertension

1. Pulmonary arterial hypertension (PAH)

- Sporadic or idiopathic (IPAH)
- Familial (FPAH)
- Associated with (APAH)
    - Collagen vascular disease
    - Congenital systemic-to-pulmonary shunts
    - Portal hypertension, HIV infection, Drugs and toxins
    - Others (thyroid disorders, glycogen storage disease, Gaucher disease, HHT, hemoglobinopathies, myeloproliferative disorders, splenectomy)
- Associated with significant venous or capillary involvement
    - Pulmonary veno-occlusive disease (PVOD)
    - Pulmonary capillary hemangiomatosis (PCH)
- Persistent pulmonary hypertension of the newborn

2. Pulmonary hypertension with left heart disease

- Left-sided atrial or ventricular heart disease
- Left-sided valvular heart disease

3. Pulmonary hypertension associated with lung diseases and/or hypoxemia

- Chronic obstructive pulmonary disease
- Interstitial lung disease and developmental abnormalities
- Sleep-disordered breathing and alveolar hypoventilation disorders
- Chronic exposure to high altitude

4. Pulmonary hypertension due to chronic thrombotic and/or embolic disease

- Thromboembolic obstruction of proximal and distal pulmonary arteries
- Non-thrombotic pulmonary embolism (tumor, parasites, foreign material)

5. Miscellaneous

- Sarcoidosis, Histiocytosis X, etc.

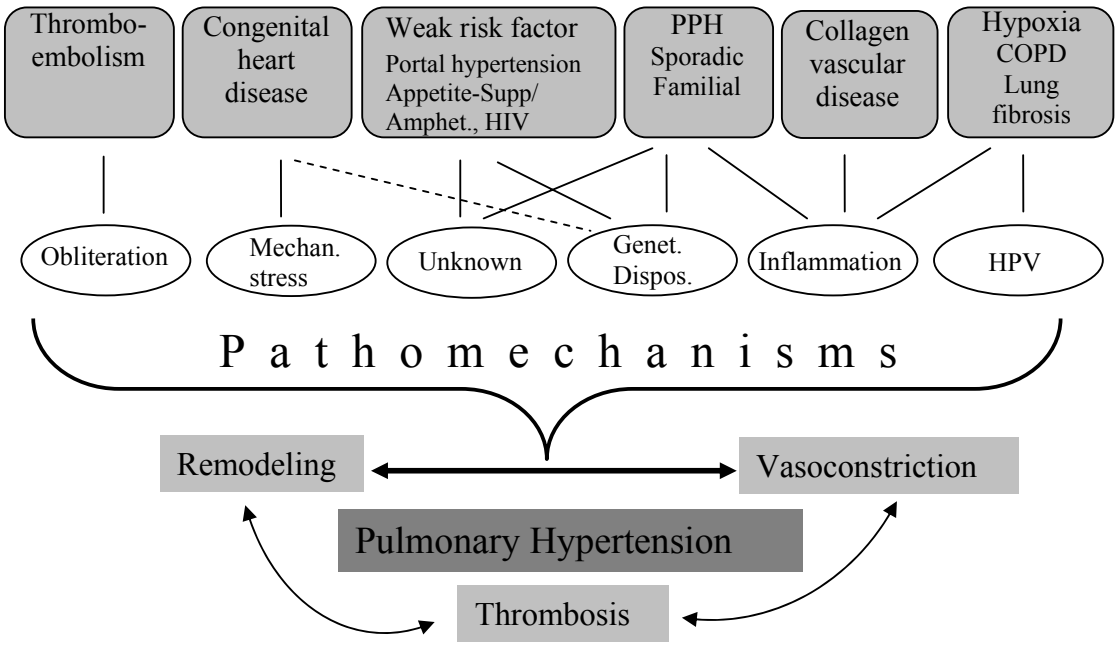


Figure 1. Most frequent causes and triggers which lead to pulmonary hypertension through different pathomechanisms.

Hypoxic pulmonary vasoconstriction (HPV), inflammation, mechanical stress, primary obliteration and idiopathic factors lead to vasoconstriction and pulmonary vascular remodeling. Vasoconstriction and vascular remodeling are hallmarks of pulmonary hypertension. In situ thrombosis of the small pulmonary arteries often aggravates the evolution of the diseases (Adapted from Olschewski H. and Seeger W.[15])

1.3.2 Pulmonary arterial hypertension (PAH)

Pulmonary arterial hypertension is a disease affecting the pulmonary vascular endothelium and is manifested by a progressive elevation in pulmonary vascular resistance followed by right ventricular failure and death. It is a complex disease caused by diverse etiological or associated risk factors. It includes three main classes namely, idiopathic pulmonary hypertension (IPAH), familial pulmonary hypertension (FPAH) and PAH related to risk factors or associated conditions (APAH, see Table 4). IPAH, as the name implies, is a disease of an unknown etiology. It is characterized by a mean pulmonary arterial pressure of >25mmHg at rest or >30mmHg during exercise and by an absence of other causes such as parenchymal lung disease, chronic thromboembolic disease, left-sided valvular or myocardial disease, congenital heart disease, or systemic connective tissue disease[28, 29].

It is a rare and fatal disease with the median survival being approximately 2.8 years in untreated adults. Ernst Romberg was first to describe an IPAH patient in 1891[30], which was later termed as primary pulmonary hypertension (PPH)[31]. PPH was the term used previously to describe what is now known as IPAH. Later, Dresdale and colleagues reported a case terming it PPH in 1951[32]. Afterwards, the case was known to be the first documented case of familial pulmonary arterial hypertension (FPAH).

FPAH has drawn attention and interest over the past years because of the identification of gene responsible for PAH. The gene, formerly known as PPH1, encodes for bone morphogenetic protein type II receptor (BMPR II)[33, 34]. BMPRII has been found to localize at locus 2q33 of chromosome 2[35]. Mutations and deletion in the gene for BMPR II are thought to account for 60% of familial cases of IPAH and possibly 10-26% of sporadic cases[34, 36, 37].Family studies have revealed that the disease is inherited in an autosomal dominant fashion with low penetrance. The relative risk of developing the disease in an affected family is as low as 15-20% in most families but as high as 80% in others[38]. Trembath and colleagues reported families with hereditary hemorrhagic telangiectasia (HHT). They found members of the families with pulmonary hypertension, not always associated with the clinical manifestation of HHT[39, 40]. Genetic investigation revealed mutations in activin-like kinase type-1 (ALK1) receptor gene. Interestingly, both genes encoding BMPRII and ALK1 belong to the members of the TGF-β superfamily. Hence, it seems likely that other members may contribute to the disease.

Clinically, most patients with PAH display exertional dyspnea, indicating their inability to increase cardiac output during exercise. Exertional chest pain, syncope and edema reflect severity of pulmonary hypertension and impaired right heart function. Pathologically, IPAH is defined by the obstruction of small pulmonary arteries associated with plexiform lesions, medial hypertrophy, concentric laminar intimal fibrosis, fibrinoid degeneration and thrombotic lesions. The disease progression is accompanied by increase in pulmonary vascular resistance followed by increase in load on right heart. Subsequently, cardiac output falls and RV failure ensues. The mean age at diagnosis of IPAH is 36, although it can occur at any age.

1.3.3 Chronic thromboembolic pulmonary hypertension (CTEPH)

Thromboembolism is one of the important factors associated with pulmonary hypertension. CTEPH refers to the pulmonary hypertension characterized by intraluminal thrombus organization and fibrous stenosis or complete obliteration of pulmonary arteries resulting into increased vascular resistance and progressive right heart failure. Chronic thromboembolism has emerged as a leading trigger for severe pulmonary hypertension. Originally, it was believed that 0.1% to 0.5% of patients who survived an episode of acute pulmonary embolism developed CTEPH[41]. However, the true incidence and prevalence are not known accurately as it is hampered by the observation that up to two thirds of these patients have no history of clinically overt acute pulmonary embolism[42]. Studies on CTEPH have suggested that the initiating event leading to progressive pulmonary vascular remodeling may be the pulmonary embolism, either as a single or as recurrent episodes. However, the reasons for incomplete resolution of pulmonary emboli permissive to recurrent episode of pulmonary embolism have not been identified. The normal pulmonary vasculature is predominantly fibrinolytic. Tissue type plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1) secretion by pulmonary vascular endothelial cells is not different between lungs from CTEPH patients and donor lungs[43, 44]. This suggests an absence alterations in the fibrinolytic system in patients with CTEPH[45]. However, an elevated level of factor VIII has been found in CTEPH[46] as well as in patients with other forms of pulmonary hypertension[47]. Regarding other hemostatic alterations, prothrombotic activities attributable to abnormal erythrocytes or platelet activation have also been implicated[48, 49].

Based on the experimental findings and associated risk factors of CTEPH, inflammation has been hypothesized to be involved in maintaining prothrombotic state and impairing resolution of thromboemboli in CTEPH[48, 50]. This hypothesis is supported by the elevated plasma levels of the proinflammatory cytokines and its correlation with magnitude of pulmonary hypertension in patients with CTEPH[51]. Experimental data have linked angiopoetin-1, a signaling molecule involved in angiogenesis and smooth muscle cell proliferation, to pulmonary hypertension, and angiopoetin-1 has been found to be upregulated in the lungs from CTEPH patients[52].

In addition, plasma levels of endothelin-1 in patients and upregulation of type B endothelin receptors on pulmonary arterial smooth muscle cells has been demonstrated[53]. Hence, an alternative hypothesis suggests that a primary arteriopathy of pulmonary vessels and secondary in situ thrombosis are attributable to the pulmonary vascular occlusion[54]. Regarding the genetic basis, the genetic link for CTEPH has not yet been determined. However, the expression of BMPR-1A, a transmembrane protein required for BMPR-II signaling, is markedly downregulated in lungs from patients with CTEPH as well as in other forms of pulmonary hypertension[55]. The available data suggest that the molecular mechanisms for pulmonary vascular remodeling in CTEPH appear to be similar to those seen in severe pulmonary hypertension of other etiology. However, the in-depth insight into the detail of the pathogenesis is still poorly understood and requires further studies.

1.4 Animal models of pulmonary hypertension/ vascular remodeling

1.4.1 Monocrotaline-induced pulmonary hypertension

Monocrotaline, a pyrrolizidine alkaloid, can be obtained by extracting the seeds of *Crotalaria Spectabilis*. This phytotoxin is used experimentally to produce pulmonary vascular syndrome in rats characterized by proliferative pulmonary vasculitis, pulmonary hypertension (PH) and cor pulmonale[56, 57]. Following a single subcutaneous or intra-peritoneal injection in rats, it causes vascular injury and inflammation, particularly endothelial injury during the initial sub acute phase (first week). Pulmonary hypertension and vascular remodeling develop at 3-4 weeks post injection. Monocrotaline (MCT) must first be activated by the liver to the putative electrophile monocrotaline pyrrole (MCTP)[58, 59]. Short term stabilization of MCTP by red blood cells facilitates subsequent transport to the lung[60], where MCTP elicits vascular insult. Monocrotaline induces severe pulmonary hypertension, characterized by massive wall thickening of pulmonary arteries accompanying a dramatic increase in media cross-sectional area and a reduction of lumen area[61]. The monocrotaline-induced pulmonary hypertension is by far the strongest model of experimental pulmonary hypertension and shares characteristics with many forms of pulmonary hypertension in human, particularly with PPH. It has been widely used for the preclinical studies to investigate several pharmacological compounds such as prostacyclin analogues and phosphodiesterase-5 inhibitor[62, 63]. Moreover, another monocrotaline-induced rat model of severe PAH has recently been reported[64].

In this model, occlusive neointimal lesions in distal pulmonary ateries have been described to develop in endothelin B receptor deficient rat treated with monocrotaline. Nevertheless, species differ in their susceptibility to develop monocrotaline-induced pulmonary hypertension. Mice, in particular, are resistant to the pulmonary vascular effects of monocrotaline.

1.4.2 Hypoxia-induced pulmonary hypertension/vascular remodeling

Most animals under chronic hypoxic condition reliably develop pulmonary hypertension and structural remodeling of pulmonary vessels[65-67]. It is therefore that chronic hypoxic exposure has been commonly used as a stimulus to induce pulmonary hypertension reproducibly in laboratory animals. Particularly, small animals such as rodents and chickens are employed.

Chronic hypoxic condition can be achieved either by normal air at hypobaric pressure (320 mmHg) or by oxygen-poor air at normal pressure (10% oxygen). Around 50% increase in the mean pulmonary arterial pressure and a doubling in weight of the right ventricle has been observed in rats under hypoxic environment for 2-3 weeks[65]. Pulmonary artery muscularization is another important pathological feature. Both muscular and non-muscular arteries undergo chronic hypoxia-induced muscularization leading to doubling of muscular arterial wall thickness and partial muscularization of normally non-muscular distal pulmonary arteries[13, 65]. These vascular changes are similar to those seen in patients with pulmonary hypertension caused by obstructive and restrictive diseases or living at high altitude. However, the hypoxia-induced pulmonary hypertension is only partially stable. The hemodynamic changes during chronic hypoxic exposure have been shown to be resolved within around 10 days after the animals are brought to normal air[68]. Interestingly, muscularization of small pulmonary arteries reverses more slowly (1 month), whereas large vessels regress only partially[14, 69, 70]. Hypoxia-induced pulmonary vascular remodeling in rats differs from that induced by monocrotaline. In general, the remodeling may not be induced as strong by hypoxia as it is by monocrotaline. Angiotensin II has been shown to play a pivotal role in hypoxia-induced but not in monocrotaline-induced pulmonary artery remodeling[61]. Clearly, hypoxic pulmonary vascular remodeling may not completely mimic the strong vascular remodeling as observed in severe human pulmonary hypertension.

However, it has remained a convenient model to study the key process of distal pulmonary artery muscularization. Recently, a rat model of severe pulmonary hypertension, characterized by occlusion of precapillary pulmonary artery lumen by endothelial cell proliferation, upon inhibition of VEGF receptor 2 and chronic hypoxic exposure has been reported[71].

The hypoxia-induced pulmonary hypertension model has been adapted to mice in which analytical techniques and tools are well established and available. In addition, the possibility to employ genetically engineered mice provides a huge potential to study the mechanisms of pulmonary vascular remodeling. Investigation into mice genetically engineered for a specific metabolic pathway or other factors is an effective approach to determine the function of specific gene products, particularly when pharmacological inhibitors for such gene products or factors are unavailable or lack specificity. Similar to chronic hypoxic rats, chronic hypoxic mice do not exhibit as strong vascular remodeling as is observed in pulmonary hypertension in human patients. Another well-characterized model for hypoxia-induced pulmonary hypertension is the chronically hypoxic newborn calf[66]. This model seems to induce strong alteration in hemodynamics and structure of the pulmonary vasculature. Hypoxic calves develop suprasystemic pulmonary hypertension with exuberant medial and adventitial thickening, and the lesions are close to that seen in patients. It is likely that newborn pulmonary circulation is more susceptible to hypoxia

Interestingly, transgenic mice overexpressing S100A4/Mts1, a calcium binding protein, have recently been reported to develop pulmonary arterial changes resembling human plexogenic arteriopathy with intimal hyperplasia in about 5% of their population[72, 73]. Moreover, a regulation of S100A4/Mts1 by serotonin transporter and receptor has also been reported[74]. S100A4/Mts1 mice revealed a greater RVSP and RVH at baseline, which increased further upon their exposure to chronic hypoxia and was sustained after 3 months “recovery” in room air. Unfortunately, S100A4/Mts1 mice failed to develop more severe pulmonary vascular disease[75], suggesting a need for further investigation to develop a more robust mouse model that resembles human pulmonary hypertension.

1.5 Plasminogen activation system

The plasminogen activator (PA)/plasmin system represents very efficient proteolytic machinary, and its enzymatic cascade participates in the control of fibrin degradation and tissue remodeling. It involves the serine proteases urokinase type plasminogen activator (u-PA), tissue type plasminogen activator (t-PA), plasminogen activator inhibitors (PAIs), plasmin and u-PA receptor (uPAR). Plasmin is generated from plasminogen upon proteolytic cleavage by u-PA and t-PA. Plasmin has broad substrate specificity and acts as an effector protease in the plasminogen activation cascade. The proteolytic activity of plasmin can degrade intravascular fibrin as well as extracellular matrix proteins such as fibrinogen, fibronectin and vitronectin, and activate matrix metalloproteinases[76]. Among the PAs, t-PA is mainly involved in intravascular fibrinolysis. Besides, t-PA has been reported to operate in stress-induced neuronal plasticity and participates in neuronal plasticity such as in memory and learning[77, 78]. On the other hand, u-PA exerts not only proteolytic but also intracellular signaling functions by binding to its high affinity receptor (uPAR) on cell surface. This endows u-PA with the ability to perform functions such as cell migration and tissue remodeling, in addition to fibrinolysis. Interestingly, plasminogen can also become membrane-bound. The occurrence of receptors for plasminogen and u-PA on the same cell results in the formation surface-associated plasmin. Thus, it generates broad-spectrum proteolytic activity, which is restricted to cell surface and protected from circulating inhibitors, such as α2-antiplasmin. Overall, u-PA has been shown to be involved in pericellular proteolytic, cell migratory, adhesive and more recently characterized chemotactic functions by virtue of its proteolytic as well as intracellular signaling function.

1.5.1 Urokinase (u-PA) and its variants

Various cell types such as vascular endothelial and SMCs, epithelial cells, fibroblasts, monocytes/macrophages and cancer cells secrete u-PA[79, 80]. u-PA, secreted as a single chain polypeptide (scu-PA), has molecular weight of ~54 kDa and 411 amino acid[81]. scu-PA can convert plasminogen into plasmin. However, it has no peptidase activity to synthetic substrates. A proteolytic cleavage at K158-I159 peptide bond in scu-PA gives rise to the highly active two chain u-PA (tcu-PA) held together by a single peptide bond.

The high molecular weight tcu-PA (HMW tcu-PA) possesses protease activity against both synthetic substrates and plasminogen[82]. Out of the two chains, the N-terminal A-chain (light chain) includes growth factor domain (GFD) and kringle domain (KD), whereas the C-terminal B-chain (heavy chain) contains the serine protease domain (PD). A further cleavage of urokinase at K135-K136 releases the amino terminal fragment (ATF) and generates a catalytically active low molecular weight urokinase (LMW u-PA)[81]. The ATF (1-135 amino acids) comprises the GFD and the KD, whereas the LMW u-PA (135-411 amino acids) comprises the protease domain (PD). Thus, proteolytic cleavage gives rise to several variants of u-PA (figure 2).

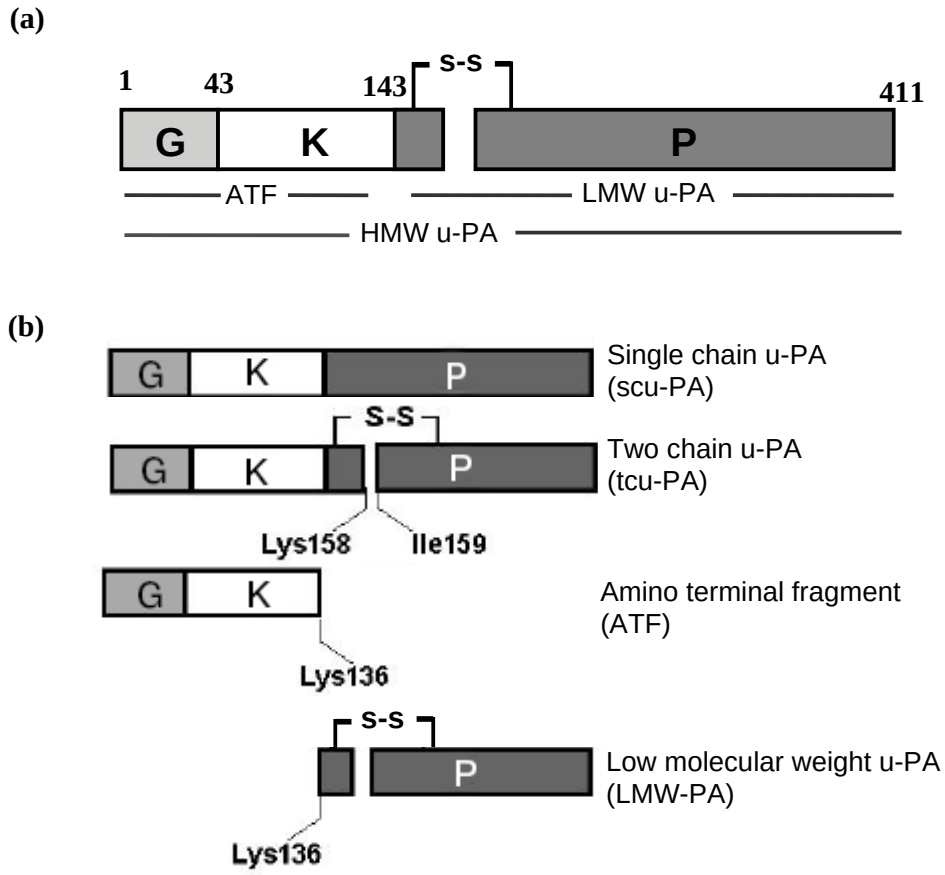


Figure 2. Variants of urokinase upon proteolytic cleavage.

(a) Domain structures of the urokinase (u-PA) and (b) Different variants of u-PA are shown. G, growth factor-like domain; K, kringle domain; P; protease domain; HMW, high molecular weight; LMW, low molecular weight; ATF, Amino-terminal fragment (Adapted and modified from Stepanova V.V. and Tkachuk V.A., 2002[83]).

1.5.2 Urokinase (u-PA) and its receptor (uPAR)

Urokinase binds to its specific cell surface receptor (uPAR) through the N-terminal growth factor-like domain. uPAR, a cysteine-rich glycoprotein with a molecular mass of ~55 kDa, was first identified on human monocytes and on U937 line cells[84]. uPAR is organized into three homologous molecular domains (D1, D2, D3) joined by linker sequences. Full length uPAR binds efficiently to u-PA through its domain D1, which encompasses the u-PA binding site. uPAR is linked covalently to the outer layer of the cell membrane via a glycosyl-phosphatidylinositol (GPI) anchor attached to its carboxyterminal end located in domain 3[85]. Localization of uPAR on the cell surface shows a specific pattern. Similar to other GPI-anchored proteins, uPAR is concentrated in special intrusion of the plasma membrane, the caveolae. The proteolytic cleavage of D1-D2 linker region generates truncated forms of GPI-uPAR (c-uPAR). Receptor shedding has also been observed in case of uPAR. Both full-length and cleaved uPAR can be shed, thus generating soluble uPAR forms. GPI-specific phospholipase C or D can cleave and release the receptor from the plasma membrane[86]. Soluble uPAR forms have been found in biological fluids, both in vitro and in vivo[87]. The full length scu-PA as well as tcu-PA can bind to uPAR on the cell surface. The role of u-PA binding to u-PAR is not just limited to localize u-PA on the cell surface. Activation of receptor-bound scu-PA by plasmin is more efficient than that of free urokinase[88], thus enhancing the pericellular proteolytic activity. However, binding of u-PA to its receptor is species-specific i.e. human u-PA does not bind to murine uPAR and murine u-PA does not bind to human uPAR[89].

uPAR also acts as a receptor for vitronectin (VN)[90, 91]. However, the intact uPAR is required for efficient vitronectin binding as its cleavage prevents interaction with ligand[92]. uPAR binding to vitronectin concentrates proteolytic activity on the cell surface and extracellular matrix by trapping soluble urokinase receptor-urokinase complexes[93]. The uPAR/vitronectin interaction can be modulated not only by u-PA but also PAI-1. The presence of u-PA enhances binding affinity between uPAR and vitronectin [94], whereas uPAR binding to VN is inhibited by PAI-1, because both uPAR and PAI-1 bind to the overlapping regions on VN, close to the integrin binding site[95].

The u-PA\uPAR system has intracellular signaling function as well. Since the GPI-anchored uPAR does not contain a transmembrane domain, it cannot per se transduce signals into the intracellular space. Intracellular signaling through uPAR, hence, depends on its cooperation with other transmembrane adaptor proteins. Integrins represent a set of molecules that can interact with uPAR on the cell surface. Several integrin families such as β1, β2, β3 and β5 have been identified[96, 97]. Integrins are ubiquitous, heterodimeric, membrane-spanning cell surface receptors that are capable of interacting with ECM proteins such as vitronectin, collagen, fibrin, laminin, and fibronectin. The interaction occurs via the integrin recognition motif Arg-Gly-Asp (RGD). The cytoplasmic domains of integrins are linked to components of the cytoskeleton and are implicated in the triggering of discrete intracellular signaling events[98]. The lateral association and functional interactions between the u-PA/uPAR system and integrins crucially affect the adhesive and motile cellular phenotype. Thus, this dual activity of uPAR in a proteolytic and/or non-proteolytic fashion together with the initiation of intracellular signaling is believed to influence cellular behaviour in many physiological and pathophysiological processes such as angiogenesis, inflammation, wound repair and tumor progression/metastasis [99, 100] (Figure 3).

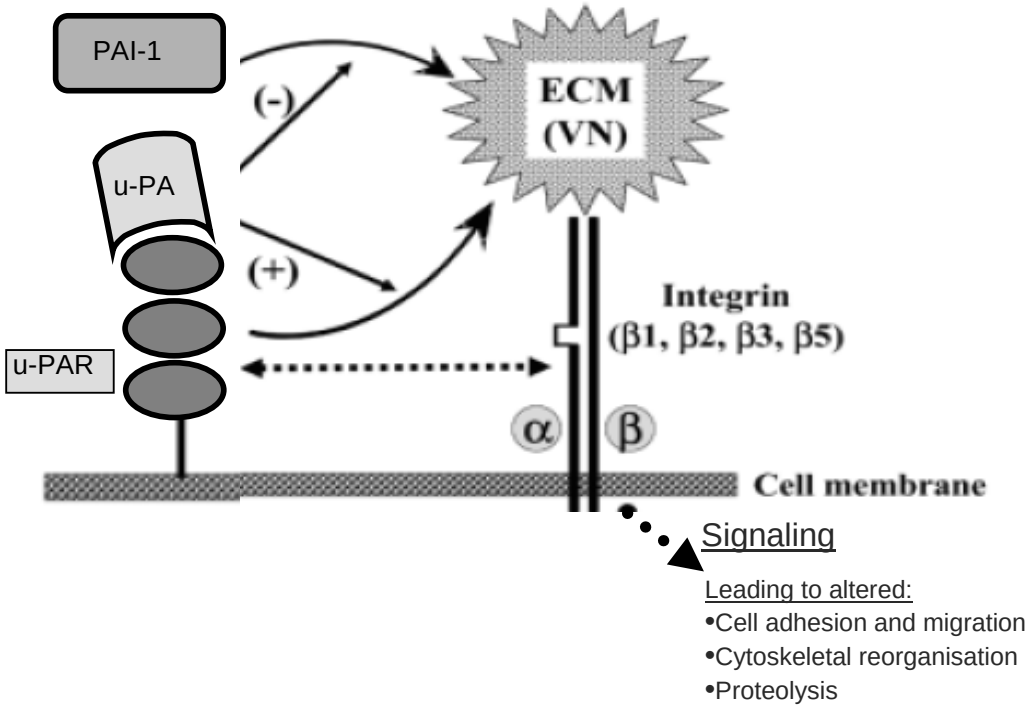


Figure 3. Interaction between u-PA/uPAR system and Integrins

The interaction between u-PA/uPAR system with integrins has been shown to alter not only the integrin adhesive function but also triggers signal transduction pathways, thereby affecting cell adhesion/migration and cytoskeletal reorganization etc. Moreover, functional crosstalk between the u-PA/uPAR system and integrins occurs by virtue of the capacity of uPAR and PAI-1 to bind to the ECM component vitronectin. By this, uPAR and PAI-1 compete mutually and with integrins for interaction with VN thus interfering with cell adhesion. Within this adhesion scenario, u-PA acts as an important modulator by dissociating of PAI-1 from VN (-) and enhancing uPAR-VN binding affinity (+). (Adapted and modified from Reuning U. et.al.[99])

Gene-targeted animal models provide a valuable tool to study components of u-PA/uPAR system and other interacting molecules in vivo[101]. Interestingly, constitutive knockout mice of u-PA or uPAR gene survive, suggesting that the mice adapt to the absence of u-PA by a redundant systems or that u-PA/uPAR system is not essential for particular function in vivo. It is likely that the role of u-PA in vivo is mainly the generation of plasmin, whereas uPAR might have only a negligible role in enhancing plasmin generation. Still, the exact molecular nature of certain u-PA/uPAR-triggered signal transmission routes remains to be established[102].

In addition to uPAR, scu-PA and tcu-PA also bind to receptors of the low density lipoprotein receptors (LDLR) family namely, the LDLR-relative protein/α_2 macroglobulin receptor (LRP//α_2-MR) and the very low density lipoprotein receptor (VLDLR)[103-105]. Moreover, u-PA devoid of growth factor-like domain can also bind to the surface of SMC and other cells either through the kringle domain or protease domain, suggesting presence of an additional receptor on the plasma membrane. Besides, it indicates that variants of u-PA can affect cellular function independently or in cross-talk with uPAR/CD87[83].

1.5.3 Inhibitors of urokinase

Plasminogen activator inhibitor-1 (PAI-1) is the principle physiological inhibitor of urokinase. Like other plasminogen activator inhibitors, PAI-1 is a member of the serine protease inhibitor (Serpin) gene family.

In addition to platelets, which contain a large pool of PAI-1 mostly in an inactive form, macrophages, endothelial cells as well as many other types of cells in culture secrete PAI-1, and it has a molecular mass of ~54 kDa [106-108]. Cells secrete active form of PAI-1, which is rapidly inactivated unless it binds to vitronectin (VN). Vitronectin stabilizes the active conformation of PAI-1[95]. PAI-1 forms complexes with single chain as well as two chain forms of both t-PA and u-PA[109]. The inhibition of plasminogen activators (PA) by PAI-1 is followed by the endocytosis. Upon binding by the PAI-1 to the receptor bound u-PA on the cell surface, this trimeric complex is endocytosed into the cell. Endocytosis occurs by means of the cell surface receptors, LRP//α_2-MR or VLDLR[104, 110]. After internalization urokinase-inhibitor complex dissociates from the uPAR in the endosomes followed by the degradation of the complex in the lysosomes, whereas the uPAR is recycled to the cell surface[111]. Thus, endocytosis serves as the regulatory mechanism by which u-PA and t-PA are cleared from the cell surface, the circulation and the extracellular space. It was also demonstrated that the scu-PA and tcu-PA, bound to the LRP/α_2-MR, can be internalized and degraded even in the absence of PAI-1[112]. However, inactivation of urokinase by the presence of PAI-1 significantly facilitates the endocytosis[113, 114] and thus, efficient internalization of receptor bound u-PA. PAI-1 has significantly higher affinity to bind to vitronectin compared to uPAR[115] and because PAI-1 recognizes and binds to the same site contained within the somatomedin domain (SMD) of the vitronectin molecule as uPAR does, it competes with uPAR for vitronectin binding[116]. However, the affinity of PAI-1 for vitronectin is markedly reduced when PAI-1 is complexed to u-PA[117]. In agreement with these data, Stefansson and Lawrence showed that active PAI-1 directly impairs smooth muscle cell (SMC) adhesion and migration by limiting the binding of vitronectin to the integrin receptor $\alpha_v\beta_3$[118]. Paradoxically, Tanaka and colleagues showed that PAI-1 could enhance, rather than impair, smooth muscle cell migration[119]. These authors suggest that the results may be explained by a reduction in the stringency of binding, which in turn allows for greater cellular motility. In addition to plasminogen activator inhibitors, the regulation of u-PA may also involve other inhibitors such as the protease nexin-1 (PN-1) and protein C inhibitor[120, 121]. These inhibitors can also inactivate urokinase activity on the cell surface. Hence, regulation of u-PA activity or plasminogen activation cascade may occur at different levels as depicted in figure 4.

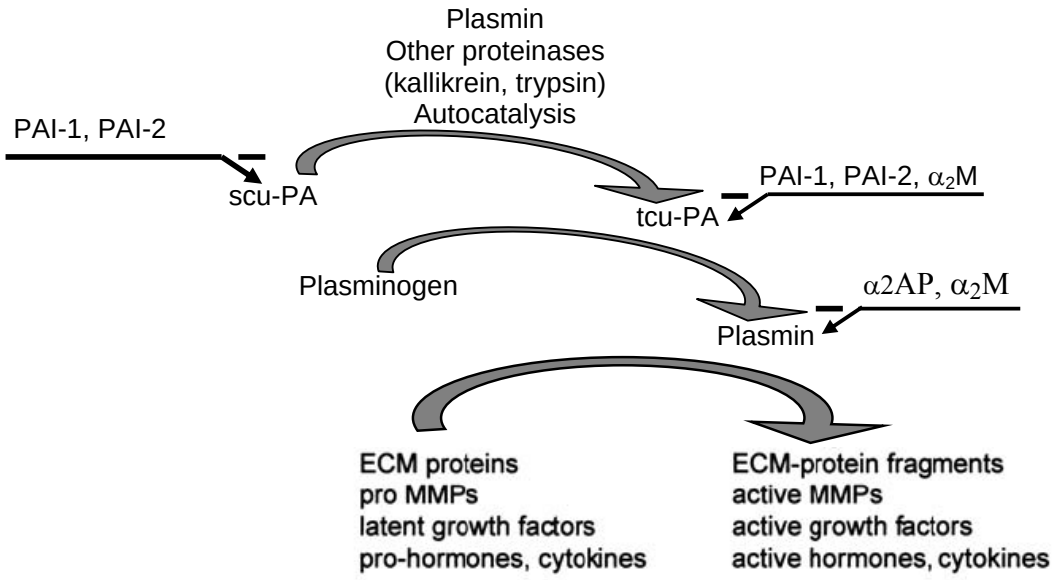


Figure 4. Plasminogen activation cascade.
Activation of scu-PA into tcu-PA by several factors including plasmin leads to activation of plasminogen into plasmin. Plasmin acts as the end effector factor to degrade ECM proteins, activate pro-MMPs and latent growth factors etc. α_2-M, alpha-2-macroglobulin; α_2AP, alpha -2-antiplasmin. (Adapted and modified from Myöhänen H and Vaheri A., 2004)

1.5.4 Coagulation and fibrinolysis in pulmonary arterial hypertension

Pulmonary arterial hypertension involves alterations in the pulmonary vasculature such as vasoconstriction, smooth muscle and endothelial cell proliferation, and thrombosis, suggesting that there may be disturbances in the normal relationships between vasodilators and vasoconstrictors, growth inhibitors and mitogenic factors, and antithrombotic and prothrombotic determinants[122]. Individuals with IPAH have been reported to exhale less nitric oxide (NO) than normal, and the production of NO in the lung is inversely related to the degree of pulmonary hypertension[123], suggesting alteration in vascular tone. It has also been shown that individuals with PAH have an increase in exhaled NO concomitant with a decrease in pulmonary artery pressure after initiation of vasodilator therapy with the prostacycline[124]. Furthermore, Christman and collaborators reported an increase in the release of thromboxane A2 and a decrease in prostacyclin in IPAH as well as secondary PAH patients[125].

Indirectly, these data suggested an imbalance of vasoconstrictor and vasodilators, indicating a reduced antithrombotic potential. The endothelium plays a key role in maintenance of normal coagulation and anticoagulation function by elaborating a variety of substances such as humoral factors, heparan sulfates, thrombomodulin, t-PA, u-PA and von Willebrand factor (vWF)[126, 127]. Endothelial dysfunction or injury may, hence, contribute to the thrombotic process, a feature that worsens pulmonary hypertension. Moreover, a relative deficiency of the antithrombotic molecules, prostacyclin and NO, and slowing of blood flow in pulmonary circulation secondary to luminal narrowing further enhances thrombogenicity[128]. Thrombus formation leads to narrowing of the pulmonary vessel lumen, and to aggravating the pulmonary hypertension(PH)[129]. Accumulating body of literatures suggest that endothelial dysfunction and haemostatic alteration with hypercoagulable state present in PH patients[130]. Several factors have been implicated in the same.

P-selectin is a glycoprotein that is expressed in α-granules of activated platelets and granules of endothelial cells. An increased P-selectin level is a marker of endothelial dysfunction and/or platelet activation and may indicate the presence of a hypercoagulable state[131]. Sakamaki and collaborators analyzed plasma from patients with primary as well as secondary PH. They found an elevated level of P-selectin and decreased level of thrombomodulin[132]. Others have also reported elevated P-selectin and thrombomodulin[133, 134]. Thrombomodulin, which is expressed with high abundance in the pulmonary circulation, is an endothelial membrane receptor for thrombin, and binding of thrombin to thrombomodulin results in the activation of protein C. In addition, the receptor-bound thrombin has no procoagulant effect. vWF, a large multimeric glycoprotein, is constitutively produced in endothelium (in the Weibel-Palade bodies) and megakaryocytes (in the α-granules of platelets). Its primary function is to bind to other proteins, particularly Factor VIII. It is important in blood coagulation. Several studies have found higher vWF level in blood plasma of patients with PH. Kawut et.al. measured vWF in PH patients and found that increased vWF levels were associated with worse survival in PH patients, suggesting that endothelial dysfunction and injury may have impact on disease course[135].

Welsh et. al. detected a decrease in soluble thrombomodulin, a rise in the PAI-1 and an elevated euglobulin lysis time in PPH patients. In SPH patients, they observed an increase in vWF antigen and fibrinogen and a decrease in fibrinolytic activity. They could also demonstrate a correlation between the loss of fibrinolytic activity and the degree of elevation of mean pulmonary artery pressure in both PPH and SPH[136], suggesting a role for the abnormalities in the coagulation mechanisms in perpetuation of pulmonary hypertension. Others have also reported an alteration in plasminogen activation system leading to a procoagulant-anti-fibrinolytic state[47, 133, 137, 138]. The gender specific analysis of the observed prothrombotic state in PPH patients showed a female specific alteration in plasma levels of the major components of the plasminogen activation system (PAI-1 and t-PA), and a basal activation of coagulation as indicated by an increase in markers of the thrombin activation system (Thrombin/antithrombin complex, prothrombin fragment)[139]. Thus, the procoagulant state in the PH patients is favored by the slowing of blood flow in the pulmonary circulation secondary to the luminal narrowing, by the presence of a relative deficiency of prostacyclin and NO, and by the switch of the hemostatic balance favouring coagulation and blocking fibrinolysis.

The available data suggest that the alteration in thrombin and plasminogen activation likely result in hypercoagulable environment conducive to in-situ thrombosis (Figure 5). Such alteration in hemostatic balance reflects a generalized diseased endothelium, which may be ineffective in maintaintaining patency of the pulmonary vasculature. This could also be the basis for the observed increase in survival of the patients with pulmonary hypertension in response to the wider use of chronic anticoagulation therapy after termination of non randomized studies[140]. Whether these alterations in the hemostatic balance may represent a primary disturbance or are the secondary response to vascular injury of different origins still remains to be explored.

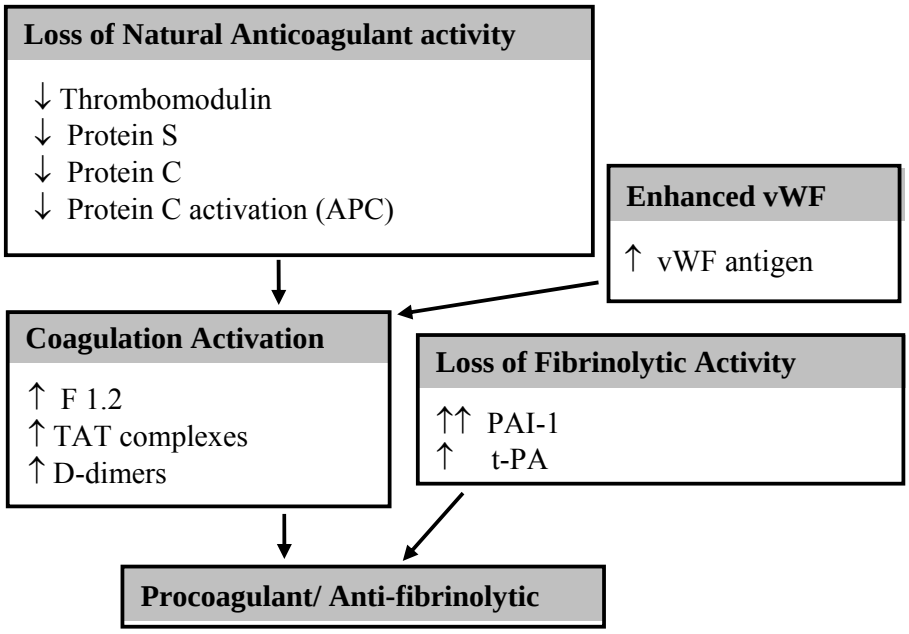


Figure 5. Abnormalities in coagulation and fibrinolysis in PAH.
As discussed in the text various abnormalities in coagulation and fibrinolysis have been observed in patients with pulmonary hypertension, which are likely attributable to favor hemostatic balance towards procoagulant state conducive to in situ thrombosis. In situ thrombosis is often associated with and aggravates pulmonary hypertension. F1.2-prothrombin fragment, TAT-thrombin/antithrombin complex, vWF-vonWilebrand factor (Adapted and modified from Welsh et.al., 1996[136]).

1.5.5 Urokinase system in pulmonary vascular remodeling

Animal models, different forms and features of pulmonary vascular remodeling, and the plasminogen activation system have been already discussed above. In this section, the urokinase system in hypoxic pulmonary vascular remodeling will be discussed. Apart from the above mentioned factors such as prostacyclin, nitric oxide, endothelin, potassium channel, serotonin and growth factors such as TGF-β superfamily members and PDGF, proteases have also been implicated in the development of pulmonary hypertension[141]. Among the proteases, the previous studies have implicated matrix metalloproteinases (MMPs) in remodeling processes owing to their function in extracellular matrix degradation and their influence in cellular events[142]. Endogenous vascular elastase (EVE), a serine proteinase, is such an example.

It has been demonstrated that EVE mediates the structural remodeling of pulmonary arteries by degrading extracellular matrix and releasing growth factors[143-145]. Moreover, inhibition of serine elastase activity could abrogate pulmonary vascular remodeling in animal models[143, 146], suggesting that it may be a key proteinase to mediate vascular remodeling. However, also an involvement of the MMP system in the process of remodeling is likely, as serine elastase may increase MMP expression through release of degraded matrix peptides[147, 148] or by inhibition of tissue inhibitors of MMPs[149].

Likewise, the plasminogen activation system has been investigated and implicated in the process of tissue and vascular remodeling. In the past, the involvement of u-PA and PAI-1 (components of fibrinolytic system) in arterial wound healing and neointima formation was investigated by employing in vivo models of arterial injury[150-157]. Initial studies on mouse model of electrically- or mechanically-induced arterial injury ascribed u-PA the role to promote neointima formation by enhancing cellular migration independent of its receptor[150, 153, 157]. The logical strategy for subsequent studies was to investigate the role of PAI-1, the principle physiological inhibitor of u-PA and t-PA. Studies employing animal models of copper- or ferric chloride-induced arterial injury yielded paradoxical findings demonstrating PAI-1 to enhance neointima formation/vascular remodeling[151, 152]. Similarly, intima-promoting role for PAI-1 has also been demonstrated in other mouse models such as carotid artery ligation and balloon carotid injury[158, 159]. Moreover, transforming growth factor-β (TGF-β), through PAI-1 dependent pathways, has been shown to induce neointimal growth[154, 156]. On the other hand, PAI-1 has been found to play an inhibitory role in neointima formation[150, 160], adding further complexity to the contribution of fibrinolytic system in neointima formation. In vitro and in vivo studies were performed to investigate cellular events induced by the components of fibrinolytic system that might explain the in vivo findings. The observation that u-PA promotes cellular proliferation and migration[161-164] supports its role to enhance neointimal growth/vascular remodeling. On the other hand, PAI-1 has also been shown to contribute to cell proliferation and migration, and to inhibit apoptosis[165-168]. This is in line with the in vivo findings that PAI-1 enhances neointima formation/ vascular remodeling.

Although the complexity seems to be deepening and hence, the consensus looks still to be far away, the contradiction is likely attributable to differing models and methods used in various studies. However, the complexity has been further worsened with two recent investigations, which employed PAI-1 knockout mice and carotid artery ligation model but yielded contradictory findings[159, 160]. This suggests that the precise role of plasminogen activation system in vascular remodeling is yet poorly understood.

Despite the numerous studies that were carried out to determine the role of fibrinolytic system in the process of remodeling in the systemic vasculature, efforts to study the role of u-PA or PAI-1 specifically in pulmonary vascular remodeling are almost negligible. The problem to study pulmonary vascular remodeling in mice is also limited by the availability of models, as hypoxia-induced pulmonary vascular remodeling is the only one available. On the other hand, clinical studies have investigated the components of coagulation and fibrinolytic system. However these studies are limited to the analysis of plasma samples from the patients with pulmonary hypertension and their primary aim was to find possible markers for the disease. Coagulation profiles of patients with primary pulmonary hypertension have revealed lower fibrinolytic and higher plasma PAI-1 activity, indicating an altered haemostasis with hypercoagulable and depressed fibrinolytic state[47, 133, 136-139]. In an experimental level, a coordinated induction of PAI-1 and inhibition of plasminogen activator gene expression in lungs and an enhanced PAI-1 antigen and activity levels in plasma were observed in mice exposed to hypoxia[169]. The coagulation profile of mice upon hypoxic exposure indicated that the elevated level of PAI-1 might mediate the pulmonary vascular response to hypoxia. Instead, knockout mice study attributed u-PA the role to mediate the pulmonary vascular response to chronic hypoxia[170]. Clearly, the available literature is not robust enough to safely judge on the precise role of u-PA in hypoxic pulmonary vascular remodeling. Besides, it is most relevant to examine the function of PAI-1, the principle physiological inhibitor of u-PA. No studies have yet examined PAI-1 directly in hypoxia-induced pulmonary vascular remodeling. Hence, a comprehensive study to understand the role of u-PA in an experimental animal model is highly desirable.

1.6 Aim of the study

In general, the aim of this study was to investigate the role of urokinase-type plasminogen activator (u-PA) and its inhibitor, plasminogen activator inhibitor 1 (PAI-1), in pulmonary vascular remodeling and subsequent right ventricular hypertrophy (RVH) by employing a mouse model of chronic hypoxia-induced pulmonary hypertension. Hence, we aimed to address the following questions.

- ☞ Expression of u-PA and PAI-1 in chronically hypoxic murine lungs
- ☞ u-PA activity in chronically hypoxic murine lungs
- ☞ Expression of u-PA and PAI-1 in human lungs from patients with IPAH and CTEPH as well as donor lungs
- ☞ u-PA activity in human lungs from patients with IPAH and CTEPH as well as donor lungs
- ☞ Effect of inhibition of u-PA activity in chronic hypoxia-induced pulmonary vascular remodeling and subsequent RVH
- ☞ Effect of the absence of u-PA and PAI-1 gene in chronic hypoxia-induced pulmonary vascular remodeling and subsequent RVH
- ☞ Effect of continuous u-PA infusion in chronic hypoxia-induced pulmonary vascular remodeling and subsequent RVH

2 MATERIALS

2.1 Chemicals, Reagents, Injecting solution and substances

Substance or reagents	Trade names or kits	Company
Chromogenic substrate (H-D-Val-Leu-Lys-pNA·2HCl)	S-2251	Chromogenix-Instrumentation laboratory SpA, Italy
HMW-urokinase	Human urokinase	American Diagnostica, USA
Plasminogen	Plasminogen	Chromogenix-Instrumentation laboratory SpA, Italy
Tissue lysis buffer for RNA isolation	RNAzol	Wak-Chemie Medical, Germany
DNA polymerase	Hot start Taq® DNA polymerase	Qiagen
Reverse transcriptase	Omniscript® Reverse Trascriptase Kit	Qiagen
PCR primers	Primers	Metabion International AG, Germany
Protein concentration determination kit	BCA™ Protein Assay kit	Pierce, USA
Tissue-Tec	Tissue-Tek®O.C.T.™	Sakura, The Netherland
t-PA	Actilyse	Dr. Karl Thomae GmbH, Germany
HMW tcu-PA	Actosolv	Hoechst GmbH, Germany
ECL reagents	ECL immunodetection kit	Amersham pharmacia biotech
Sodium hydroxide 1N (1mol/l)	Sodium hydroxide	Merck Darmstadt, Germany
Chlorhidric acid 1N (1mol/l)	Chlorhidric acid	Merck Darmstadt, Germany
Ketaminhydrochloride 100 mg/ ml	Ketamin®	Pharmacia Erlangen, Germany
Lidocainhydrochloride 2%	Xylocain®	Astra Zeneca Wedel, Germany
Sterile isotonic Saline solution (0.9% NaCl)	Physiological Saline solution	Baxter S.A. München, Germany
50% O2, 50%N2	Ventilation gas	Air Liquid (ehem. Messer) Siegen, Germany
Enrofloxacine oral solution	Baytril 2,5%®	Bayer Vital GmbH Leverkusen, Germany
u-PA inhibitor	CJ-463	Curacyte AG, Munich, Germany

2.2 Consumables

Substance or reagents	Trade names or kits	Company
Nitrocellulose membrane	Hybond ECL membrane	Amersham parmacia Biotech
Micro-well plate	Micro-well plate	Nunc, Denmark
Single use syringes 1ml, 2ml, 5ml, 10ml	Inject Luer®	Braun Melsungen, Germany
Needles 26G (0,9mm x 25mm)	BD Microlance 3®	Becton Dickinson Germany
Medical adhesive bands	Durapore®	3M, St. Paul, MN, USA
Cannula for vein catheter support 22G and 20G	Vasocan Braunüle®	Braun Melsungen, Germany
Gauze 5 x 4 cm	Purzellin®	Lohmann und Rauscher Rengsdorf, Germany
Single use gloves	Transaflex®	Ansell Surbiton Surrey, UK
Gauze	Gauze balls size 6	Fuhrman Verrbandstoffe GmbH Much, Germany
Perfusor-tubing 150 cm	Original-Perfusor®-tubing	Braun Melsungen, Germany
Combi-Stopper	Combi-Stopper	Intermedica GmbH Kliein-Winternheim, Germany
Stopcock for infusion therapy and pressure monotoring	Discofix®-	Braun Melsungen, Germany
Napkins	Napkins	Tork Mannheim, Germany
Threads Nr. 12	Surgical threads	Coats GmbH Kenzingen, Germany
Surgical threads non-absorbable Size 5-0	ETHIBOND EXCEL®	Ethicon GmbH Norderstedt, Germany
Surgical threads with needle Size 5-0, 6-0 and 7-0	ProleneTM	Ethicon GmbH Norderstedt, Germany
Surgical instruments	Surgical instruments	Martin Medizintechnik Tuttlingen, Germany
Heating pad	Thermo-Lux®	Witte und Suttor Murrhardt, Germany
Tracheal cannula	from BD Microlance 3 15or 20G shortened to 1,5cm	Becton Dickinson Germany
Osmotic mini pump	Alzet OMP (Model 2004)	Durect Corporation, CA, USA

2.3 Systems, machines and softwares

System, machines and software	Trade names or kits	Company
Ventilator for mice	SAR830A/P Ventilator	IITH Inc. Life Science Woodland Hills, CA, USA
PET-Tubes with different diameters	Tygon®	Saint-Gobain Performance Plastics Charny, France
Computer and Monitor		
Transducer	Combitrans Monitoring Set Mod. II for arterial Blood Pressure Measurement	Braun, Melsungen, Deutschland
PCR machine	GeneAmp® PCR system 2400	Applied Biosystem
Transfer machine	Trans-Blot® SD semi-dry Electrophoretic transfer blot	Bio-Rad
ELISA reader	Tecan Spectrafluor plus,	MTX Lab Systems, Inc.
Software for densitometry	AlphaEase	AlphaInnotech
Software (ELISA reader)	Magellan	Tecan, Inc
Software (vascular morphometry and scanning)	Leica Qwin	Leica, Germany
Centrifuge (hematocrit measurement)	Adams autocrat centrifuge	Clay Adams, Parsippany, NY, USA.

2.4 Materials for histology

Substance or reagents or appliances	Trade names or kits	Company
Parafilm	Parafilm	American National Can Menasha, Wisconsin, USA
Automated microtome	RM 2165	Leica Microsystems, Nussloch, Germany
Flattening table	HI 1220	Leica Microsystems,Nussloch, Germany
Flattening bath for paraffin sections	HI 1210	Leica Microsystems, Nussloch, Germany
Tissue embedding machine	EG 1140H	Leica Microsystems, Nussloch, Germany
Cooling plate	EG 1150C	Leica Microsystems, Nussloch, Germany
Tissue processing automated machine	TP 1050	Leica Microsystems, Nussloch, Germany

Stereo light microscope	DMLA	Leica Microsystems, Nussloch, Germany
Digital Camera Microscope	DC 300F	Leica Microsystems, Nussloch, Germany
Ethanol, 70%, 95%, 99,6%	Ethanol	Fischer. Saarbrücken, Germany
Isopropanol (99,8%)	Isopropanol	Fluka Chemie, Buchs, Swiss
Methanol	Methanol	Fluka Chemie, Buchs, Swiss
Formaldehyde alcohol free (≥37%)	Formaldehyde	Roth, arlsruhe, Germany
Roti-Histol (Xylolersatz)	Formalin	Roth, Karlsruhe, Germany
Xylol	Xylol	Roth, Karlsruhe, Germany
Hydrogen peroxide	Hydrogen peroxide30% pro analysi	Merck,Darmstadt, Germany
Cover slips 24x36mm	Cover slips	Menzel, Germany
Tissue embedding cassettes	Universal-embedding cassettes	Leica Microsystems, Nussloch, Germany
Histological glass slices	Superfrost Plus®	R. Langenbrinck, Emmendingen, Germany
Microtom blades	Microtom blades S35	Feather, Japan (über Produkte für die Medizin AG, Köln, Germany)
Paraffin embedding medium	Paraplast Plus®	Sigma Aldrich, Steinheim, Germany
Pikric acid	Pikric acid	Fluka Chemie, Buchs, Swiss
Mounting medium	Pertex®	Medite GmbH, Burgdorf, Germany
Sodium Chloride (NaCl)	Natriumchloride pro analysi	Roth, Karlsruhe, Germany
Di-sodiumhydrogenphosphate dihydrate	Di-Natriumhydrogenphosphat Dihydrat, pro analysi	Merck, Darmstadt, Germany
Potassium hydrogen phosphate	Kaliumdihydrogenphosphat pro analysi	Merck, Darmstadt, Germany
Trypsin	Digest All 2®	Zytomed, Berlin, Germany
Avidin-Biotin-Blocking reagent	Avidin-Biotin-Blocking Kit	Vector/ Linaris, Wertheim-Bettingen, Germany
Goat Serum	Normal Goat Serum	Alexis Biochemicals, Germany
Substrat Kit	Vector VIP Substrat Kit	Vector/ Linaris, Wertheim-Bettingen, Germany
Substrat Kit	DAB Substrat Kit	Vector/ Linaris, Wertheim-Bettingen,
Avidin with fluroscein	Fluorescein avidin DC	Vectastinkit, Vector Laboratories
Methylgreen	Methylgreen	Vector/ Linaris, Wertheim-Bettingen, Germany

2.5 Antibodies

Antibodies	Dilution (application)	Company
Mouse anti-human anti-alpha-smooth muscle Actin; Clone 1A4 monoclonal,	Dilution 1:900 (IHC)	Sigma Aldrich Steinheim, Germany
Rabbit anti-human anti-von Willebrand factor, polyclonal	Dilution 1:900 (IHC)	Dako Cytomation Hamburg, Germany
Rabbit anti-mouse u-PA IgG fraction	Dilution 1:1000 (IHC)	Loxon, Germany
Rabbit polyclonal anti-PAI-1 antibody (H-135)	Dilution 1:50 (IHC)	Santacruz Biotechnology
Rhodamin-conjugated donkey anti-rabbit antibody	Dilution 1:100 (IHC)	Jackson Immunoresearch
Rabbit anti-mouse u-PA IgG fraction	Dilution 1:1000 (WB)	Loxon, Germany
Rabbit polyclonal actin antibody	Dilution 1:3000 (WB)	Abcam
Rabbit anti-mouse PAI-1 polyclonal antibody (MI48034)	Dilution 1:2000 (WB)	Innovative research
Monoclonal antibody against uPA B-chain	Dilution 1µg/ml (WB)	American Diagnostica
HRP conjugated Secondary antibodies (anti-rabbit or anti-mouse or anti-goat)	Dilution 1:2000 (WB)	Dako cytomation, Denmark.
Biotinylated Secondary anti-mouse and anti-rabbit antibody	Dilution 1:250 (Vectastain Elite ABC Kits) (IHC)	Vector/ Linaris Wertheim-Bettingen, Germany

3 METHODS

3.1 Animals

Adult u-PA knockout ($Plau^{tm1Mlg}$, Jackson Laboratories), PAI-1 knockout ($Serpin1^{tm1Mlg}$, Jackson Laboratories) and their wild type genetic background mice C57Bl/6N were procured from Charles River, Germany. Mice were given free access to water and food, and were kept under controlled temperature (~ 22°C) and light (12/12-hour light/dark cycle) throughout the experimental period. The experimental protocol for mouse experiment is depicted in Figure 6. The methods for exposure of mice to hypoxia or normoxia and their treatment with different substances have been discussed in detail in separate headings below. All experiments were performed as per the institutional guidelines that comply with national and international regulations. Approval from the local ethic commission was obtained for the experiments and registered as GI 20/10 Nr. 46/2004.

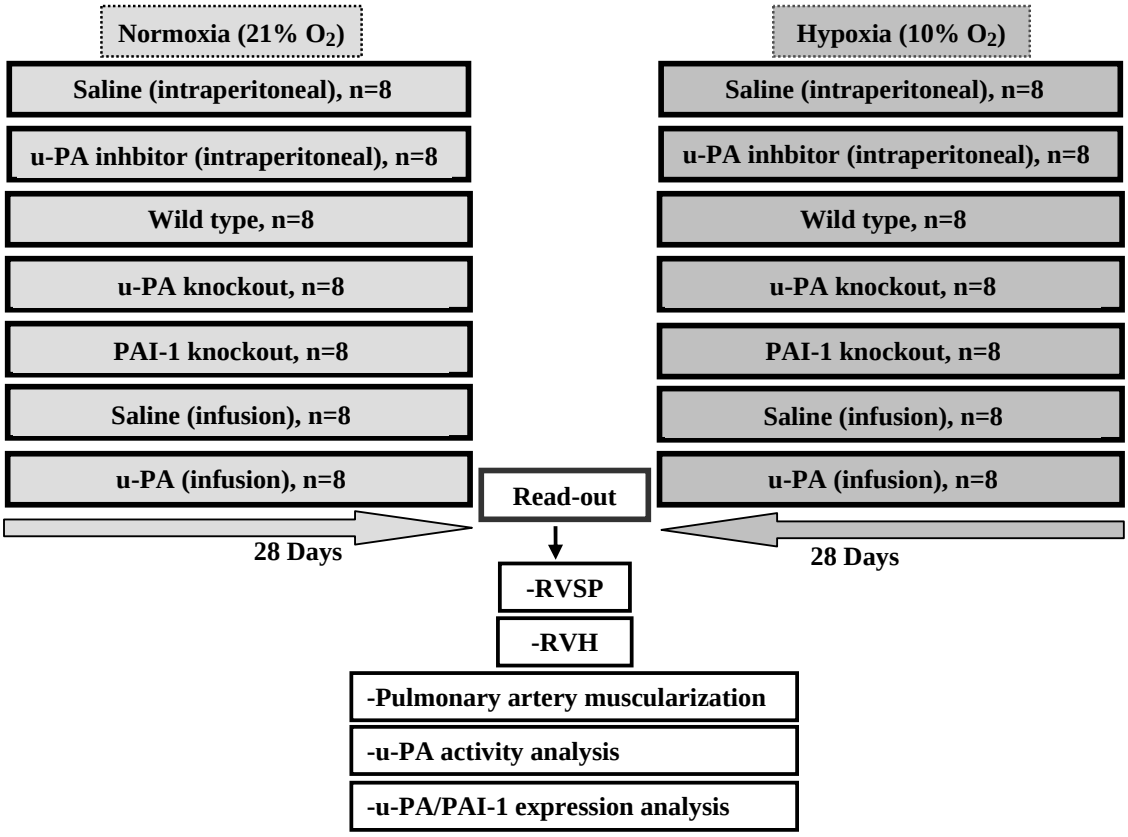


Figure 6. Schematic protocol for mouse experiment.
Mice were kept for 28 day under normoxia and hypoxia, followed by obtaining the read-outs and required tissue samples. RVSP-right ventricular systolic pressure, RVH-right ventricular hypertrophy, n- number of mice in the group.

3.2 Induction of pulmonary hypertension in mice by hypoxia

Pulmonary hypertension was induced in mice by exposure to hypoxia (10 % inspired O_2 fraction, FIO_2 10%) in a normobaric chamber for 28 days as described previously[171]. Constant level of hypoxia was maintained with the aid of an auto-regulatory control unit (model 4010, O_2 controller, Labotect; Göttingen, Germany) supplying either nitrogen or oxygen. Excess humidity in the system was prevented by condensation in a cooling system. CO_2 was continuously removed by soda lime. Cages were opened for food and water supply and for cleaning. The chamber temperature was maintained at 22–24°C. Control animals were placed in similar conditions in a normoxic chamber with a normal oxygen environment (21% inspired O_2 fraction, FIO_2 21%). Thus, the automatic system was in place to maintain the chamber environment as hypoxic or normoxic.

3.3 Treatment of mice with u-PA and u-PA inhibitor

Mice were treated with u-PA and specific u-PA inhibitor throughout the experimental period. Benzylsulfonyl-D-Ser-Ser-4-amidinobenzylamide (CJ463), described previously as inhibitor 26[172], was used as u-PA inhibitor. Stock solution of CJ463 (0.5 mg/100 µl) was prepared in 0.9% NaCl. Intraperitoneal injection of CJ463 was given to mice at a dose of 20 mg/kg body weight (BW) twice a day. Control mice received the same volume of 0.9% NaCl. Human two-chain u-PA was infused intravenously into mice via Osmotic Mini-pump (OMP). Subcutaneous implantation of OMP was performed by aseptic surgery 24 hours before mice were exposed to hypoxia or normoxia. Two doses of u-PA as 1427 and 7135 U/day were delivered through the OMP. Control mice received saline via OMP.

3.4 Hemodynamic and right ventricular hypertrophy (RVH) measurement in mice

For general anesthesia mice were given intraperitoneal injection of ketamine and xylazine combination (100 mg/kg and 15 mg/kg body weight respectively). The anesthetic mixture was prepared as 20 µl ketamine/20 µl xylazine/40 µl NaCl and the required volume as per the dose was injected. The anesthetized animals were placed on a heating pad in order to maintain the body temperature within the physiological range. Tracheotomy was performed to ventilate the animals artificially with 10ml/kg body weight and a frequency of 120 per second.

A positive end expiratory pressure (PEEP) of 1.0 cm H_2O was used throughout the experiment, while inspiratory oxygen (FIO_2) was set at 0.5. Through a small opening in the chest a 26-guaze stainless steel needle attached to a fluid-filled force transducer was inserted into the right ventricle to measure the right ventricular systolic pressure (RVSP). The transducer was calibrated at zero at the level of hillum before every measurement. RVSP was recorded for 10 minutes and the saved data was printed for analysis. Total blood was collected directly from right ventricle for hematocrit measurement and plasma separation. Hematocrit was measured immediately by capillary centrifugation technique. The capillary tube containing the whole blood was spun in an Adams Autocrit Centrifuge for about 5 minutes and hematocrit value was noted. Plasma was separated from the citrated blood by centrifuging at 1500 g for 10 minutes. After centrifugation, the upper phase containing plasma was carefully separated and stored at -20°C in clean eppendorf tubes until used for further analysis. The heart was dissected under a dissection microscope. The right ventricle (RV) was separated from left ventricle and septum (LV+S). After separation, the RV and LV+S were placed on glass slides and dried for one week at room temperature. The right ventricle and left ventricle plus septum were weighed to obtain the right ventricle to left ventricle plus septum ratio (RV/LV+S), as an index of right ventricular hypertrophy.

3.5 Histology and morphometric analysis of murine lungs

After completion of hemodynamic measurement the murine lungs were first flushed with sterile saline solution at a constant pressure of 22 cm H_2O above the pulmonary hillum in order to get rid of blood. They were then perfused with phosphate-buffered paraformaldehyde through pulmonary artery and with saline through trachea with a constant pressure of 22 and 11 cm H_2O respectively. The heart and the lungs were removed en block. The heart was subject to dissection for RVH measurement as described. The lungs were stored in phosphate buffered paraformaldehyde for the next 24 hours and then in 0.1 mol/L phosphate buffer till dehydration process. The individual lung lobe was placed in histological cassettes and was dehydrated in an automated dehydration station followed by embedding in paraffin blocks. 3 μm sections of the lung lobes in a transversal anatomical plan were prepared. Immunostaining of the lung sections was done using the standardized protocol. Mouse anti-α-SMC actin antibody and polyclonal rabbit anti-human v-WF antibody were used as primary antibodies. All reagents from the kits including the corresponding biotinylated secondary antibodies were used as suggested by the supplier (Avidin-biotin blocking kit, Vectastain ABC kit, VIP and DAB substrate kits).

Counterstaining was done with methyl green. Given below is the protocol summarized in tabular form (Table 5).

Table 5. Double Immunostaining protocol for paraffin embedded murine lung section

Incubation time (minute)	Reagents	Preparation for next step
10	Rotihistol	
10	Rotihistol	
10	Rotihistol	
5	Ethanol absolute 99.6%	
5	Ethanol absolute 99.6%	
5	Ethanol 96%	
5	Ethanol 70%	-Prepare H_2O_2 (3%)
15	H2O2-Methanol (3%)	
5	H2O	-Trypsin ⇒ RT (i.e. bring to room temperature)
5	PBS	-Prepare Trypsin
10	Trypsin (Incubate at 37°C)	
5	PBS	-Avidin Blocking Reagent⇒ RT
15	Avidin Blocking	
5	PBS	-Biotin Blocking Reagent ⇒ RT
15	Biotin Blocking	
5	PBS	
15	10% BSA	-Prepare Mouse Ig Blocking Reagent (MIgBR) ⇒ RT (M.O.M. Kit)
5	PBS	
60	Mouse Ig Blocking (1)	-Prepare Protein Blocking Reagent, PBR (2)
2 x 2	PBS	
5	M.O.M. PBR (2)	-Prepare α-actin Ab
30	Primary Ab (α-actin)	-Prepare M.O.M. biotinylated IgG reagent (3), -Prepare ABC reagent
2x2	PBS	
10	M.O.M. biotinylated IgG reagent (3)	
2x2	PBS	
5	ABC reagent (4)	
2x5	PBS	-Prepare VIP Substrate
3-4	Vector VIP substrate	-Check color intensity on white sheet
5	water	
5	PBS	Avidin Blocking Reagent ⇒ RT
15	Avidin Blocking	

Contd.

5	PBS	Biotin Blocking Reagent ⇒ RT
15	Biotin Blocking	
5	PBS	
15	10% BSA	
5	PBS	-Prepare Blocking Serum (Vectastain Kit)
20	Blocking Serum	-Prepare Primary Ab
30	Pab (vWF), 37°C	
5	PBS	-Prepare Biot. 2ndary Ab (Rabbit Kit)
30	Biotinylated Sec Ab	
5	PBS	- Prepare ABC reagent
30	ABC reagent	
5	PBS	-Prepare DAB substrate:
½	DAB Substrate Kit	-Check color intensity on white sheet
5	H2O	
3	Methylgreen	- Apply methyl green at 60°C and
1	Distilled water	
2	Ethanol 96%	
2	Ethanol 96%	
5	Isopropylalcohol	
5	Isopropylalcohol	
5	Rotihistol	
5	Rotihistol	
5	Xylol	
Apply cover slip using gluing agent		

The sections were examined under light microscope using computer based image processing system and pulmonary vascular morphometry was performed as described previously[173]. The computer software recognizes the brown and purple staining of endothelium and smooth muscle cells respectively, and analyzes the purple staining along the vessel periphery. At 40X magnification, 80-100 intra-acinar vessels (20-70 μm) accompanying either alveolar duct or alveoli were analyzed in each mouse by an observer blinded to the treatment.

Vessels were categorized as non-muscular (<5%) and muscular (>5%) based on α-SMC actin staining and were quantified as percentage of all vessels. The percentage of non-muscular (NM) and muscular (M) arteries was expressed as the ratio (NM/M). Furthermore, the immunostained sections were used to count alveoli and pulmonary arteries (15-50μm external diameter). The sections were scanned randomly at x20 magnification using scanning software. Vessels and alveoli from five fields in each lung section and five lungs from each experimental group were counted by two independent investigators. The number of vessels per 100 alveoli was calculated and analyzed.

3.6 Characterization of human lungs obtained from transplant programme

Human lung tissues were obtained from donor lungs and patients, both IPAH and CTEPH undergoing lung transplantation. IPAH (n = 5) and CTEPH (n = 5) diagnosis was set up on clinical data and confirmed by histopathological examination of explanted lung tissue. In addition, donor lungs were characterized by histology and found to be free from major inflammatory changes. Lung tissues were snap-frozen after explantation for mRNA and protein extraction or were embedded in paraffin blocks for immunohistochemistry. Detail of lung tissue preparation for gene expression or protein analysis is discussed in the following section. The study protocol for tissue donation was approved by the Ethik-Kommission am Fachbereich Humanmedizin der Justus-Liebig-Universitaet Giessen of the University Hospital Giessen and Marburg (Giessen, Germany) in accordance with national law and with Good Clinical Practice/International Conference on Harmonization guidelines.

3.7 Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis

For gene expression analysis, total RNA was isolated from both human and mouse lung tissues using guanidine-thiocyanate acid phenol (RNAzol, Wak-Chemie Medical, Germany). Similar protocol was followed for RNA isolation from lung tissue samples from human (h) and the half of the right lung from mice (m). The concentration of RNA was determined spectrophotometrically. The protocol used for RNA isolation is as follows.

- Homogenize the lung tissue with RNAsol (3 ml., depending on the quantity of tissue). The homogenate can be stored at –80°C till next step.

- Add Chloroform ($CHCl_3$) @10% of the volume of RNAsol (i.e. 300 μl chloroform), and immediately vortex briefly but strongly.

•Incubate for 20 minutes on Ice.

•Centrifuge @5000 rpm at 4 °C for 45 minutes (It separates RNA).

•Transfer the upper phase containing RNA to a new eppendorf tube.

•Add an equal quantity of Isopropanol into the eppendorf as the volume of upper phase is transferred.

•Invert the tube a couple of time to mix with Isopropanol, and incubate it for 1 hour at -20°C (It precipitates RNA).

•Centrifuge @13000 rpm at 4°C for 20 minutes (It makes the pellet of RNA precipitate).

•Wash pellet with 70% Ethanol (ice cold). This step can be done twice, each time mixing with about 1 ml of ethanol and Centrifuging to make the pellet.

•Centrifuge @13000 rpm at 4°C for 20 minutes.

•Remove ethanol carefully, and let the RNA in the eppendorf tube to dry at room air (performed under hood).

•Mix the pellet properly with RNAse free water (30-50 µl, depending on the mass of the pellet).

•Measure the RNA concentration and store RNA at -80°C

2 µg of RNA was used as template to synthesize first strand cDNA in a reverse transcription reaction using commercial kit (Omniscript RT kit). The cDNA was amplified by PCR using hot start DNA polymerase. Numbers of PCR cycles were optimized so that analysis of PCR products could be carried out within the linear range of amplification. PCR products were electrophoresed in 2% agarose gels. The PCR products were scanned and densitometric analysis of the PCR products was performed by the image analysis software. Integrated density value (IDV) for PCR products of u-PA or PAI-1 in each sample was normalized against the IDV for β-actin of the same sample. The normalized IDV in hypoxia was expressed as percentage of normalized IDV in normoxia.Similarly, the normalized IDV for u-PA or PAI-1 in case of the human lungs tissues (IPAH and CTEPH) was expressed as percentage of normalized IDV in the healthy donor lungs. The following are the primers used.

Table 6. Primers used for the PCR amplification of cDNA

(c)DNA	Primer sequence (5' ⟶ 3') (S: Sense primer; AS: Anti-sense primer)
mu-PA	S: TTC CAG TGT GGC CAG AAG G AS: CCA GGC TGT CTT CCC TGT AG
mPAI-1	S: CCT GGT GCT GGT GAA TGC AS: CTG GTC ATG TTG CCC TTC C
mMMP-2	S: GGC GAG TAC TGC AAG TTC C AS: CGG TCT CGG GAC AGA ATC C
mβ-actin	S: CTA CAG CTT CAC CAC CAC AG AS: CTC GTT GCC AAT AGT GAT GAC
hu-PA	S: ACT CTG CCA CTG TCC TTC AG AS: CGG TGC CTC CTG TAG ATG G
hPAI-1	S: GCT GGT GCT GGT GAA TGC AS: CCT GGT CAT GTT GCC TTT CC
hβ-actin	S: ACC CTG AAG TAC CCC ATC G AS: CAG CCT GGA TAG CAA CGT AC

3.8 Western blot analysis

The snap frozen lung tissue samples from both mice and human were processed for western blot analysis. The right halves of the lung tissues from mice (normoxic and hypoxic) and the lung tissues from human (IPAH, CTEPH and donor) were pulverized by mortar and pestle**.** The pulverized tissue was treated with lysis buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 1% TritonX100, 0.5% Natrium deoxycholat, pH to 7.4) containing protease inhibitor (PMSF, 1 mM final concentration). The ground tissue treated with lysis buffer was incubated for about an hour on ice, followed by centrifugation at 13000 rpm at 4°C for 10 minutes. The supernatant was obtained and used for protein concentration determination by Bicinchonic acid technique. The lung tissue samples (50 µg protein) were separated by SDS-PAGE (10%). The separated proteins were then transferred from SDS-PAGE to Hybond ECL nitrocellulose membranes using a semi-dry blotting technique. The membranes were blocked with 5% non-fat dry skim milk in TBST (20 mmol/L Tris, 150 mmol/L NaCl, pH 7.4, 0.1% Tween-20). The membranes were incubated with primary antibodies at 4°C overnight. The following primary antibodies were used.

Rabbit anti-mouse u-PA IgG fraction, monoclonal antibody against u-PA B-chain, rabbit polyclonal actin antibody, rabbit polyclonal anti-PAI-1 antibody, rabbit anti-mouse PAI-1 polyclonal antibody and mouse monoclonal anti β-actin antibody. Incubation with primary antibody was followed by washing with TBST (3 washing for 15 minutes each). The membranes were then incubated with secondary antibody for about 90 minutes at room temperature. Biotinylated anti-rabbit or anti-mouse secondary antibodies were used followed by detection of immune complex with an enhanced chemiluminescence (ECL) immunodetection kit. During each incubation and washing step, the membranes were subject to gentle shaking. The membranes were stripped by incubating with stripping buffer (100 mM β-mercaptoethanol, 2% Sodiumdodecyl sulphate, 62.5 mM Tris) at 60°C for about an hour under gentle shaking followed by washing with TBST and blotting for β-actin.

Blot intensity was quantified by densitometric analysis using AlphEase software (Alpha Innotech). Integrated density value (IDV) for u-PA or PAI-1 in each sample was normalized against the IDV for β-actin in the corresponding sample. The normalized IDV in hypoxia was expressed as percentage of normalized IDV in normoxia. Similarly, the normalized IDV in diseased lungs (IPAH, CTEPH) was expressed as percentage of normalized IDV in healthy donors.

3.9 Casein gel zymography

The lung tissue samples (both human and mice) prepared for protein analysis were used for casein gel zymography. Lung homogenates (15 µg protein) were separated by SDS-PAGE (10%) containing casein (1mg/ml) (Sigma) and plasminogen (5µg/ml). In addition, u-PA and pre-stained protein standard (SeeBlue plus, Invitrogen) were also loaded. Upon completion of electrophoresis, the gel was incubated at 37°C in 1% Tween 80 for 2 hours. The gel was then incubated at 37°C with PBS containing 0.1% Tween 80 followed for 16 hours. The gels were stained with Coomassie blue. Destaining was done with 10% acetic acid to visualize the lytic zones. To confirm that the caseinolytic activity was u-PA dependent, another gel was incubated in buffer containing the specific u-PA inhibitor, CJ463 (0.5mM). To check the specificity of CJ463, t-PA was also loaded in a gel with or without incubation with buffer containing CJ463.

The part of the gels containing pre-stained protein standard was carefully cut and removed after electrophoresis and before processing the remaining part of the gel. The pre-stained protein marker was used for identifying molecular weight.

3.10 Immunohistochemistry

3.10.1 Cryo-preserved murine lung

Wild type mice (n=6) were exposed to hypoxia and normoxia for 4 weeks as mentioned. The lungs were flushed with physiological saline solution and processed as follows. Right lungs were cryo-preserved for RNA and protein analysis, and left lungs were used for immunohistochemistry. Tissue-Tek was filled in a 1 ml syringe and warmed at 60°C in order to increase the fluidity followed by infusion of Tissue-Tek into left lung carefully so as to inflate the lung, while avoiding leakage. The Tissue-Tek infused lungs were snap-frozen and stored at -80°C. The cryo-preserved lungs were sectioned to obtain 5 µm thick sections. Single immunofluorescent staining was performed as described by supplier. Briefly, following fixation of tissue sections with cold acetone for 10 minutes, they were air dried and washed with TBS (50 mM Tris, 0.15 M NaCl, pH 7.6). Further steps for blocking, washing and incubation with primary antibody were performed as described before (section 3.5, Histology and morphometric analysis). Rabbit anti-mouse u-PA IgG fraction and rabbit polyclonal anti-PAI-1 were used as primary antibodies. The secondary antibody was Rhodamin-conjugated donkey anti-rabbit antibody. Serial sections were stained using polyclonal rabbit anti-human Von-Willebrand Factor antibody to identify the blood vessels. Biotinylated anti-rabbit secondary antibody was then used followed by washing and incubating with fluorecein avidin DC for 5 minutes. Finally, after washing with TBS buffer (50 mM Tris, 0.15 M NaCl, pH 7.6) tissue sections were incubated with DAPI (1µg/ml) for 10 minutes to stain nucleus. Sections were examined and photomicrographs were obtained.

3.10.2 Paraffin-embedded human lungs (donors, IPAH and CTEPH)

The formalin-fixed and paraffin-embedded human lung tissues from patients, both IPAH and CTEPH, and donors (n=3) were subjected to sectioning to obtain 3 µm thick tissue sections. Immunostaining was performed using the avidin-biotin complex (ABC) peroxidase method as described in detail in section 3.5.

Sections were incubated with monoclonal anti-human u-PA antibody (20µg/ml concentration) for 1 hour at 37°C, and with polyclonal rabbit anti-PAI-1 antibody overnight at 4°C. Biotinylated anti-mouse IgG and anti-rabbit IgG were used respectively as secondary antibodies. Negative control sections for staining received no primary antibody. Visualization of antigen-antibody complex was performed using a chromogenic substrate for peroxidase linked to secondary antibody (VIP substrate kits). Peroxidase-substrate reaction products elicit purple or violet color. Counterstaining was done with methyl green.

3.11 u-PA activity analysis in plasma and lung homogenates

u-PA activity in plasma and lung homogenates was determined using a chromogenic substrate (S-2251) as described[174], with some modification. Briefly, serial dilution of standard stock solution (1000 U/ml) of HMW u-PA, plasminogen (50 µg/ml) and substrate (2mM) were prepared in Tris buffer (100 mM Tris, 0.5% Tween-20 and 0.1% BSA, PH 7.6). Equal volume (50 µl) of plasma samples and serially diluted standards were pipetted into a micro-well plate, followed by addition of 50 µl of plasminogen and 50 µl of Tris buffer into the samples and standard. Finally, substrate (50 µl) was added making a total volume of 200 µl in each well. The plate was incubated at room temperature with gentle shaking. As the highest standard developed color, the absorbance was read at 405 nm in an ELISA reader. A reading was also taken before adding substrate to measure the background absorbance. A standard curve was obtained from the standard to confirm the linearity between u-PA activity and substrate degradation. The u-PA activity was calculated by computer software (Magellan) and expressed as unit of activity per unit sample volume or per unit protein mass in the sample loaded. Before calculating the activity, background absorbance was subtracted from the final absorbance. For lung homogenates, all samples (containing equal quantity of protein) were diluted with buffer to have equal final volume and 50 µl (containing 6.25 µg protein in mouse lung homogenate and 20.8 µg protein in human lung homogenate preparation) was loaded into the well and u-PA activity was measured as described for plasma samples.

3.12 Data analysis

Data are expressed as mean ± SEM. Statistical analysis of the data from mice experiments such as right ventricular systolic pressure (RVSP), right ventricle/ left ventricle plus septum (RV/LV+S) ratio, non-muscular/ muscularized (NM/M) vessels ratio was performed by one-way ANOVA and subsequent Neuman-Keul test. A value of $P<0.5$ was considered to be statistically significant. T-test was used to perform statistical analysis of the data from molecular biological experiments.

4 RESULTS

4.1 Expression of u-PA and PAI-1 in the lungs of patients with CTEPH and IPAH as compared to donor lungs

In the human lung homogenates, the expression of u-PA and PAI-1 was analyzed both at mRNA and protein level. In addition, u-PA activity was also analyzed.

4.1.1 u-PA and PAI-1 expression at mRNA level

We first investigated the u-PA and PAI-1 expression at transcript level in diseased and healthy lungs. Semi-quantitative RT-PCR analysis revealed an enhanced expression of u-PA in both IPAH (x 2) and CTEPH (x 1.5) lungs, whereas elevated transcript level of PAI-1 was found only in IPAH (x 1.4) as compared to the healthy donor lungs (Figure 7). Surprisingly, PAI-1 transcript in CTEPH lungs was comparable to that in the healthy donor lungs (Figure 7). Overall, IPAH lungs showed significant induction of u-PA and PAI-1 as compared to the donor, whereas in CTEPH lungs the induction appeared somewhat less pronounced.

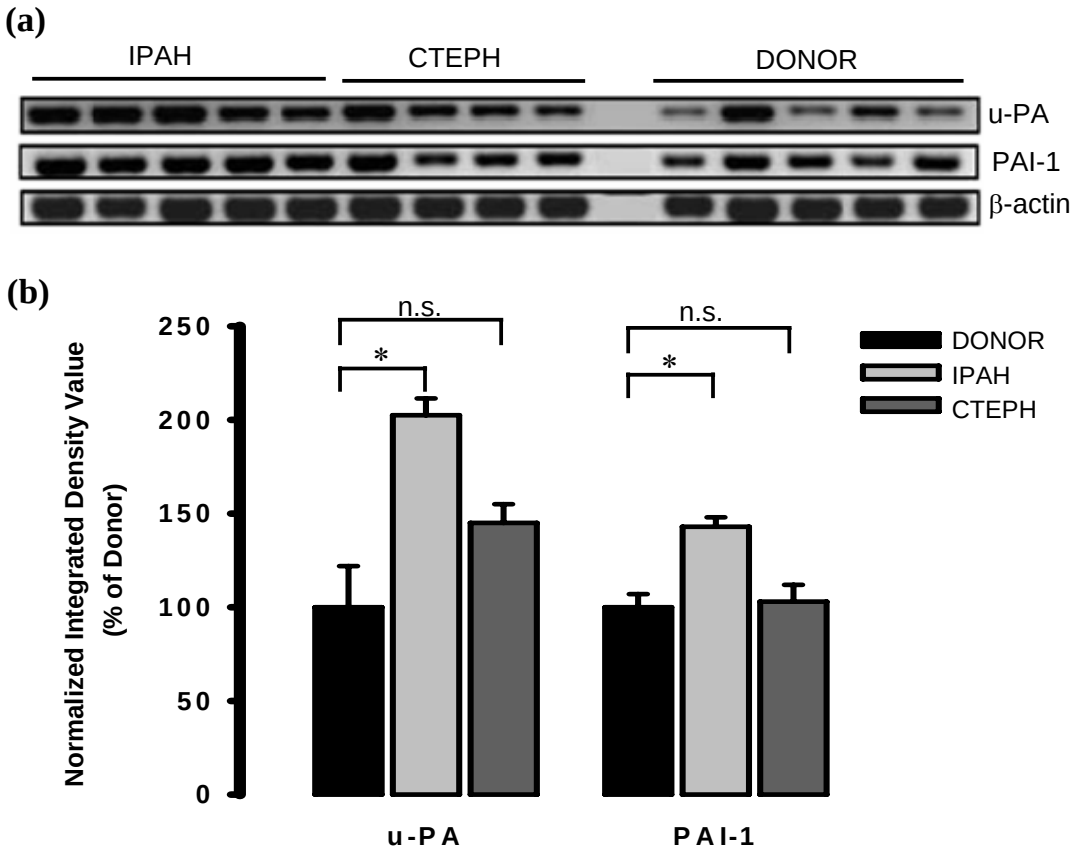


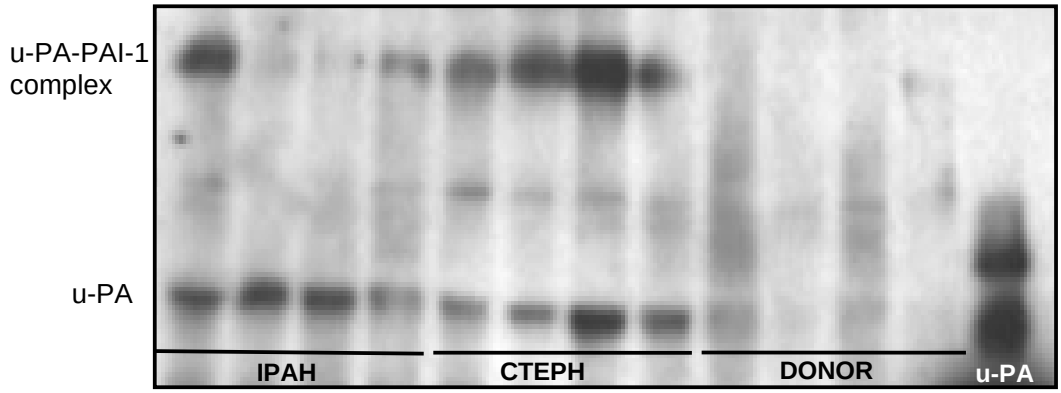
Figure 7. u-PA and PAI-1 gene expression in the lungs of patients with CTEPH and IPAH.

The lung samples from healthy donors (n=5) and patients with CTEPH (n=4) and IPAH (n=5) were homogenized, total RNA was isolated and RT-PCR was performed as described in methods. (a) RT-PCR products of the lung homogenates upon agarose gel electrophoresis. (b) Densitometric analysis of band intensity of PCR product. Normalized (against β-actin) integrated density value (IDV) of band intensity in pulmonary hypertension patients as percentage of normalized IDV of healthy donors are given (lower panel). Bar represents mean ± SEM. *P<0.05. n.s., non-significant (unpaired T-test). The PCR was done two times.

4.1.2 Expression of u-PA and PAI-1 at protein level

We then performed western blot analysis to check the expression of u-PA and PAI-1 at protein level. A clear upregulation of both u-PA and PAI-1 proteins was observed in IPAH and CTEPH as compared to healthy donors (figure 8). In addition, a complex between u-PA and PAI-1 was also detected in the diseased lungs, predominantly in CTEPH when a non-reducing gel was probed for u-PA (figure 8a). Furthermore, densitometric analysis of blot intensity revealed a significant elevation of u-PA and PAI-1 in IPAH (x 2.6 and x 3 respectively) and CTEPH (~ x 2 and x 4 respectively) as compared to healthy donor lungs (figure 8c). Interestingly, u-PA and PAI-1 complex were also significantly upregulated in IPAH (x 2.8) and CTEPH (x 4) (figure 8c). The u-PA and PAI-1 transcript level data corroborated with that of the protein level except for PAI-1 in CTEPH, where we observed higher PAI-1 protein level despite the absence of induction at transcript level.

(a)



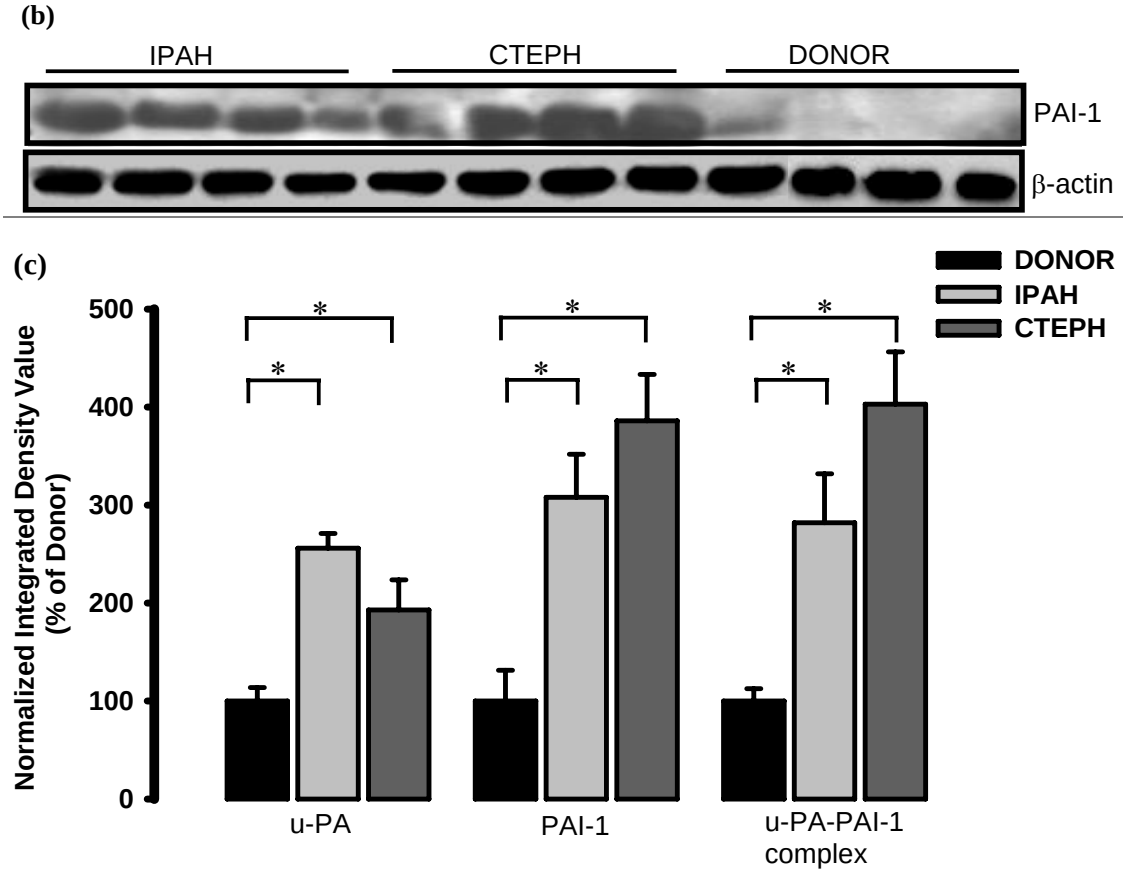


Figure 8. u-PA and PAI-1 protein in the lungs of patients with CTEPH and IPAH.
The lungs samples from healthy donors (n=4) and patients with CTEPH (n=4) and IPAH (n=4) were homogenized and protein concentration was determined as described in methods. Western blot analysis of the lung homogenates under (a) non-reducing and (b) reducing conditions. (c) Densitometric analysis of blot intensity. Normalized (against β-actin) IDV of blot intensity of pulmonary hypertension patients as percentage of normalized IDV of the healthy donors are given. Bar represents mean ± SEM. *P<0.05 (unpaired T-test). The western blot was performed twice.

4.1.3 u-PA activity in the lungs of CTEPH and IPAH patients as compared to donor lungs

We sought to investigate if the higher induction at protein level would lead to a change in u-PA activity. u-PA activity in the lung homogenates was assessed by casein gel zymography as well as chromogenic substrate assay. Interestingly, an enhanced u-PA activity was evident in IPAH (x 1.2) as well as CTEPH (x 2.1) compared to healthy donor lungs as revealed by casein gel zymography (figure 9a, 9b left bar graph).

Moreover, chromogenic substrate assay performed on the same lung homogenates also showed an enhanced u-PA activity in the lungs of IPAH (x 1.2) and CTEPH (x 12) as compared to healthy donors (figure 9b right bar graph). Overall, the results showed that u-PA activity was enhanced in CTEPH and IPAH lungs as compared to healthy donor lungs, and the enhanced activity was in agreement with the enhanced protein level. However, the u-PA activity data did not corroborate precisely with the pattern of protein level observed in IPAH and CTEPH.

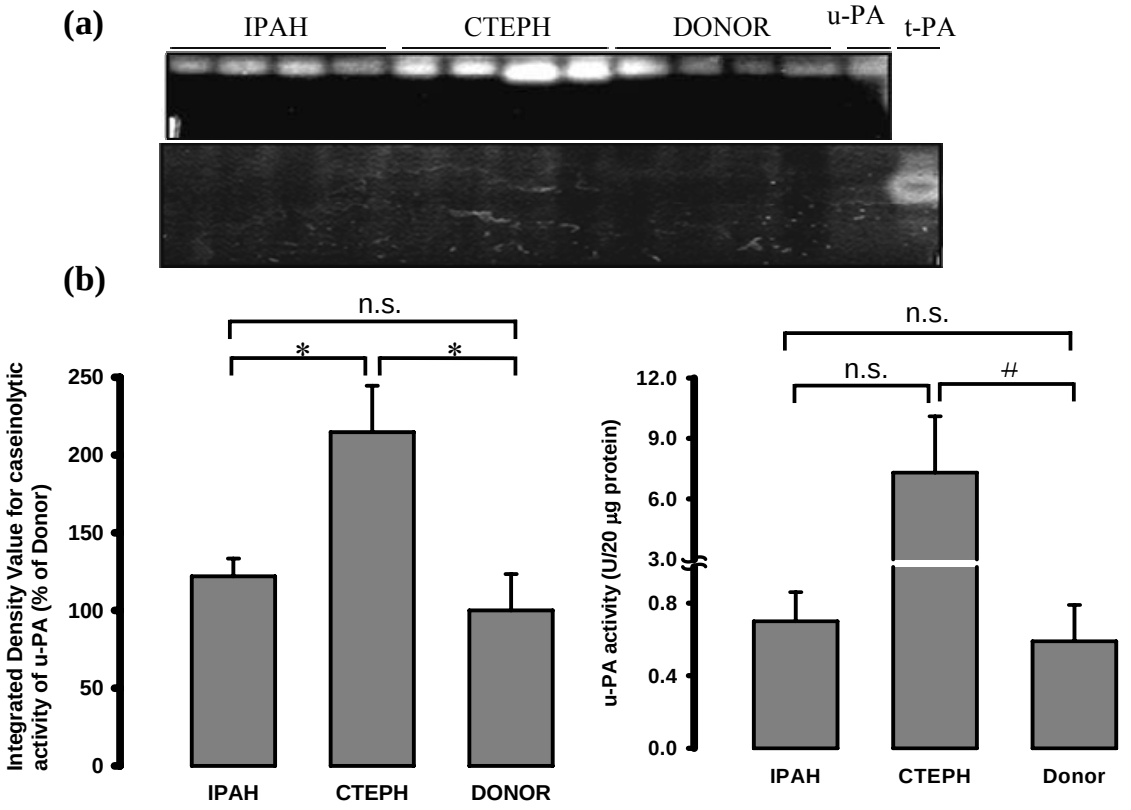


Figure 9. u-PA activity in the lungs of patients with IPAH and CTEPH

The lung homogenates from IPAH, CTEPH and donors were used for casein gel zymography (n=4) and chromogenic substrate assay (n=5). (a) Casein gel zymographs. The lower gel was incubated with buffer containing u-PA inhibitor (CJ463) as described. The caseinolytic zone due to t-PA activity is evident even in the presence of CJ463. (b) Densitometric analysis of lytic zones. IDV of u-PA activity in patients as percentage of the IDV in healthy donors are given (left bar graph). u-PA activity in (Unit/ 20µg of protein) as measured from chromogenic substrate assay is given (right bar graph). Bar represents mean ± SEM. *P<0.05 ; #P=0.049 n.s., non-significant (unpaired T-test) . The experiments were done at least twice.

4.1.4 Localization of u-PA and PAI-1 in the lungs from IPAH, CTEPH and donors

Our observation of an upregulation of u-PA and PAI-1 protein, and their complex formation prompted us to look for the spatial distribution of u-PA and PAI-1 in IPAH, CTEPH and donor lungs. In donor lungs, positive staining for u-PA and PAI-1 was observed in epithelial, vascular endothelial (intima) as well as media cells and macrophages. As expected, lungs from both IPAH and CTEPH patients showed extensive vascular remodeling as compared to donor lungs. Enhanced immunoreactivity for both u-PA and PAI-1 was evident in CTEPH and IPAH versus donor lungs, although quantitative analysis was not performed (figure 10). Moreover, immunostaining revealed a tendency towards differential distribution pattern of u-PA and PAI-1 under conditions of pulmonary hypertension as compared to donor lungs. Overall, a more pronounced staining for u-PA was encountered in the interstitial and alveolar compartments, whereas staining for PAI-1 appeared to be more prominent in the vascular compartment.

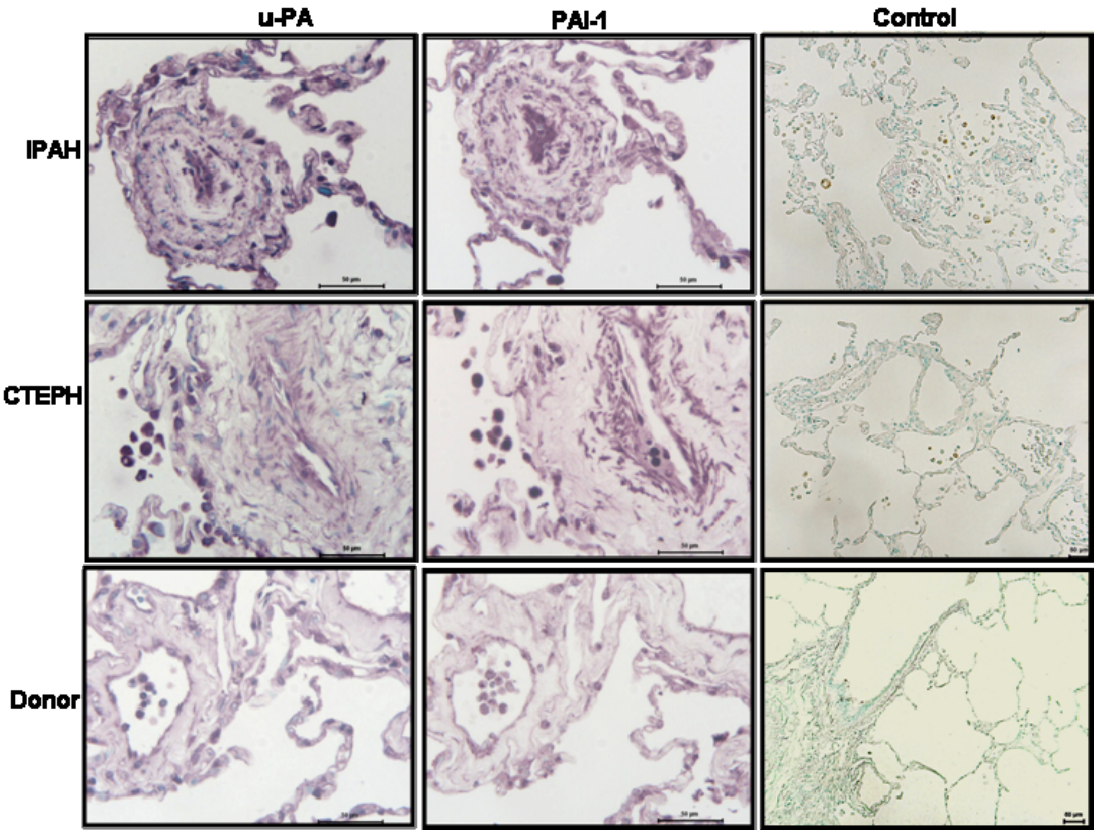


Figure 10. Localization of u-PA and PAI-1 in the lungs of IPAH, CTEPH and Donors.
3 μm thick sections of paraffin-embedded lungs from donor as well as patients with IPAH and CTEPH were immunostained for u-PA and PAI-1 as described in methods. Representative photomicrographs of immunostained lung sections are shown (x40 magnification). Control (negative control) sections were stained with identical protocol except that the primary antibody was omitted (x10 magnification). Scale = 50μm.

4.2 Expression of u-PA and PAI-1 in chronically hypoxic mouse lungs

After analyzing u-PA and PAI-1 regulation in CTEPH and IPAH versus donor lungs, we investigated the regulation of these factors in murine model of chronic hypoxia-induced pulmonary vascular remodeling. The chronically hypoxic mouse lungs were investigated for expression of u-PA and PAI-1 at both mRNA and protein level. In addition, u-PA activity in the same lung homogenates was also analyzed.

4.2.1 u-PA and PAI-1 gene expression under hypoxia

The lung homogenates from mice that were exposed to hypoxia or normoxia for 28 days were analyzed. Semi-quantitative RT-PCR showed a clear upregulation of both u-PA and PAI-1 genes under hypoxia (figure 11a). Densitometric analysis revealed a significant induction of u-PA and PAI-1 transcripts under hypoxia ($P < 0.05$ vs. normoxia) (figure 11b).

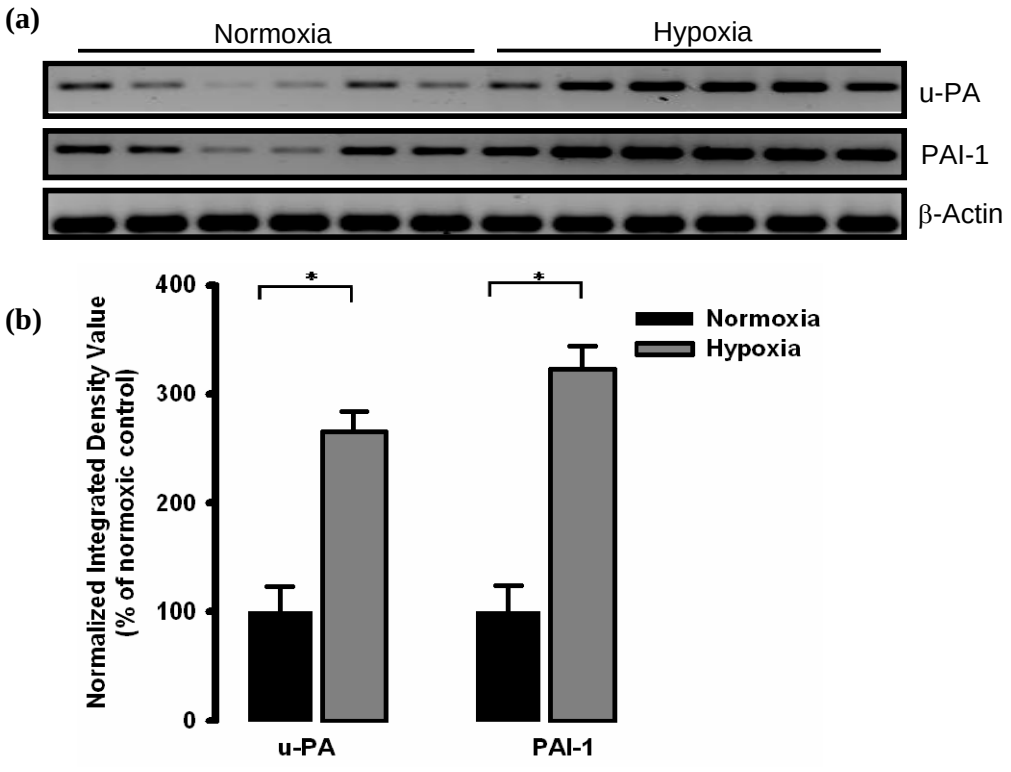


Figure 11. u-PA and PAI-1 gene expression in chronically hypoxic lungs.
Mice were exposed to hypoxia or normoxia (28 days). The lungs from the exposed mice were obtained and homogenized. (a) RT-PCR products of RNA extracted from lung homogenates (n=6). (b) Densitometric analysis of the band intensity. Normalized (against β-actin) IDV of PCR products in hypoxia as percentage of the normalized IDV in normoxia are. Bar represents mean ± SEM. *P<0.05. (Unpaired T-test). The experiments were done twice.

4.2.2 u-PA and PAI-1 protein level under hypoxia

We also checked whether the enhanced u-PA and PAI-1 gene expression would result in correspondingly increased protein levels during hypoxia. Western blot showed an increase in u-PA and PAI-1 protein levels (figure 12a), although not as strong as at transcription level. Densitometric analysis of the blot intensity revealed a mild induction (≈1.5 fold) of u-PA and PAI-1 proteins under hypoxia figure 12b). The mild induction, despite the high level of transcripts, in chronically hypoxic lungs suggests that a regulation likely operates at post-transcriptional or translational level.

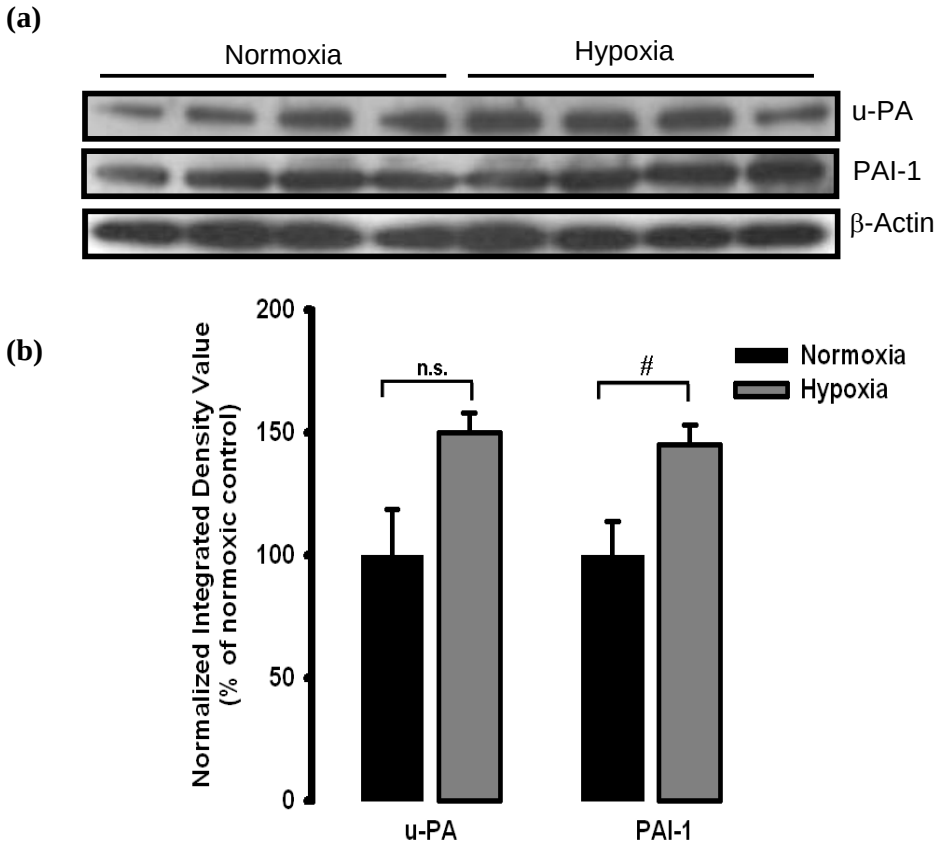


Figure 12. u-PA and PAI-1 protein in chronically hypoxic lungs.

Mice were exposed to hypoxia or normoxia (28 days) and lung samples were obtained. The lungs were homogenized and protein concentration was determined as described in methods.

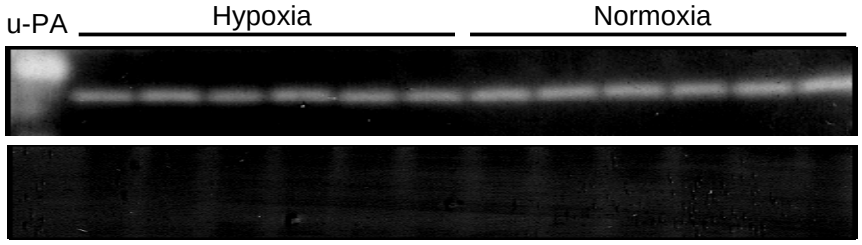
(a) Western blot performed on the lung homogenates (n=4) are shown.

(b) Densitometric analysis of the blot intensity. Normalized (against β-actin) IDV (lower panel) in hypoxia as percentage of normalized IDV in normoxia are given. Bar represents mean ± SEM. $^{\#}$P=0.0493; n.s., non- significant (unpaired T-test). The experiments were done twice.

4.2.3 Influence of hypoxia on lung u-PA activity

We then asked for the consequences of the observed upregulation of u-PA and PAI-1 on u-PA activity. u-PA activity in the lung homogenates was analyzed by casein gel zymography and chromogenic substrate assay. Interestingly, a significant, 30-50% reduction of u-PA activity ($p<0.05$ vs. normoxia) was observed in lung homogenates from hypoxic mice (Figure 13a, 13b, 13c left bar graph). We also checked lung homogenates from chronic hypoxic PAI-1 KO mice for u-PA activity by chromogenic substrate assay. Despite the higher baseline u-PA activity under normoxia, a reduced u-PA activity (~20%) was observed in PAI-1 mice under hypoxia (Figure 13c right bar graph). This suggests that the reduced u-PA activity may be attributable to other factors and not only PAI-1. Overall, the results showed that u-PA was negatively regulated at functional level under hypoxia albeit the induction at transcript and protein level.

(a)



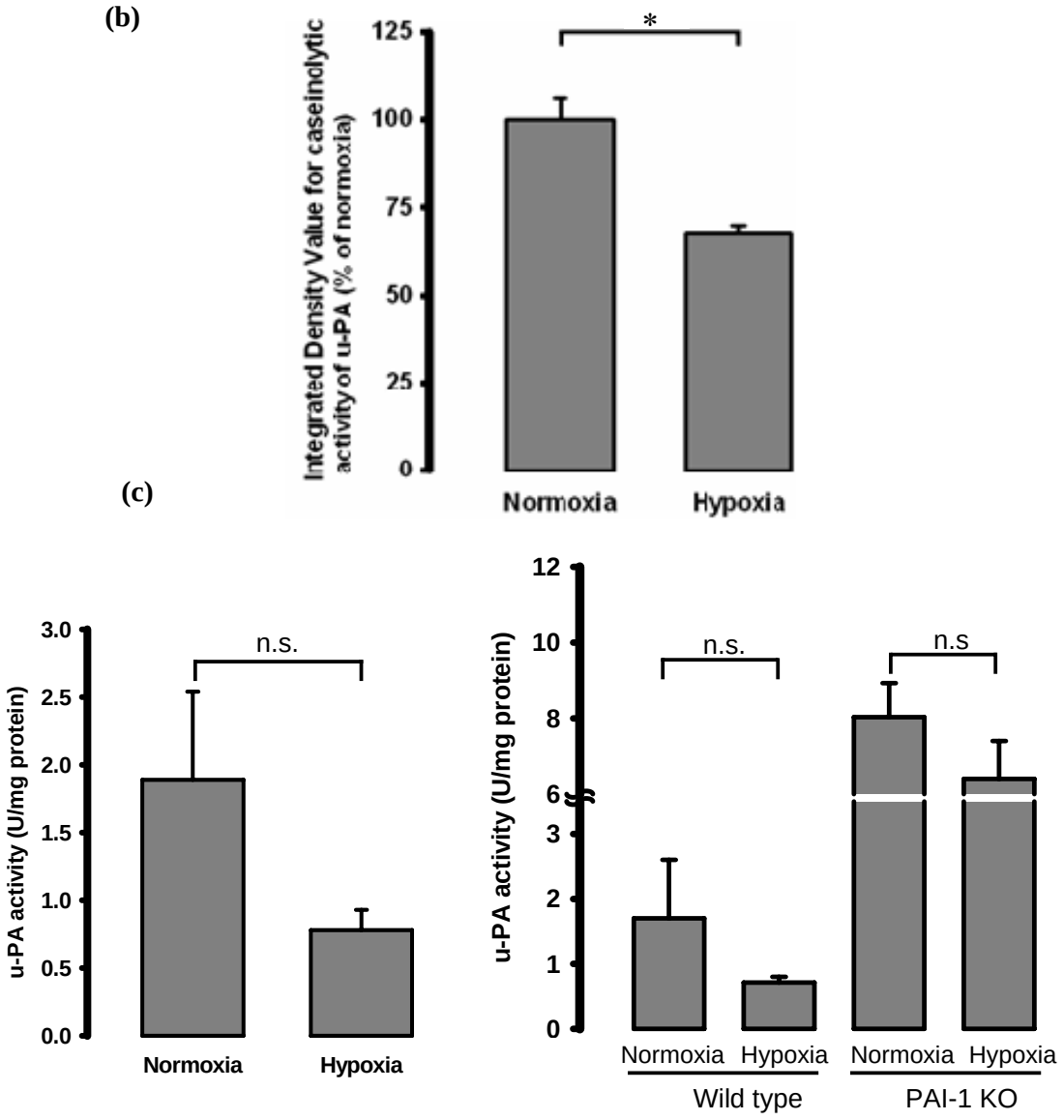


Figure 13. u-PA activity in chronically hypoxic lungs.

The lung homogenates used for RT-PCR and WB were used for casein gel zymography and chromogenic substrate assay (n=6). (a) Casein gel zymographs. The lower gel was incubated with buffer containing CJ463 as described. (b) Densitometric analysis of lytic zones. IDV of u-PA activity in hypoxia as percentage of the IDV in normoxia are given. (c) u-PA activity (Unit/ mg of protein) as measured from chromogenic substrate assay for wild type mice alone (left bar graph) and together with PAI-1 KO mice (right bar graph) are given. Bar represents mean ± SEM. n.s., non-significant (unpaired T-test).

4.2.4 Localization of u-PA and PAI-1 under hypoxia

We were interested to characterize the spatial distribution of u-PA and PAI-1 in the lung tissue to know where at the tissue level the hypoxia-associated modulation of u-PA was taking place. Immunohistochemical analysis of normoxic lungs revealed localization of u-PA mostly in vessels, bronchi and alveolar septae and PAI-1 immunoreactivity in bronchial epithelium, vessels and – to only a minor extent – also in septae. Under hypoxic conditions, u-PA and PAI-1 staining pattern was similar to control but staining intensity appeared increased in the vessel walls (Figure 14). Overall, the vessels, septae and bronchi seemed to be the predominant source of u-PA and PAI-1.

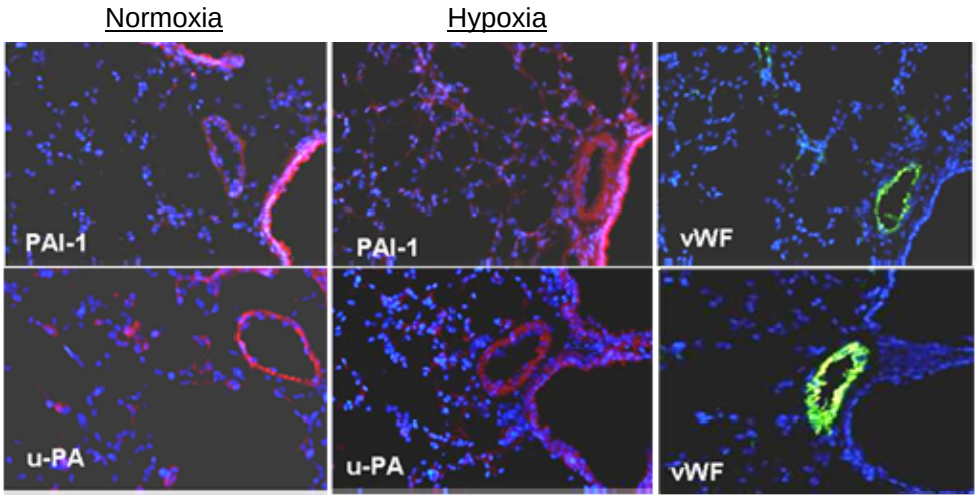
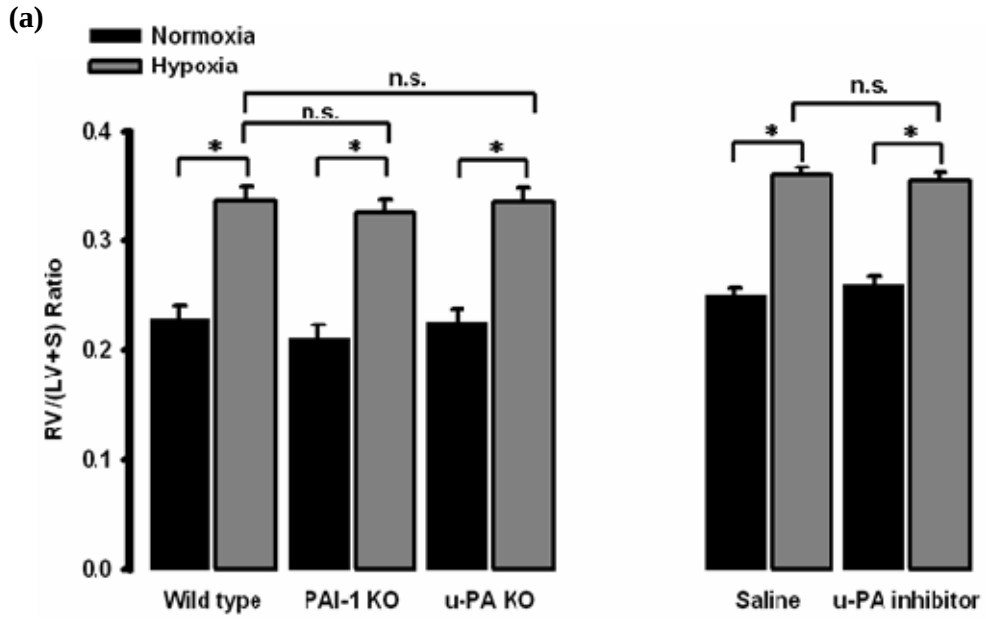


Figure 14. Localization of u-PA and PAI-1 in chronically hypoxic murine lungs.
Left Lungs from the mice exposed to hypoxia or normoxia (28 days) were infused with Tissue-Tek and stored at -80^{o}C. Immunohistochemical staining was performed on cryosections of the lung tissues as described in methods. Representative photomicrograph of lung sections immunostained for u-PA, PAI-1 and vWF. Immunostaining for vWF was done on the serial sections to identify blood vessels, and it also served as control.

4.3 Hypoxia-induced right ventricular hypertrophy (RVH) in wild type, u-PA and PAI-1 deficient mice as well as in u-PA inhibitor (CJ463) and u-PA treated mice

After analyzing the hypoxia-induced changes of u-PA and PAI-1 at molecular level, we employed this animal model to investigate the role of u-PA in pulmonary vascular remodeling. A comprehensive approach was applied. u-PA and u-PA inhibitor treated as well as u-PA and PAI-1 knockout mice were investigated.

Exposure of wild type mice to 28 days of hypoxia (10%) did result in pulmonary vascular remodeling, indirectly proved by a significant increase in the right ventricular to left ventricular plus septum [RV/(LV+S)] ratio (figure 15a) as well as in the right ventricular systolic pressure (RVSP) (Figure 15b). Surprisingly, exposure of u-PA and PAI-1 knockout mice to hypoxia did not affect the hypoxia-induced RV/(LV+S) ratio and RVSP, suggesting that the absence of either u-PA or PAI-1 did not interfere with the magnitude of right ventricular hypertrophy (figure 15a, 15b). Similarly, daily treatment with the highly specific urokinase inhibitor CJ463 by intraperitoneal injection did not exert any influence on the natural course of right ventricular hypertrophy. This was evident by the significant increase in RV/(LV+S) ratio and RVSP under hypoxia (figure 15a, 15b) and suggests that loss of u-PA activity did not impair the chronic hypoxia-induced pulmonary vascular remodeling. In addition to the above mentioned marker of right heart hypertrophy i.e. [RV/(LV+S)], we also analyzed the left ventricle plus septum weights alone to ensure that they had no influence on the observed [RV/(LV+S)] quotient. We did not observe any significant difference in the weight of the left ventricle plus septum between normoxic and hypoxic mice, thus excluding any left ventricular reason for the change in [RV/(LV+S)] quotient as noted above (Table 5). These results suggest that neither inhibition of u-PA nor absence of u-PA or PAI-1 did affect development of pulmonary vascular remodeling.



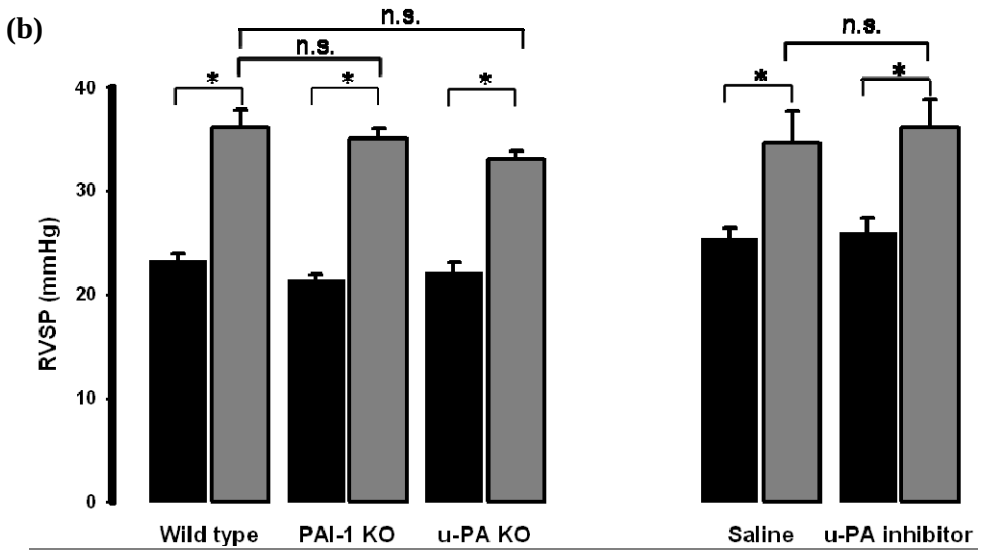


Figure 15. RV/(LV+S) and RVSP of wt, u-PA and PAI-1 KO mice as well as in CJ463 treated mice. Wild type (n=8), u-PA knockout (n=8) and PAI-1 knockout mice (n=5) were exposed to hypoxia or normoxia (28 days). Wild type mice (n=8) in the treatment groups received either saline or CJ463 throughout the hypoxic or normoxic exposure. Intraperitoneal injection of 100 µl of saline or CJ463 (20 mg/kg BW) was given twice a day. Hemodynamic and right heart hypertrophy measurements were performed as described in methods. (a) RV/(LV+S) ratio and (b) RVSP are given. Bar represents mean ± SEM. *P<0.05; n.s.- non-significant; KO- knockout. Experiment with knock out mice (u-PA and PAI-1) was done twice.

Table 7. Hematocrit, BW and LV+S of mice under hypoxia or normoxia (28 days).

	Hematocrit (%)	BW (g)	LV+S (mg)
Normoxia			
Wild type	36 ± 0.9	25.1 ± 0.8	20.2 ± 0.5
PAI-1 (-/-)	32 ± 0.5	29.1 ± 1.9	23.8 ± 1.7
uPA (-/-)	35 ± 1.2	23.1 ± 1.3	21.4 ± 1.5
Saline WT (i.p.)	33 ± 0.5	25.8 ± 0.4	19.4 ± 0.5
Saline WT (Inf.)	36 ± 2.1	27.5 ± 0.5	22.5 ± 0.8
u-PA inhibitor (i.p.)	32 ± 0.8	24.6 ± 0.7	18.5 ± 0.5
u-PA (Inf.)	36 ± 1.2	27.9 ± 0.4	22.3 ± 0.8
Hypoxia			
Wild type (WT)	57 ± 0.7	19.5 ± 0.8	20.0 ± 0.3
PAI-1 (-/-)	54 ± 1.2	23.7 ± 0.9	22.3 ± 1.1
uPA (-/-)	59 ± 0.8	22.2 ± 1.0	21.1 ± 1.0
Saline WT (i.p.)	56 ± 1.2	20.0 ± 0.3	18.7 ± 0.9
Saline WT (Inf.)	57 ± 1.2	22.5 ± 0.6	20.8 ± 0.6
u-PA inhibitor (i.p.)	56 ± 0.9	19.7 ± 0.2	18.3 ± 1.2
u-PA (Inf.)	59 ± 0.6	22.9 ± 0.5	22.4 ± 0.7

Values are mean ± SEM, WT-wild type, i.p.- Intraperitoneal, Inf- Continuous intravenous infusion through Osmotic mini pump, BW-body weight, (-/-) - knockout

We genotyped the mice used in the experiment to ensure that the gene was absent in the knockout mice (figure 16a, 16b). In addition, pilot experiments were conducted to confirm that the u-PA inhibitor was effective in our experimental set up. As expected, we could verify that by such application mode of CJ463 plasmatic u-PA activity would be greatly reduced **(**figure 17). In addition, application of the same dose as used herein was recently found to suppress primary tumor growth in a heterotopic Lewis lung carcinoma model [Ruppert et al, personal communication].

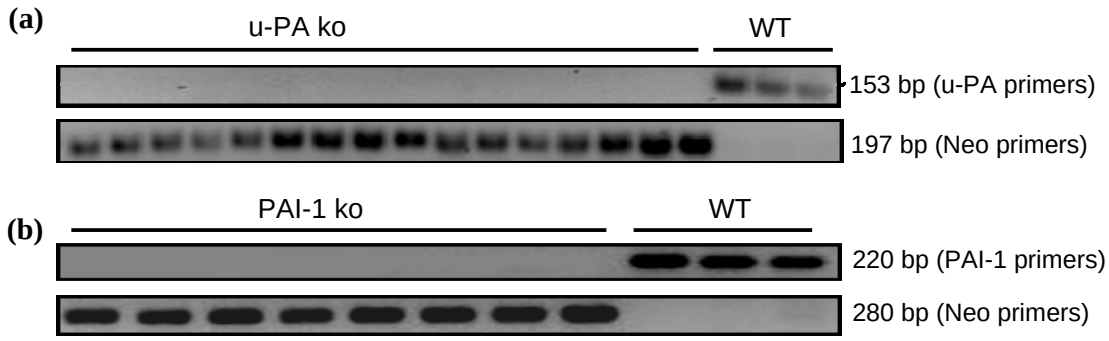


Figure 16. PCR genotyping of u-PA and PAI-1 knockout mice.
(a). PCR products obtained using u-PA and Neo-specific primers and (b). PCR products obtained using PAI-1 and Neo-specific primers are shown. bp- base pair, ko-knockout, WT-wild type.

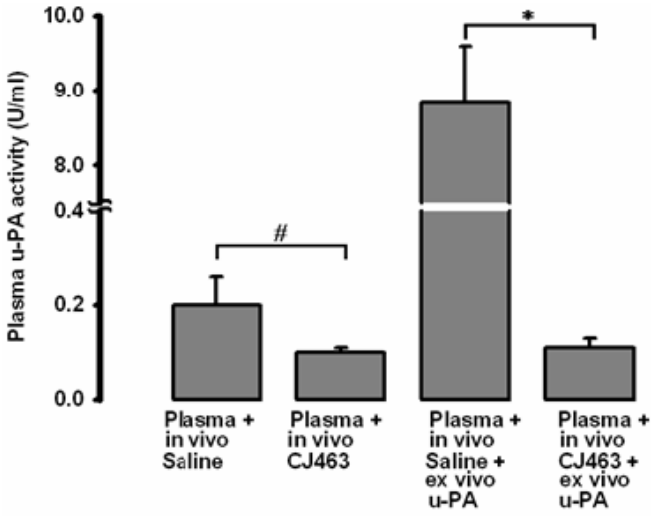
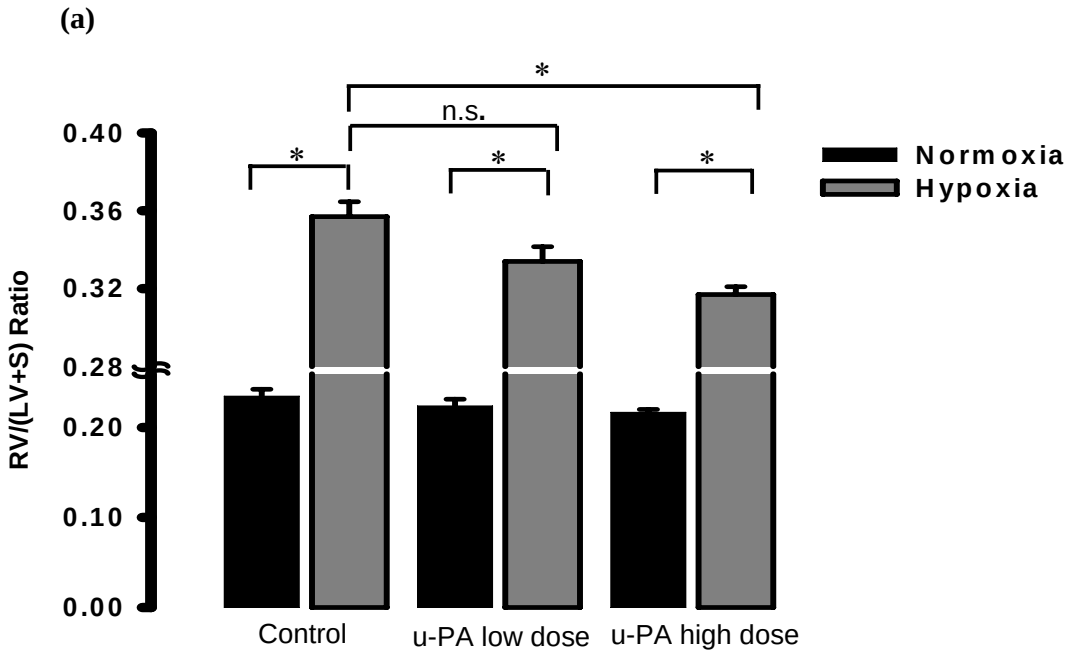


Figure 17. u-PA activity in plasma upon u-PA inhibitor (CJ463) treatment.
Mice were given intraperitoneal injection of CJ463 and saline as described. Plasma samples from CJ463 or saline treated mice, with and without adding u-PA ex vivo, were analyzed for u-PA activity using chromogenic substrate as described. Plasma u-PA activity (U/ml) is given. Bar represents mean ± SEM. *P=0.0003 and #P=0.12 (T-test) (n=3).

After investigating the effect of modulating endogenous u-PA on the chronic hypoxia-induced right ventricular hypertrophy, we sought to study if application of exogenous u-PA will have any effect. We administered u-PA as a continuous infusion by implanting osmotic mini pump (OMP) subcutaneously into the mice as described in methods. In contrast to those observations on u-PA inhibitor treated and knockout mice, chronic infusion of u-PA via OMP turned out to have a mild and dose-dependent, beneficial effect on the extent of right heart hypertrophy (figure 18a, 18b). We found an increase in plasmatic urokinase activity in pilot experiments 1 week after exogenous urokinase application via OMP. In addition, long-term stability of the u-PA preparation had been investigated in advance and was shown to be, on average, 50% at d14 and 20% at d28 of the initially provided activity upon incubation at 37°C in vitro. Accordingly, an elevation of u-PA activity could be observed at d28 especially in the high dose as compared to saline treated mice (Figure 19).



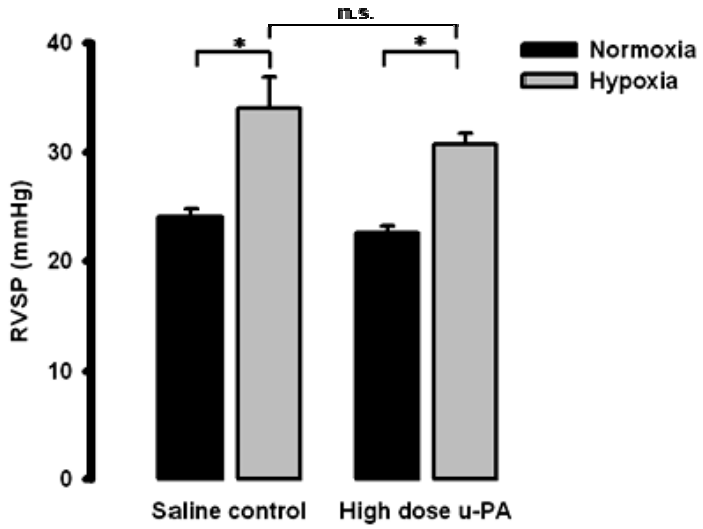


Figure 18. RV/ LV + S and RVSP of chronically hypoxic mice receiving continuous u-PA infusion.

Mice (n=8) were given infusion throughout their exposure to hypoxia or normoxia (28 days). Surgical implantation of Osmotic mini-pump (OMP) was performed for u-PA and saline infusion, and hemodynamic as well as right ventricular hypertrophy measurements were done as described in methods. (a) RV/(LV+S) and (b) RVSP are given. The lower dose group RVSP was similar to the saline control and is not shown. Bar represents mean ± SEM. *P<0.05; n.s.-non-significant.

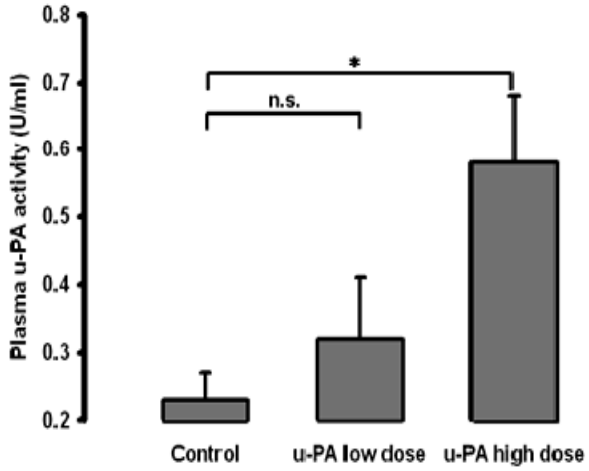


Figure 19. Plasma u-PA activity of mice receiving continuous u-PA infusion.

Mice received u-PA infusion through surgically implanted Osmotic mini-pump (OMP) throughout their exposure to normoxia/hypoxia (28 days). Control mice received saline. u-PA activity was measured at day 28 by chromogenic substrate as described. Representative plasma u-PA activity (U/ml) is given. Bar represents mean ± SEM. *P<0.05 (n=5-6); n.s.-non-significant (unpaired T-test).

4.4 Hypoxia-induced muscularization in wild type, u-PA and PAI-1 deficient mice as well as in CJ463 and u-PA treated mice

We then investigated the degree of chronic hypoxia-induced pulmonary vascular remodeling under various conditions as used for right ventricular hypertrophy. As anticipated, all hypoxic groups, irrespective of treatment or genotypes, had enhanced immunoreactivity for α-SMC actin at day 28, suggesting an increased muscularization of pulmonary arteries (figure 20). Staining intensity seemed to be somewhat less pronounced in the experimental group receiving high dose u-PA infusion. Again not surprisingly, the ratio of non-muscularized vs. muscularized (NM/M quotient) vessels was greatly depressed in wild type mice in response to chronic hypoxia (figure 21). In full accordance with the unaffected extent of right heart hypertrophy in the u-PA and PAI-1 knockout and the CJ463 treated mice, this NM/M quotient also turned out to be unaffected in these groups. In contrast, chronic infusion of the high dose of u-PA resulted in a significant increase of the NM/M quotient (Figure 21). These results suggested that hypoxia-induced pulmonary arterial muscularization was not impaired either by inhibition of u-PA activity or in the absence of u-PA or PAI-1, but was significantly depressed in the mice receiving a high dose of u-PA.

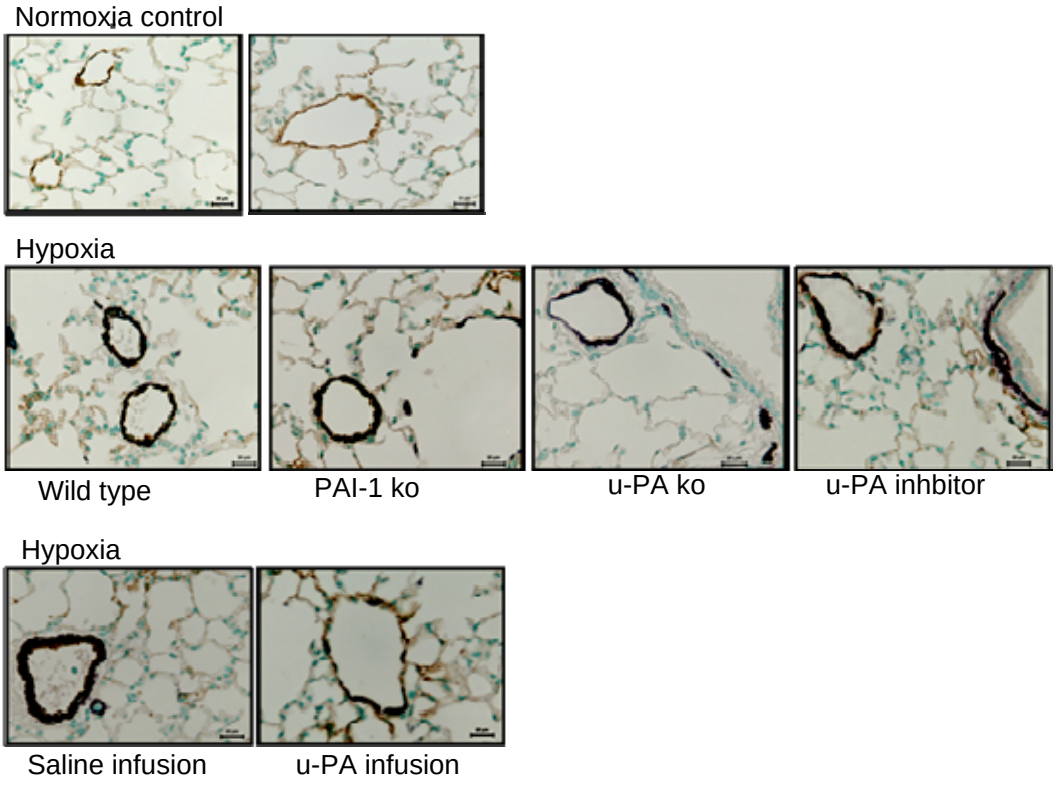


Figure 20. Immunohistochemical analysis of pulmonary vessel muscularization in wild type, u-PA and PAI-1 ko as well as CJ463 and u-PA treated mice.

Lung sections of the mice exposed to hypoxia or normoxia (28 days) were immunostained for α-SMC actin (purple, arrow) and vWF (brown, arrow head) to identify muscular and endothelial layers of vessels respectively. Representative photomicrographs of lung sections are shown. (Scale bar=20 μm, x 40 magnification), ko -knockout.

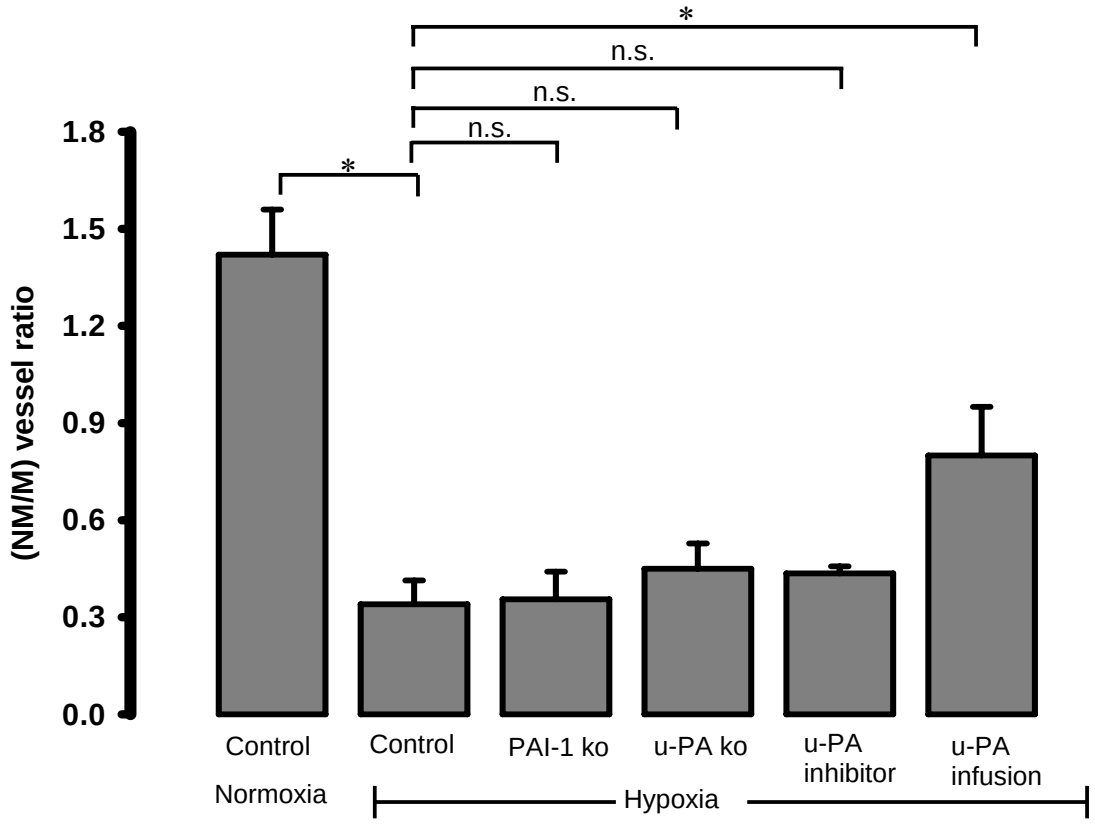


Figure 21. Morphometric analysis of pulmonary vessel in wild type, u-PA and PAI-1 knockout as well as CJ463 and u-PA treated mice.

The immunostained lung sections (for α-SMC actin and vWF) were subject to morphometric analysis as described in methods. Non-muscular (NM) to muscular (M) vessel ratios (NM/M) are given. Bar represents mean ± SEM. *P<0.05; n.s.- non-significant. There was no significant difference among the normoxic control groups independent of treatments or genotypes, and among the hypoxic wild type and saline control groups (n = 4-6).

4.5 Attenuation of hypoxia-induced loss of pulmonary vessels upon u-PA infusion

As mentioned, chronic hypoxia leads to vascular rarefaction. We sought to investigate if u-PA infusion has any influence on the chronic hypoxia-induced changes in pulmonary vascular density. Analysis of distal pulmonary arteries revealed a significant reduction in number of arteries per 100 alveoli in hypoxic control mice (1.1±0.13) compared to normoxic control (2.07±0.3) ($P< 0.05$) (figure 22), indicating hypoxia-induced loss of peripheral pulmonary arteries. Interestingly, no significant reduction in arteries was found in hypoxic mice receiving u-PA infusion (1.88±0.19) ($P> 0.05$ vs. normoxic control) (Figure 22). The result suggests a beneficial effect of u-PA infusion in preserving peripheral pulmonary arteries under hypoxia.

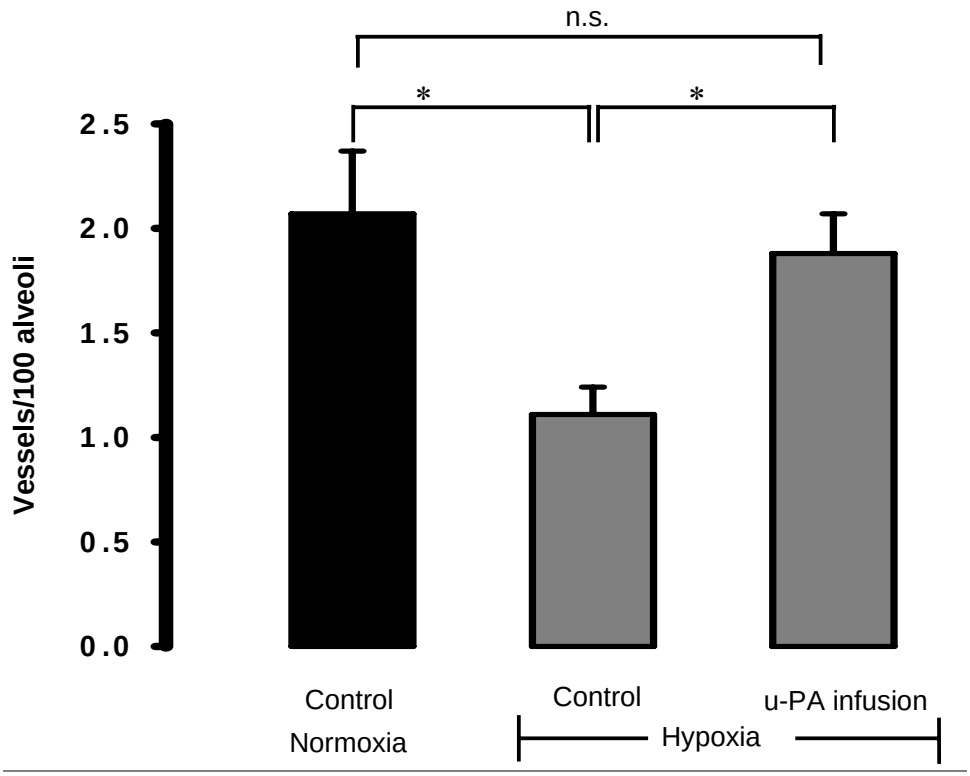


Figure 22. Attenuation of loss of pulmonary vessels in hypoxic mice upon u-PA infusion.

Mice receiving saline or u-PA infusion through OMP were exposed to hypoxia or normoxia (28 days). Immunostaining of lung sections and counting of pulmonary arteries and alveoli were performed as described in Methods. Arteries/100 alveoli are given. Bar represents mean ± SEM. *$P<0.05$; n.s.- non-significant (n=5).

5 DISCUSSION

In the present study, we firstly analyzed u-PA and PAI-1 expression, and u-PA activity in lung homogenates from patients with different forms of pulmonary hypertension (IPAH, CTEPH) as well as from mice exposed to chronic hypoxia. The expression and activity pattern in hypoxic murine lungs was not identical to that observed in IPAH and CTEPH patients. Secondly, by employing a mouse model of chronic hypoxia-induced pulmonary hypertension, we demonstrated that neither the inhibition or absence of u-PA nor the absence of PAI-1 exerted a major effect on the course of pulmonary vascular remodeling and RVH under hypoxia. Somewhat contradictory, we could observe a beneficial role of a permanent u-PA infusion on pulmonary vascular remodeling in chronically hypoxic mice. In view of our results, the following aspects need to be discussed:

5.1 Regulation of u-PA and PAI-1 in the lungs of patients with pulmonary hypertension and in murine lungs in response to chronic hypoxia

5.1.1 u-PA and PAI-1 in the lungs from patients with IPAH and CTEPH

In our study, expression analysis clearly revealed an induction of u-PA and PAI-1 at transcript and protein level in IPAH and CTEPH versus donor lungs, except that in CTEPH lungs, PAI-1 transcript was comparable to donor lungs. In agreement with the enhanced u-PA and PAI-1 protein, an elevated level of u-PA-PAI-1 complex was also detected in lungs of PH patients. Moreover, analysis of plasminogen activator activity showed enhanced u-PA activity in the lung homogenates from patients, both in CTEPH and IPAH, despite the increased PAI-1 expression on protein level. Immunohistochemical analysis revealed an enhanced immunoreactivity for u-PA and PAI-1 and forwarded a differential distribution pattern of u-PA and PAI-1 under conditions of pulmonary hypertension as compared to donor lungs. In detail, a more pronounced staining for u-PA was observed in the interstitial and alveolar compartments as compared to the vascular compartment, where it was largely confined to the endothelium. In contrast, PAI-1 tended to localize more in the vascular compartment and, in here, in the media of the vessels. These results will be discussed in separate sections followed by a summary.

PAI-1 as well as u-PA expression in the lungs from IPAH patients

Our findings with regard to PAI-1 are in line with previous clinical studies, in which higher plasma PAI-1 antigen as well as activity levels were detected in patients with IPAH[47, 136, 137, 175]. Moreover, Hoeper and collaborators found considerably higher arterial PAI-1 than mixed venous PAI-1 level in PPH patients[47], suggesting the lung to be a major source of PAI-1 under these conditions. This, in turn, strongly supports our observation that PAI-1 protein is induced in the lungs of patients with IPAH. In addition, alterations of the fibrinolytic system in patients with pulmonary hypertension were characterized in other studies by quantifying t-PA and PAI-1 in plasma. Despite a significant gender-based difference not only of t-PA and PAI-1 but also of u-PA in normal human plasma[176], no difference in plasma levels of t-PA antigen and activity was detected between PPH patients and healthy control individuals. In this patient category, however, performance of a peripheral venous occlusion test resulted in a weaker increase in mean circulating t-PA activity as compared to control subjects[138]. The authors reasoned that an impaired fibrinolytic capacity in PAH patients may underlie this finding. Other authors have ascribed such impairment of the fibrinolytic system to the elevated concentration of PAI-1[137]. With regard to u-PA in IPAH patients, we observed an upregulated transcript and protein level in the lung homogenates. Moreover, u-PA has yet not been extensively studied under such clinical conditions.

PAI-1 and u-PA expression in the lungs from CTEPH patients

A higher basal antigen but similar activity level of t-PA and PAI-1 was detected in plasma from CTEPH patients[44], whereas an unaltered endothelial fibrinolytic potential was observed in thrombus free area of pulmonary arteries from CTEPH patients[177] as compared to the donors. Taking together, these findings did not suggest any alteration in net fibrinolytic potential in patients with CTEPH. However, we observed an enhanced PAI-1 protein level in the lung homogenates of CTEPH patients similar to IPAH patients. Our result is in agreement with the findings of a clinical study by Huber and collaborators, who found a comparable prothrombotic situation in patients with primary pulmonary hypertenstion as well as in patients with CTEPH[138]. Likewise our results are supported by the findings of elevated PAI-1 expression in pulmonary artery of patients with pulmonary thromboembolism, as demonstrated by immunohistochemical analysis[178].

Surprisingly enough, PAI-1 transcripts in CTEPH lungs was comparable to donor lungs and did not correlate with the protein level in our study. This suggests that PAI-1 might be differentially regulated at translational/ posttranslational levels under conditions of pulmonary hypertension. Such differential regulation might involve stabilization of PAI-1 by vitronectin[179], induction of PAI-1 by u-PA in epithelial cells[180] and the ability of PAI-1 to transform into other immunologically detectible conformations[181, 182], leading to the higher PAI-1 protein level in the diseased lungs. Alternatively, the enhanced pulmonary PAI-1 protein level in CTEPH could be explained by remote production in alternate tissues such as liver[183, 184] and adipose tissue[185] and accumulation in the pulmonary circulation.

With regard to u-PA in CTEPH patients, we observed an upregulation in both transcript and protein level in the lung homogenates. Our results did agree with Lang and collaborators, who demonstrated an elevated expression of u-PA by immunohistochemical and in situ hybridization analysis of pulmonary artery specimens from patients with pulmonary thromboembolism[178]. However, u-PA has not been extensively studied in the lungs from patients with CTEPH in such clinical studies.

Taken together, we observed enhanced protein and activity of u-PA in lung homogenates from IPAH and CTEPH patients as compared to donor lungs. At a quick glance our findings appear contradictory to these previous reports[47, 133, 137, 139] which suggested reduced plasminogen activator activity due to enhanced anti-fibrinolytic activity. However, difference in sampling (lung homogenate versus plasma) may well underlie such differences. In this regard, changes in the peripheral circulation may not adequately reflect local changes within the pulmonary circulation, as has been suggested previously. Even within the pulmonary circulation, differences in view of plasminogen activator regulation have been reported[186]. Likewise, we have to consider that analysis of u-PA and PAI-1 expression and activity in lung homogenates does not provide detailed information on the compartment- or cell-specific distribution within the lung. Modulation of coagulation system in cell- and compartment-specific manner has been demonstrated in vivo in endotoxin-challenged murine lungs[187].

In this line, we also observed different spatial distribution of u-PA and PAI-1 in the lungs from patients with IPAH and CTEPH. PAI-1 tended to be localized more in endothelium of the vascular compartment, whereas u-PA appeared to be preferatially distributed in interstitial and alveolar compartment.This indicates a disparate distribution of u-PA and PAI-1 protein in CTEPH and IPAH versus donor lungs. Our results thus suggest that the enhanced plasminogen activator activity observed in lung homogenates from IPAH and CTEPH patients could originate from the interstitial and alveolar compartments. Increased turnover of u-PA and PAI-1, and higher plasminogen activator activity have been further supported by our observation that complex of u-PA and PAI-1, in addition to free u-PA, was also detectable in the diseased lungs. Recently, Hoeper and coworkers have suggested that the molecular mechanisms involved in pulmonary vascular remodeling in CTEPH appear to be similar to those seen in severe pulmonary hypertension of other etiology[45]. In line with this concept, the similar alteration in u-PA and PAI-1 in both disease categories as observed in this study suggest that the components of the fibrinolytic system might be involved in a similar fashion in the disease processes, both of IPAH and CTEPH.

5.1.2 u-PA and PAI-1 in the murine lungs in response to chronic hypoxia

On the experimental level, we observed an enhanced u-PA and PAI-1 gene expression in murine lungs exposed to chronic hypoxia and this corresponded with an enhanced protein level. Interestingly, casein gel zymography and chromogenic substrate assay on the same lung homogenates showed a reduction in u-PA activity under hypoxia. Immunohistochemical analysis revealed that u-PA and PAI-1 were mostly localized in vessels, bronchi and alveolar septae. Under hypoxic conditions, u-PA and PAI-1 staining pattern was similar to control but staining intensity appeared to be increased in the vessel walls.

Previously, an enhanced PAI-1 expression was reported in the lungs of mice exposed to short-term hypoxia[169], and we could expand this observation in the current study where we found persistently elevated PAI-1 levels in chronically hypoxic murine lungs. Taking together, this suggests that an induction of PAI-1 gene persists throughout the hypoxic exposure, probably driven by hypoxia responsive transcription factors such as hypoxia inducible factor-1α (HIF-1α), early response gene-1 (Egr-1) and CCAAT/enhancer binding protein α (C/EBPα)[188-190].

With regard to u-PA expression, a downregulation of u-PA was demonstrated in the lungs from mice under short-term hypoxic exposure[169]. However, we found an induction of u-PA gene expression in murine lungs kept under hypoxia for 28 days, indicating a possible difference in u-PA regulation dependent on the duration of hypoxic exposure.

Protein levels of u-PA and PAI-1 were increased under hypoxia, but these changes were not as significant as compared to the induction at mRNA level. Such discrepancy may be attributable to posttranscriptional/ translational regulation. In this regard, the co-localization of u-PA and PAI-1 as observed in this murine study, in concert with known upregulation of urokinase receptor (uPAR) under hypoxia[191, 192], might result in rapid degradation of u-PA complexed to its inhibitor upon internalization into the cells via uPAR[193]. Despite the slightly increased u-PA protein levels we observed a reduced u-PA activity in chronically hypoxic murine lungs as compared to normoxic control lungs by means of casein gel zymography, and this was further confirmed by chromogenic substrate assay for plasminogen activator activity. Thus, our data suggest a shift towards reduced u-PA activity under chronic hypoxia and hence a suppressed fibrinolytic potential in hypoxic lungs.

Overall, the expression of u-PA and PAI-1 at transcript and protein level in the lungs from chronic hypoxia-exposed mice showed some similarities to that in IPAH and CTEPH lungs. PAI-1 as well as u-PA was observed to be induced both in the experimental and clinical lung samples. Strikingly, the u-PA activity in the lung homogenates from the patients with pulmonary hypertension was clearly higher compared to that from hypoxic mice, which may- in part- be related to the differential expression pattern of u-PA in the human CTEPH/ IPAH versus the chronically hypoxic murine lungs. In addition, a complex heterogeneity of etiological/environmental factors is involved in development of pulmonary arterial hypertension (PAH), with hypoxia being just one environmental factor associated with an increased risk of the development of PAH[122, 194]. Unlike in clinical PAH, in the experiemental model of chronic hypoxia relatively mild vascular remodeling is induced that is also reversible upon return to normoxia[68]. Other models such as transgenic mouse[75] or monocrotaline-injected rat[57, 195] models of PH have been suggested to more closely represent the histopathology and molecular changes observed in PAH.

Although monocrotaline injection alone does not produce a severe vascular remodeling as observed in PAH, pneumonectomized rats that receive monocrotaline do develop severe pulmonary hypertension with neointimal formation[19]. Recently, a rat model of severe pulmonary hypertension, characterized by occlusion of precapillary pulmonary artery lumen by endothelial cell proliferation, upon inhibition of VEGF receptor 2 and chronic hypoxic exposure has been reported[71]. Moreover, another rat model of severe PAH, characterized by the development of occlusive neointimal lesion in distal pulmonary ateries, has also been described[64]. Hence, the experimental model itself may- in part- explain the observed differences in view of regulation of fibrinolysis between chronic hypoxic murine lungs and patients with IPAH/CTEPH. Unfortunately, we were not able to establish a murine model of monocrotaline-based PH and there is no other murine model of severe PH available so far.

5.2 Development of hypoxia-induced pulmonary vascular remodeling and RVH in wild type, u-PA and PAI-1 knockout and u-PA inhibitor (CJ463) treated mice

Despite being upregulated on mRNA level, u-PA activity in lung tissue was found to be downregulated under conditions of chronic hypoxia for 28 days. A further reduction of u-PA activity by means of a u-PA specific low molecular weight inhibitor (CJ463) or complete absence of u-PA in case of the u-PA knockout mice did not result in any difference in RVH under these conditions. Accordingly, we could also not observe any change in the muscularization pattern of the pulmonary arteries in response to hypoxia in these three groups. Together, although being differentially regulated complete suppression of the endogenous u-PA activity seem not to result in any difference in the development of pulmonary vascular remodeling and RVH in the currently applied model of chronic hypoxia. As evident from the analysis of u-PA activity in wt and PAI-1 knockout mice, the net u-PA activity of lung tissues ranged between absence of u-PA activity (u-PA ko), ~2 (wt) and ~8 (PAI-1 ko mice) U/mg protein and our data would imply that there is no modulation of RVH by any mechanism conceivable within this range of u-PA activity.

Our data are in sharp contrast to those of Levi and collaborators[170] who observed a clearly less pronounced extent of pulmonary vascular remodeling and right ventricular hypertrophy in u-PA knockout mice exposed to chronic hypoxia.

On the basis of their results, these authors suggested that u-PA based plasmin generation plays a detrimental role in pulmonary vascular remodeling by enhancing and facilitating smooth muscle cell proliferation and migration. At a quick glance, the discrepancy between their and our data is not easily explainable. However, Levi and coworkers reported values of RV/(LV+S) for normoxic (≈ 0.3) and hypoxic (>0.5) wild type mice that are tremendously higher than any previously reported value in this model from our[173] or other groups[146] and, as such, raise questions as to the correctness of the method they applied. Usually, such high values of RV/(LV+S) are not encountered in murine models of hypoxia-induced pulmonary hypertension, rather they are observed in animal models with a much severe form of pulmonary vascular remodeling such as in the model of monocrotaline-induced pulmonary hypertension[143, 173]. In addition, there are clear methodological differences in view of the pressure used for lung fixation, size and number of the vessels examined, and method of analysis. For fixation of the lungs the authors applied very high pressures of 30 and 100cm H_2O via trachea and right atrium respectively. Verhoeffs-van Gieson elastica stain was used to categorize the vessels into different degree of muscularization regardless of defined vessels size. On the other hand, we stained for von Willebrand′s factor and α-smooth muscle cell (SMC) actin to perform computer based morphometric analysis as reported by our group[173, 196] and others[146]. Importantly, we analyzed smaller size (within defined range), and considerably large number of vessels. Finally, by analyzing the efficacy of an u-PA inhibitor at a dose that has recently been shown to retard growth of ectopic Lewis Lung Carcinoma tumors due to inhibition of angiogenesis (Ruppert et.al., unpublished observations) we added a second experimental group of u-PA downregulation that forwarded similar results as compared to the u-PA knock out mice.

Our data from u-PA knockout and u-PA inhibitor treated mice suggested possibilities for redundant mechanisms mediating (mal)adaptive response of pulmonary vasculature to chronic hypoxia, unlike the proposed mechanism based on plasmin-induced activation of MMPs[197]. Indeed, there are reports proving plasmin-independent activation of MMP system. Lijnen and collaborators, by using gene-deficient mice, demonstrated that in vivo activation of proMMP-2 occurred independently of plasmin-(ogen), and activation of proMMP-9 might occur via plasmin-dependent or plasmin-independent (MMP-mediated) mechanism[198].

In addition, hypoxia-derived radicals could also activate MMPs[199, 200], followed by MMP-induced proteolysis leading to growth factor release, cell proliferation and migration[201, 202] and finally, to vascular remodeling. Moreover, MMP expression may be enhanced by an endogenous vascular elastase induced upon hypoxic exposure through release of degraded matrix peptides[147, 148] or by inhibition of tissue inhibitors of MMPs[149] and hence, may lead to vascular alteration.

Rregardless of mechanism yet to be elucidated, a vascular serine elastase distinct from u-PA has been demonstrated to be involved in chronic hypoxia-induced pulmonary vascular remodeling[144, 146]. It was found that hypoxia- as well as monocrotaline-induced pulmonary vascular remodeling was abrogated upon inhibition of this serine elastase activity[143, 146]. After 12 hours of hypoxic exposure, a transient increase in serine elastase activity was documented in murine lungs[146]. In this regard, the procoagulant environment in pulmonary vasculature in response to hypoxia might be followed by the induction of serine elastase, suggesting a possible role for early hypoxia-induced haemostatic imbalance in facilitating serine elastase-mediated pulmonary vascular alterations. The predominantly procoagulant milieu within vasculature leading to enhanced thrombin generation could favour transmigration leakage of serum factors through the leaky endothelial barrier[203] and thus promote structural remodeling[146].

Plasminogen activator inhibitor (PAI-1) has been shown to inhibit apoptosis and to augment the proliferation of vascular smooth muscle cells (VSMCs)[167, 204 165] in addition to promoting cellular migration[166]. This suggests a detrimental role for PAI-1 in vascular remodeling. Indeed, absence or presence of PAI-1 has been shown to exert a deep influence on neointima formation in animal models of arteriosclerosis, however, with contradictory findings[151, 152, 205, 206]. Our data are also somewhat contradictory to these recent reports on the contribution of PAI-1 to neointima formation in models of arteriosclerosis. However, it has to be kept in mind that the currently employed model of hypoxia-induced pulmonary vascular remodeling is not associated with neo-intimal formation. It is rather associated with media thickening and muscularization of previously not muscularized vessels. To this end, our currently applied model is not directly comparable those models as reference above.

Moreover, PAI-1 has not yet been directly investigated in chronic hypoxia-induced pulmonary vascular remodeling. This study, to our knowledge, is thus the first to investigate the PAI-1 knockout mice in a murine model of hypoxia-mediated pulmonary vascular remodeling.

5.3 Attenuation of hypoxia-induced pulmonary vascular remodeling and RVH in u-PA treated mice

Interestingly, we observed a significant attenuation in chronic hypoxia-induced RVH and muscularization of distal pulmonary arteries upon continuous u-PA infusion. Several explanations for this efficacy of exogenous u-PA application in the context of missing modulation by using u-PA and PAI-1 knockout mice are to be discussed.

1. Efficient dissolution of intravascular fibrin and thus avoidance of thrombosis.

An altered hemostasis, with predominant procoagulant and anti-fibrinolytic potential of pulmonary vasculature favoring in situ thrombosis, has been reported in humans with PAH [133]. This procoagulant milieu favours disease progression. In an animal model of hypoxia-induced pulmonary hypertension, the pulmonary vasculature contributes to a procoagulant milieu due to induction of tissue factor (TF) as well as PAI-1[207, 208] and thus, the hypoxic vasculature has higher tendency towards intravascular fibrin deposition and thrombosis[166,209]. The efficient dissolution of intravascular fibrin and thus avoidance of thrombosis by continuous infusion of u-PA might partially explain the beneficial effect observed in the current study. However, we do not have direct evidence in this regard and there is still uncertainity also in the literature about the true site and extent of intravascular clotting processes in this model.

2. Alteration in neointima formation and SMC characteristics

Alterations in vascular smooth muscle cell (VSMC) characteristics may play a key role in the vascular remodeling process such as medial thickening. Enhanced proliferation and migration, and reduced apoptosis are such features associated with altered VSMC phenotype.

Interestingly, studies investigating influence of u-PA[161-164] and PAI-1[165-168] on VSMC characteristics yielded quite paradoxical findings in view of their role in VSMC proliferation and migration. In this regard, the dual role of urokinase receptor system by virtue of its ability to interact not only with u-PA but also with vitronectin and the ability of PAI-1 to modulate these interactions might provide some explanation for the contradictory findings on cell migration[100, 210, 211]. Furthermore, such paradoxical observations were also made in studies involving experimental animal models. Depending on the animal model of arteriosclerosis being used, both u-PA[150, 153, 157] as well as PAI-1[151, 152,158, 159] were found to promote neointima formation, suggesting their role in VSMC proliferation and migration. The underlying reasons for these contradictory findings are yet not settled. At best, the data suggest that depending on the presence or absence of u-PA or PAI-1, model- or compartment-specific factors may influence VSMC proliferation and migration leading to neointima formation. In the context of the herein presented study, however, exogenous application of excess u-PA might represent a very dominant signal and induce excessive proteolytic activity by plasminogen activation dependent proteases, namely plasmin and MMPs. It could be speculated that excessive proteolytic activities may have resulted in increased apoptosis of VSMC, as suggested in recent reports [212-217]. However, we could not directly investigate the influence of u-PA infusion on VSMC apoptosis.

3. Liberation of protective growth factors

In the rat model of monocrotaline-induced pulmonary hypertension, supplementation with hepatocyte growth factor (HGF) has been shown to suppress media thickening and accumulation of extracellular matrix. Furthermore, an increased apoptosis and a decreased proliferation of VSMCs, in addition to a significantly increased lung vessel density, was suggested as underlying reason[218]. Recently, HGF has been shown to inhibit PDGF-dependent VSMC proliferation and to induce apoptosis of myofibroblast-like stromal cells in vitro[218, 219]. Moreover, HGF also possesses angiogenic properties[220]. The activity status of HGF, however, is partially depending on the plasminogen activation system. The release of extracellular matrix bound HGF as well as activation of pro-HGF into active HGF has been shown to be also induced by plasmin[221-223]. Thus, the observed beneficial effect of exogenous u-PA application in the current study might partly be attributable to protective role of plasmingen activation dependent HGF activity.

4. Influence on vessel formation/Loss of vessels

The plasminogen activation system has been extensively studied in animal models of turmor growth and metastasis, where hypoxic microenvironment and angiogenesis are key features. In tumor models, blocking or deleting u-PA has been shown to reduce metastasis or tumour progression[224, 225] suggesting that u-PA likely contributes to angiogenesis and tumor growth. Paradoxically, PAI-1 has also been reported to promote tumor growth and angiogenesis[226-228]. In contrast to tumor models, loss of vessels has been consistently demonstrated in chronic hypoxia-induced pulmonary vascular remodeling[143, 146]. The only exception to this is the recent finding by Hyvelin et al. who demostrated a Rho kinase dependent capillary angiogenesis in chronically hypoxic rat lungs[229]. This discrepancy might be attributable to the differences in methodology and vessel size investigated. Moreover, angiogenesis does not seem to be a key event in hypoxic pulmonary vasculature, and thus the model in the current study is not directly comparable to a tumor model. Nevertheless, we observed an attenuation of chronic hypoxia-induced loss of pulmonary vessels by continuous infusion of exogenous u-PA. This could probably be explained by multiple effects of exogenous u-PA such as maintaining the normal fibrinolytic potential of vasculature by counteracting hypoxia-induced procoagulant milieu, by inducing excessive proteolytic activity leading to VSMC apoptosis and thus reduced muscularization of distal pulmonary vessels and by liberating other protective factors like HGF. Although we did not investigate further on these possible mechanisms, the maintainance of the distal pulmonary vasculature, as observed in the current study, offers as apparent explanation for the beneficial effect of continuous u-PA infusion.

Putative explanation for the current findings

As already discussed in detail, the putative explanation for the current findings can be summarized as follows.

a. When u-PA is absent or inhibited, the remodeling process is unchanged. This could be attributable to higher PAI-1 levels in the concerned area of the hypoxic vasculature, indicating that the endogenous u-PA .activity is not high enough to exert any influence on the remodeling process.

b. In case of PAI-1 knock out, there may be theoretically an increased plasmin generation. However, other factors such as α2-antiplasmin and other serpins may still block plasmin generation or plasmin activity. In addition, spatial differences in the u-PA vs PAI-1 expression may also limit the potential benefit of blocking PAI-1.

c. When a large amount of exogenouse u-PA is infused, the only putative explanation for the observed beneficial effect in view remodeling of hypoxic pulmonary vasculature may be the induction of a high local u-PA gradient along the vessel wall, thereby counteracting local PAI-1 and other plasminogen activator/plasmin inhibitor acitivities.

6 SUMMARY

Pulmonary hypertension, a devastating disease of complex and multifactorial pathogenesis, is characterized by sustained elevation in pulmonary artery pressure, pulmonary vascular remodeling and subsequent progressive right heart hypertrophy. The structure of the pulmonary vascular bed severely altered. A marked elevation in plasma levels of plasminogen activator inhibitor (PAI)-1 has consistently been reported in patients with severe primary pulmonary hypertension, and accordingly, an induction of PAI-1 and diminution of plasminogen activator (PA) have been described in hypoxia-exposed murine lungs. Hence, as also suggested by the clinical efficacy of warfarin treatment in PAH, alterations of the hemostatic balance towards predominance of procoagulant and antifibrinolytic activity in the pulmonary vascular compartment might potentially play an important role in the pathogenesis of pulmonary hypertension. In the present study, we aimed to investigate the effects of a modulation of u-PA system in pulmonary vascular remodeling. We firstly analyzed u-PA and PAI-1 expression, and u-PA activity in lung homogenates from patients with different forms of pulmonary hypertension (IPAH, CTEPH) as well as from mice exposed to chronic hypoxia. Secondly, we investigated the potential role of u-PA in pulmonary vascular remodeling in a mouse model of hypoxia-induced pulmonary hypertension by employing wild type, u-PA and PAI-1 knock out (KO), specific u-PA inhibitor (CJ463)-treated and continuously u-PA-infused mice.

Overall, the expression of u-PA and PAI-1 at transcript and protein level in the lungs from chronic hypoxia-exposed mice showed some similarities to that in the lungs from patients with IPAH and CTEPH. u-PA was induced at protein level in the lung homogenates from the patients as compared to donor lungs. However, the u-PA activity was either increased (CTEPH) or unchanged (IPAH). In line with the difference in u-PA activity, a different spatial distribution of u-PA and PAI-1 was observed in the lungs from patients with IPAH and CTEPH. On the other hand, reduced u-PA activity was observed in the lungs from chronically hypoxic mice, suggesting a differential regulation of u-PA activity in the lungs from hypoxic mice and patients with pulmonary hypertension.

By employing u-PA ko and specific u-PA inhibitor treated mice, we demonstrated that neither the inhibition of u-PA activity nor the absence of u-PA exerted a major effect on the course of chronic hypoxia-induced pulmonary vascular remodeling and RVH. Moreover, pulmonary vascular remodeling and subsequent RVH was not impaired also in PAI-1 ko mice exposed to chronic hypoxia. To further clarify the potential role of u-PA, we applied exogenous u-PA infusion into mice exposed to chronic hypoxia. Somewhat contradictory, we could observe a beneficial role of a permanent u-PA infusion on pulmonary vascular remodeling in chronically hypoxic mice.

Our results suggest that endogenous regulation of u-PA and PAI-1 does not alter the course of pulmonary vascular remodeling induced by chronic hypoxia. This could probably be attributable to existence of redundant factors and regulation of u-PA functions at multiple levels in vivo. However, exogenous application of u-PA did attenuate pulmonary vascular remodeling, probably by yielding a high endoluminal to vascular wall u-PA gradient and thus excessive proteolytic activities. Further studies to precisely delineate the underlying mechanism are warranted. Our findings may have an important implication for future investigation of plasminogen activation system based therapeutic strategies with regard to pulmonary hypertension.

7 ZUSAMMENFASSUNG

Pulmonaler Hochdruck, eine Erkrankung mit komplexer und multifaktorieller Pathogenese, ist charakterisiert durch eine Erhöhung des pulmonal-arteriellen Druckes, Umbauprozesse der Gefäßwand und progredienter Rechtsherzhypertrophie. Eine signifikante Erhöhung der Plasmaspiegel des plasminogen activator inhibitor (PAI)-1 wurde bei Patienten mit schwerem Lungenhochdruck beschrieben, verbunden mit einer Induktion von PAI-1 und einer Abnahme des plasminogen activator (PA). Aus diesem Grund könnte eine Dominanz prokoagulatorischer und antifibrinolytischer Mediatoren im pulmonalen Gefäßsystem eine bedeutende Rolle in der Pathogenese des Lungenhochdrucks spielen, was durch den erfolgreichen klinischen Einsatz von Warfarin bestätigt wird. In der vorliegenden Studie untersuchten wir die Effekte einer Modulation des u-PA Systems auf Umbauprozesse des pulmonalen Gefäßsystems. Zu Beginn analysierten wir die u-PA und PAI-1 Expression und u-PA Aktivität in Lungenhomogenaten von Patienten mit verschiedenen Formen von Lungenhochdruck (IPAH, CTEPH) und Mäusen, welche chronischer Hypoxie ausgesetzt wurden. Im folgenden untersuchten wir einen möglichen Einfluss von u-PA auf pulmonale Gefäßumbauprozesse im Tiermodell des durch chronische Hypoxie ausgelösten Lungenhochdrucks mit Hilfe von Wildtyp, u-PA und PAI-1 knockout Tieren, Behandlung mit einem spezifischen u-PA Inhibitor (CJ463) und kontinuierlichen u-PA Infusionen.

Zusammenfassend zeigen sich beim Vergleich der u-PA und PAI-1 mRNA und Protein Expression von Mäusen, welche chronischer Hypoxie ausgesetzt wurden, und Lungen von Patienten mit IPAH und CTEPH gewisse Gemeinsamkeiten. Im Vergleich zu Gesunden zeigen Lungen von Patienten mit Lungenhochdruck eine erhöhte u-PA Proteinexpression, wohingegen die u-PA Aktivität entweder erhöht (CTEPH) oder unverändert (IPAH) war. Ferner konnten wir eine unterschiedliche Verteilung von u-PA und PAI-1 in Lungen von Patienten mit IPAH und CTEPH beobachten. Im Kontrast zu diesen Daten war die u-PA Aktivität in Mäusen, welche chronischer Hypoxie ausgesetzt wurden, stark reduziert.

Durch den Einsatz von u-PA knockout Mäusen und einem spezifischen u-PA Inhibitor konnten wir zeigen, dass weder eine Inhibition der u-PA Aktivität noch ein Fehlen von u-PA Einfluss auf den Verlauf der durch chronische Hypoxie induzierten pulmonalen Gefäßumbauprozesse hat. Ferner waren diese Gefäßumbauprozesse bei PAI-1 knockout Tieren nicht beeinträchtigt. Um die Rolle von u-PA genauer aufzuklären applizierten wir Hypoxie-exponierten Mäusen u-PA Infusionen und beobachteten überraschenderweise einen günstigen Effekt auf pulmonale Gefäßumbauprozesse.

Unsere Studie zeigt, dass eine Regulation von u-PA und PAI-1 keinen Einfluss auf den Verlauf der durch chronische Hypoxie induzierten Gefäßumbauprozesse hat. Dies spricht für die Existenz weiterer Faktoren und eine differentielle Regulation der u-PA Funktionen. Infusionen von u-PA konnten in unseren Experimenten die Gefäßumbauprozesse aufhalten, wahrscheinlich durch verstärke proteolytische Aktivität. Die Ergebnisse unserer Studie sollen Grundlage für weitere Untersuchungen zur therapeutischen Modulation des PA-Systems bei Patienten mit Lungenhochdruck sein.

8 ABBREVIATION

ALK1	Activin-like kinase type-1
ANOVA	Analysis of variance
ATF	Amino terminal fragment
BMPR II	Bone morphogenetic protein type II receptor
CD	Cluster of différentiation
COPD	Chronic obstructive pulmonary disease
CTEPH	Chronic thromboembolic pulmonary hypertension
EVE	Endogenous vascular elastase
FPAH	Familial pulmonary arterial hypertension
GFD	Growth factor domain
HHT	hereditary hemorrhagic telangiectasia
HMW	High molecular weight
HPV	Hypoxic pulmonary vasoconstriction
HPV	Hypoxic pulmonary vasoconstriction
ECM	Extra cellular matrix
IDV	Integrated density value
IgG	Immunoglobulin
IPAH	Idiopathic pulmonary arterial hypertension
KD	Kringle domain
KO	knockout
LMW	Low molecular weight
MCTP	Monocrotaline pyrrole
MMPs	Matrix metalloproteinases
mRNA	Messenger ribonucleic acid
NM/M	The ratio of non-muscularized vs. muscularized vessels
NO	Nitric oxide
NYHA	New York Heart Association
OMP	Osmotic Mini-pump
PAH	Pulmonary arterial hypertension
PAI	Plasminogen activator inhibitor

PAP	Pulmonary arterial pressure
PASMC	Pulmonary artery smooth muscle cell
PDGF	Platelet derived growth factor
PEEP	Positive end expiratory pressure
PH	Pulmonary hypertension
PPH	Primary pulmonary hypertension
PVR	Pulmonary vascular resistance
ROS	Reactive oxygen species
RT-PCR	Reverse Transcription- Polymerase chain reaction
RV	Right ventricle
RV/LV+S	Right ventricle per left ventricle plus septum ratio
RVH	Right ventricular hypertrophy
RVSP	Right ventricular systolic pressure
scu-PA	Single chain u-PA
SDS-PAGE	Sodium dodecyl-sulphate polyacrilamide gel electrophoresis
SEM	Standard error mean
SMC	Smooth muscle cell
SPH	Secondary pulmonary hypertension
tcu-PA	Two chain u-PA
TF	Tissue factor
TGF-β	Transforming growth factor-beta
t-PA	Tissue-type plasminogen activator
u-PA	Urokinase-type plasminogen activator
uPAR	Urokinase-type plasminogen activator receptor
VN	Vitronectin
vWF	von Willebrand factor
WHO	World Health Organization
WT	Wild type
HIF-1α	Hypoxia inducible factor-1α
PN-1	Protease nexin-1

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10 ERKLÄRUNG

"Ich erkläre: Ich habe die vorgelegte Dissertation selbständig, ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nicht veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis" niedergelegt sind, eingehalten."

11 ACKNOWLEDGMENTS

I would like to reveal my sincere acknowledgement to Prof. Dr. Werner Seeger for this wonderful opportunity of learning science in an international environment of graduate college and UGLC. Prof. Seeger, you have ever been the first and final source of inspiration for people like me…….

I express my sincere thanks to Dr. Oliver Eickelberg, Director of the Graduate Program, MBML, and his team for their constant efforts to make the program intensive, effective and a success. You, as a program director, were absolutely right to assume that students needed adequate pressure. My heartfelt appreciation to Oli and Rory ..…….

I would like to acknowledge Prof. Dr. N. Weissmann for his guidance throughout the animal experiments and Dr. M. Hecker for the "Zusammenfassung". I thank the postdoctoral fellows and technical staffs in the lab, and Karin Endres for their support. My special thanks go to MBML colleagues for their contribution to make the study an interactive, interesting and enjoying of its kind. Hey Rio and Takwi ! I must at least mention you ..……..

I would like to disclose my sincere gratitude to Prof. Dr. A. Guenther, my PI, for all his efforts in my learning. I learnt as much as I could from his scientific insight, and ever optimistic personality. I would also like to unveil my sincere gratitude to Prof. Dr. R.T. Schermuly, my Co-PI, for his support whenever I needed. I am greatly impressed by his friendly personality, punctuality and team approach of exploring science. Andreas and Ralph, I will ever remember both of you for your kind favor at right time……..

My sincere acknowledgement goes to the Institute of Agriculture and Animal Sciences (IAAS), Nepal to encourage me to pursue this study.

It goes without saying that my beloved parents ever deserve my deepest gratitude. Finally, Madhu, Babu (Kshitij) and Nani (Sadiksha), I am highly indebted to all of you. I indeed lack words that appropriately express my appreciation and feeling for your patients and moral support every moment.

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PUBLICATIONS

- Markart P, Ruppert C, Wygrecka M, Colaris T**, Dahal B,** Walmrath D, Harbach H, Wilhelm J, Seeger W, Schmidt R, Guenther A. Patients with ARDS show improvement but not normalization of alveolar surface activity with surfactant treatment: putative role of neutral lipids. **Thorax, 2007, 62 (7):588-94.**

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ABSTRACTS *(Oral and poster presentation)*

*** Dahal B.K.**, Heuchel R., Pullamsetti S.S., Ghofrani H.A., Weissmann N., Seeger W., Grimminger F. and Schermuly R.T. Platelet Derived Growth Factor Receptor-β (Pdgfr-β) Contributes To Hypoxia-Induced Pulmonary Vascular Remodeling. **Poster presented at the 4th World Symposium on Pulmonary Hypertension, Dana point, California, February, 11-14, 2008.**

*** Dahal, B.K**., Schermuly, R.T., Markart, P., Ruppert, P., Seeger, W., Weissmann, N. and Gunther A. Role of Urokinase in Vascular Remodeling in a murine model of Hypoxia-induced Pulmonary Hypertension. **Poster presented in a poster discussion session at the International Conference of the American Thoracic Society (ATS), San Diego, California, May 19- 24, 2006.**

* Ruppert C., Markart P., Nkymbeng T., **Dahal B.K**., Seeger W., Eickelberg O., Heuchel R. and Gunther A. Smad7-knockout Mice show an accelerated developement of lung fibrosis after Bleomycin exposition. **Poster presented at the Conference of the German society of Internal medicine (DGIM), Wiesbaden, Germany, April 22 - 24, 2006.**

*** Dahal, B.K**., Schermuly, R.T., Markart, P., Ruppert, P., Seeger, W., Weissmann, N. and Gunther A. Role of Urokinase in Vascular Remodeling in a murine model of Hypoxia-induced Pulmonary Hypertension. **Poster presented at the Conference of the German Society for Internal medicine (DGIM), Wiesbaden, Germany, April 22 - 26, 2006.**

* Schmidt R., B.C. Rehm, C. Ruppert, P. Markart, **B.K. Dahal**, A. Jahn, F. Rose, P. Kaese, R. Geyer, W. Seeger and A. Günther. Clearance and Incorporation of Aerosolized 13C Oleic Acid in LPS-induced Acute Lung Injury in Mice. **Poster presented at International Conference of ATS, 2005, San Diego, USA.**

*** Dahal, B.K**., Schermuly, R.T., Markart, P., Ruppert, P., Schmidt, R., Kofrei, M., Seeger, W., Weissmann, N. and Gunther A. Role of Urokinase in Vascular Remodeling in a murine model of Hypoxia-induced Pulmonary Hypertension. **Oral presentation at Annual conference of German Society for Pneumology (Cell Biology Section), Bonn, Germany, October 14-15, 2005.**

* Van Den Bergh, R., G.H. Hassanzedeh, L Brys, **B.K. Dahal**, J. Brandt, J. Grooten, F. Brombacher, G. Vanham, W. Noel, P. Bogaert, T. Boonefaes, A. Kindt, P.de Baetselier and G. Raes. Novel Markers for Alternative Activation of Macrophages: Macrophage Galactose Type C-Type Lectins 1 and 2. **Abstract presented at Scandinavian Society for Immunology, 35th Annual meeting and 20th Summer School, Aarhus, Denmark, June 13-16, 2004.**