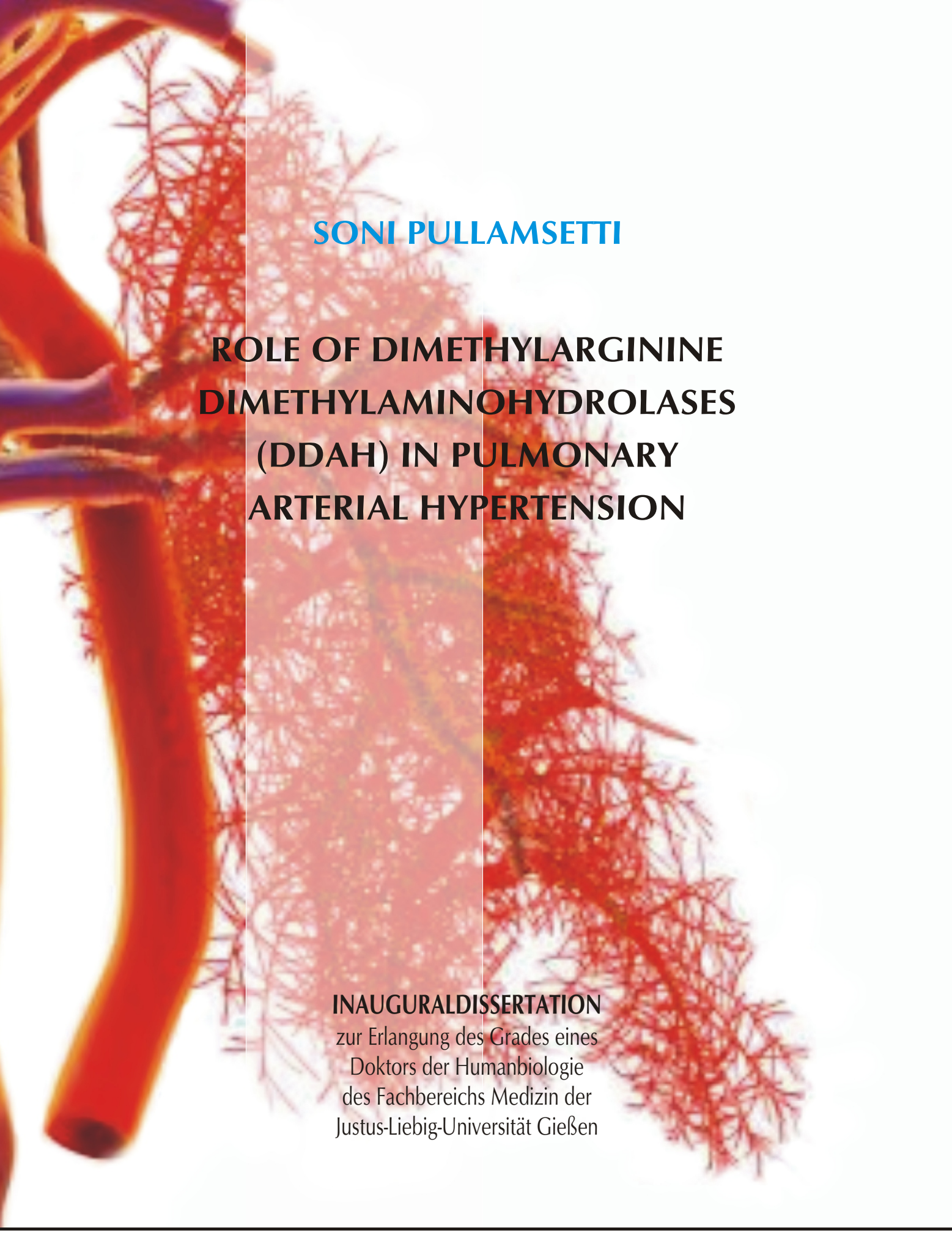

SONI PULLAMSETTI

ROLE OF DIMETHYLARGININE DIMETHYLAMINOHYDROLASES (DDAH) IN PULMONARY ARTERIAL HYPERTENSION

INAUGURALDISSERTATION
zur Erlangung des Grades eines
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vorgelegt von

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Giessen 2005

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Gutachter: PD Dr. Jürgen Bauer

Tag der Disputation: 22.03.2006

To four lucks of my life

My parents

Poorna, Subbarao

Brother Ramesh

Fiancé Raj

Table of Contents

Chapters	Page(s)

Chapters	Page(s)

Chapters	Page(s)

List of Figures

Figures	Page(s)

Figures	Page(s)

List of Tables

Glossary of Symbols

ABC	avidin-biotin-complex
Abs	antibodies
AC	adenylyl cyclase
ADMA	N_G, N_G-Asymmetric dimethylarginine
5’AMP	adenosine-5'-monophosphate
aPKA	active protein kinase A
αSMA	alpha-smooth muscle actin
ATP	adenosine 5'-triphosphate
BH_4	(6R)-5,6,7,8-Tetrahydro-L-biopterin
BW	body weight
Ca^{2+}i	intracellular calcium
CaM	calmodulin
cAMP	cyclic 3'5'-adenosine monophophate
CAT	cationic amino acid transporter
Cav-1	caveolin-1
CCB	calcium channel blocker
cDNA	complementary DNA
cGMP	cyclic 3'5'-guanosine monophophate
CI	cardiac index
Ch	channel
CO	cardiac output
CVP	central venous pressure
DAB	3, 3’-diaminobenzidine
DDAH	dimethylarginine dimethylaminohydrolase
DMA	dimethyl amine
EC	endothelial cells
ECM	extra cellular matrix
ecSOD	extracellular superoxide dismutase
EDRF	endothelium derived relaxing factor

eNOS	endothelial nitric oxide synthase
ET-1	endothelin-1
ETA	endothelin receptor A
ETB	endothelin receptor B
FAD	flavin-adenine dinucleotide
FB	fibroblast
FiO_2	inspired fraction of oxygen
FMN	flavin mononucleotide
FPAH	familial pulmonary arterial hypertension
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GTP	guanosine 5'-triphosphate
HIV	human immuno-deficiency Virus
HPLC	high performance liquid chromatography
HR	heart rate
Hsp-90	heat shock protein-90
5-HT	5-hydroxy tryptamine
5-HTT	5-hydroxy tryptamine transporter
5-HT2B	5-hydroxy tryptamine 2b receptor
HUVEC	human umbilical vein endothelial cells
IFN-γ	interferon-gamma
Ig	immunoglobulin
IL-1β	interleukin-1, beta
iNO	inhaled nitric oxide
iNOS	inducible nitric oxide synthase
IPAH	idiopathic pulmonary arterial hypertension
iPKA	inhibited protein kinase A
Kv	Voltage-gated potassium channels
LDL	low Density lipoprotein
L-NMMA	NG, monomethyl-L-arginine
LPS	lipopolysaccharide

MCT	monocrotaline
MCT-PAH	monocrotaline induced pulmonary hypertension
MMP	matrix metalloprotease
mPAP	mean pulmonary artery pressure
mSAP	mean systemic arterial pressure
NADPH	nicotine adenine dinucleotide phosphate
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
NOx	Nitrate/Nitrite plasma levels
NYHA	New york heart association
O^{2-}	superoxide anion
$ONOO^{-}$	peroxynitrite anion
PAP	pulmonary arterial pressure
PASMC	pulmonary arterial smooth muscle cells
PCR	polymerase chain reaction
PDE	phosphodiesterase
PDEi	Phosphodiesterase inhibitor
PGI_2	prostacyclin
PKA	protein kinase A
PKB/Akt	Protein kinase B
PH	pulmonary hypertension
PPH	primary pulmonary hypertension
PPARγ	peroxisome proliferator-activated receptor, gamma
PO_2	oxygen partial pressure
PRMT	protein-arginine methyl transferases
PVH	pulmonary venous hypertension
PVR	pulmonary vascular resistance
PVRI	pulmonary vascular resistance index
PAWP	pulmonary artery wedge pressure

RNA	ribonucleic acid
RT-PCR	reverse transcriptase-polymerase chain reaction
RV/LV+S	right ventricle to left ventricle plus septum weight ratio
RVSP	right ventricular systolic pressure
SAH	S-adenosyl-l-homocysteine
SAM	S-adenosyl Methionine
SaO_2	arterial oxygen saturation
SDMA	NG, NG- symmetric dimethylarginine
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SMC	smooth muscle cells
Smw	six minute walk test
SPH	secondary pulmonary hypertension
SVO_2	venous oxygen saturation
SVRI	systemic vascular resistance index
TNF-α	Tumor necrosis factor-alpha
V/Q	ventilation/perfusion
vWF	von Willibrand factor
WHO	World health organization
4124W	S-2-amino-4(3-methylguanidino) butanoic acid

1. Introduction

Pulmonary hypertension (PH), a chronic disorder of the pulmonary vasculature, is characterized by progressive elevation in pulmonary artery pressure and the ultimate development of right heart failure and death (1). It is a devastating disease that drastically limits physical capacity and seriously reduces life expectancy. On average, without treatment, patients had a median life expectancy of less than 2.8 years post diagnosis (2). However, the true incidence of PH is rare, 1–2/million/year and approximately 10% appear to be familial (3). Interestingly, a preponderance of females among PH patients was noted with a ratio of female to male varying between 1.7 and 3.5:1(4).

Pulmonary hypertension (PH) was first described over 100 years ago by Ernst von Romberg, a distinguished German physician and clinical scientist, in a patient with right-heart failure whose necropsy showed no obvious reason for pulmonary arteriosclerosis and diagnosed as "sclerosis" of the pulmonary arteries. In 1901, Ayerza noted the profound cyanosis associated with this disorder, and described the disorder as "cardiacos negros", but it was Dresdale and coworkers who first used the term primary pulmonary hypertension (PPH) and subsequently demonstrated the involvement of pulmonary vasoconstriction in the pathogenesis of PPH (5). Interest in so-called PPH was excited in 1967–1972 by an epidemic that was attributed to the ingestion of an appetite suppressant, aminorex fumarate (6). This epidemic “Wake-up call” to the scientific community prompted the first WHO sponsored symposium, monograph on diagnosis and treatment and a substantial progress in understanding the pathogenesis of this disorder.

1.1 Definition & Classification

Pulmonary hypertension is defined as a sustained elevation of pulmonary arterial pressure to more than 25 mm Hg at rest or to more than 30 mm Hg with exercise, with a mean pulmonary-capillary wedge pressure and left ventricular end-diastolic pressure of less than 15 mm Hg. In the past, based on etiology, pulmonary hypertensive was divided into two categories (7).

i) Primary pulmonary hypertension (PPH): describes PH without a demonstrable etiology.

ii) Secondary pulmonary hypertension (SPH): describes PH that results from a coexisting condition known to be complicated by pulmonary hypertension.

However, due to similar histopathological features and treatment responses among these two groups of patients, in 1998, during the Second World Symposium on PH held in Evian, France, a new clinical classification of PH was proposed (8).

The Evian classification consisted of five categories and had focused mainly on the basis of mechanisms, rather than the associated conditions: 1) pulmonary arterial hypertension (PAH), 2) pulmonary venous hypertension (PVH), 3) PH associated with disorders of the respiratory system or hypoxemia, 4) PH caused by thrombotic or embolic diseases, and 5) PH caused by diseases affecting the pulmonary vasculature. PAH has been further divided into idiopathic pulmonary arterial hypertension (IPAH) and, when supported by genetic evidence, familial pulmonary arterial hypertension (FPAH). Like wise, within each category are subsets that reflect diverse causes and sites of injury. This classification served as a useful guide to the clinician in organizing the evaluation of a patient with PH and developing a treatment plan.

Recently, a revised clinical classification was proposed at Venice conference in 2003 (1) (Table 1, Page 3). This classification has preserved the structure and spirit of the Evian classification. However, it includes changes that reflect recent advances in the understanding and management of PH and uses consistent terminology and defines pulmonary hypertension more precisely than previous versions.

Table 1: Clinical Classification of Pulmonary hypertension

1. Pulmonary arterial hypertension (PAH)
- 1.1. Idiopathic (IPAH)
- 1.2. Familial (FPAH)
- 1.3. Associated with (APAH):
  - 1.3.1. Collagen vascular disease
  - 1.3.2. Congenital systemic-to-pulmonary shunts**
  - 1.3.3. Portal hypertension
  - 1.3.4. HIV infection
  - 1.3.5. Drugs and toxins
  - 1.3.6. Other (thyroid disorders, glycogen storage disease, Gaucher disease, hereditary hemorrhagic telangiectasia, hemoglobinopathies, myeloproliferative disorders, splenectomy)
- 1.4. Associated with significant venous or capillary involvement
  - 1.4.1. Pulmonary veno-occlusive disease (PVOD)
  - 1.4.2. Pulmonary capillary hemangiomatosis (PCH)
- 1.5. Persistent pulmonary hypertension of the newborn

2. Pulmonary hypertension with left heart disease
- 2.1. Left-sided atrial or ventricular heart disease
- 2.2. Left-sided valvular heart disease

3. Pulmonary hypertension associated with lung diseases and/or hypoxemia
- 3.1. Chronic obstructive pulmonary disease
- 3.2. Interstitial lung disease
- 3.3. Sleep-disordered breathing
- 3.4. Alveolar hypoventilation disorders
- 3.5. Chronic exposure to high altitude
- 3.6. Developmental abnormalities

4. Pulmonary hypertension due to chronic thrombotic and/or embolic disease
- 4.1. Thromboembolic obstruction of proximal pulmonary arteries
- 4.2. Thromboembolic obstruction of distal pulmonary arteries
- 4.3. Non-thrombotic pulmonary embolism (tumor, parasites, foreign material)

5. Miscellaneous

Sarcoidosis, histiocytosis X, lymphangiomatosis, compression of pulmonary vessels (adenopathy, tumor, fibrosing mediastinitis)

(Revised classification of pulmonary hypertension (Venice 2003) from Simonneau G et al., J Am Coll Cardiol. 2004 Jun 16;43:5S-12S).

In addition, a functional classification (Table 2, Page 4) patterned after the New York Heart Association (NYHA) for heart disease was developed to allow

comparisons of patients with respect to the clinical severity of the disease process (8).

Table 2: Functional Classification NYHA

Class	Description
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.
II	Patients with pulmonary hypertension resulting in slight limitation of physical activity. They are comfortable at rest. Ordinary physical activity causes undue dyspnea or fatigue, chest pain or near syncope.
III	Patients with pulmonary hypertension resulting in marked limitation of physical activity. They are comfortable at rest. Less than ordinary physical activity causes undue dyspnea or fatigue, chest pain or near syncope.
IV	Patients with pulmonary hypertension with inability 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.

(Rich S. Primary pulmonary hypertension: executive summary. Evian, France: World Health Organization, 1998).

1.2 Histopathology

Histopathologically, the pulmonary arteries in PAH patients showed intimal thickening, medial hypertrophy, adventitial thickening, obliteration of small arteries, and occasionally, vasculitis in the walls of the pulmonary veins (Figure 1). A fascinating focal vascular structure, the plexiform lesion, is also found in many cases of PAH (9-11).

Plexiform lesion: is a focal proliferation of endothelial channel lined by myofibroblasts, smooth muscle cells and connective tissue matrix. They

represent a mass of disorganized vessels that arise from pre-existing pulmonary arteries (Figure 1A, B).

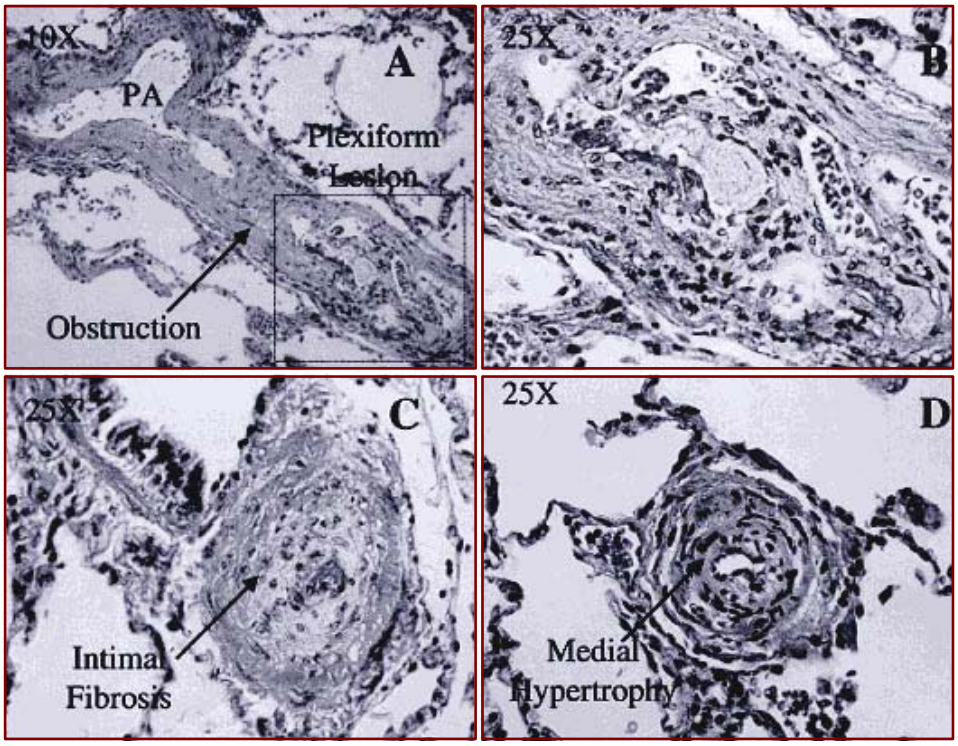

Figure 1: Histology of PAH. *(***A)**, Plexiform lesion (magnification 10X). Note plexiform lesion occurs distal to vascular obstruction, suggesting a role for angiogenesis in its origin. **(B)**, Magnification of plexiform lesion seen in A magnification 25X). **(C)**, Intimal fibrosis of small pulmonary artery causing vascular obstruction (magnification 25X). **(D)**, Medial hypertrophy (magnification 25X). (Archer S et al., Circulation. 2000)

Intimal fibrosis: is a thickening and fibrosis of intimal layer due to migration and proliferation of smooth muscle cells, fibroblast and myofibroblast into the intima. This may arise in concentric luminar, eccentric or concentric non luminar fashion. In addition, a hallmark of severe pulmonary hypertension the neointima is the formation of a layer of myofibroblasts and extracellular matrix between the endothelium and the internal elastic lamina, termed the neointima (Figure 1C).

Media hypertrophy: is an increase in the cross sectional area of the media of pre and intra acinar pulmonary arteries. This originates due to both hypertrophy

and hyperplasia of smooth muscle fibres as well as increase in connective tissue matrix and elastic fibres in the media of muscular arteries (Figure 1D).

Adventitial thickening: is a thickening of adventitia due to increased fibroblast cellularity and extracellular matrix (ECM) deposition. This is highly prominent in the small, muscular pulmonary arteries and occurs in most cases of PAH. Nevertheless, it is extremely difficult to evaluate.

All the above changes are seen typically in clinical classification IPAH; FPAH and APAH. Histopathological changes in various forms of PAH are qualitatively similar but with quantitative differences in the distribution and prevalence of pathological changes in the different components of the pulmonary vascular bed including arterioles, capillaries and veins.

1.3 Pathophysiology/Pathological mechanisms

The pathogenesis of IPAH is complex and multifactorial. Increased pulmonary vascular resistance in IPAH patients probably results from a combination of pulmonary vasoconstriction, vascular-wall remodeling and thrombosis. However, a growing body of evidence implicates the central role of endothelial dysfunction in the initiation and progression of IPAH.

1.3.1 Endothelial dysfunction/Vasoconstriction

One of the complex and multifactorial processes that contribute to the development of pulmonary hypertension involves endothelial cell dysfunction. Endothelial cells play an integral role in the maintenance of normal vascular structure and function. An injury and subsequent dysfunction of the endothelium causes altered production of endothelial mediators and growth factors that thereby facilitates vasoconstriction and pulmonary arterial smooth muscle cell (PASMC) hypertrophy, leading to pulmonary vascular remodeling and in situ thrombosis (Figure 2, Page 8) (12,13).

The mechanisms responsible for endothelial activation are yet to be fully elucidated. However, a number of stimuli, including cytokines, viral infection (HIV), free radicals, shear stress from increased pulmonary blood flow, and

alveolar hypoxia, may potentially activate vascular endothelial cells to elicit basic alterations in their local production of vasoactive and vasoconstrictive mediators. The abnormal balance of these mediators in the pulmonary vasculature culminates in the development of endothelial cell proliferation and vasoconstriction (14,15). Though, it is yet unclear whether a disturbance in humoral mediators causes pulmonary hypertension or is a result of it. Of those local mediators, nitric oxide, prostacyclin, and endothelin-1 are among the best studied and most commonly implicated in the pathogenesis of IPAH.

1.3.1.1 Nitric oxide

Nitric oxide (NO)/Endothelium derived relaxing factor (EDRF) is a potent vasodilator and an inhibitor of platelet activation and vascular smooth-muscle cell proliferation (16,17). Nitric oxide (NO) is constitutively produced in the lung endothelium by endothelial nitric oxide synthase (eNOS). Nonetheless, the expression of eNOS can be modulated by diverse stimuli such as shear stress and increased pulmonary blood flow (18).

Interestingly, patients with IPAH have low levels of NO in their exhaled breath. In fact, the severity of pulmonary hypertension correlates inversely with NO levels estimated by measurement of NO reaction products in bronchoalveolar lavage fluid (19). Furthermore, decreased levels of the eNOS have been observed in the pulmonary vascular tissue of patients with pulmonary hypertension, particularly those with IPAH (20,21). Though, controversial reports exist in concern to eNOS expression in IPAH patients, which were discussed in detail in later part of the thesis.

1.3.1.2 Endothelin-1

Endothelin-1 (ET-1) produced by human endothelial cells, is the most potent vasoconstrictor and mitogen, with the ability to induce cell proliferation in a number of cell types, including vascular smooth muscle cells. The peptide exerts its biologic effects via interacting with two G-protein-coupled receptors, ETA and ETB (22). In patients with IPAH, several derangements in ET-1 expression and

activity have been demonstrated. Patients with IPAH have been shown to have higher serum levels of ET-1 and higher arterial-to-venous ratios of ET-1 than do healthy controls. Endothelin levels have also been shown to correlate with pulmonary hemodynamics (23). In addition, lung specimens from patients with IPAH when compared to healthy donors, exhibit increased ET-1 staining of the muscular pulmonary arteries (24).

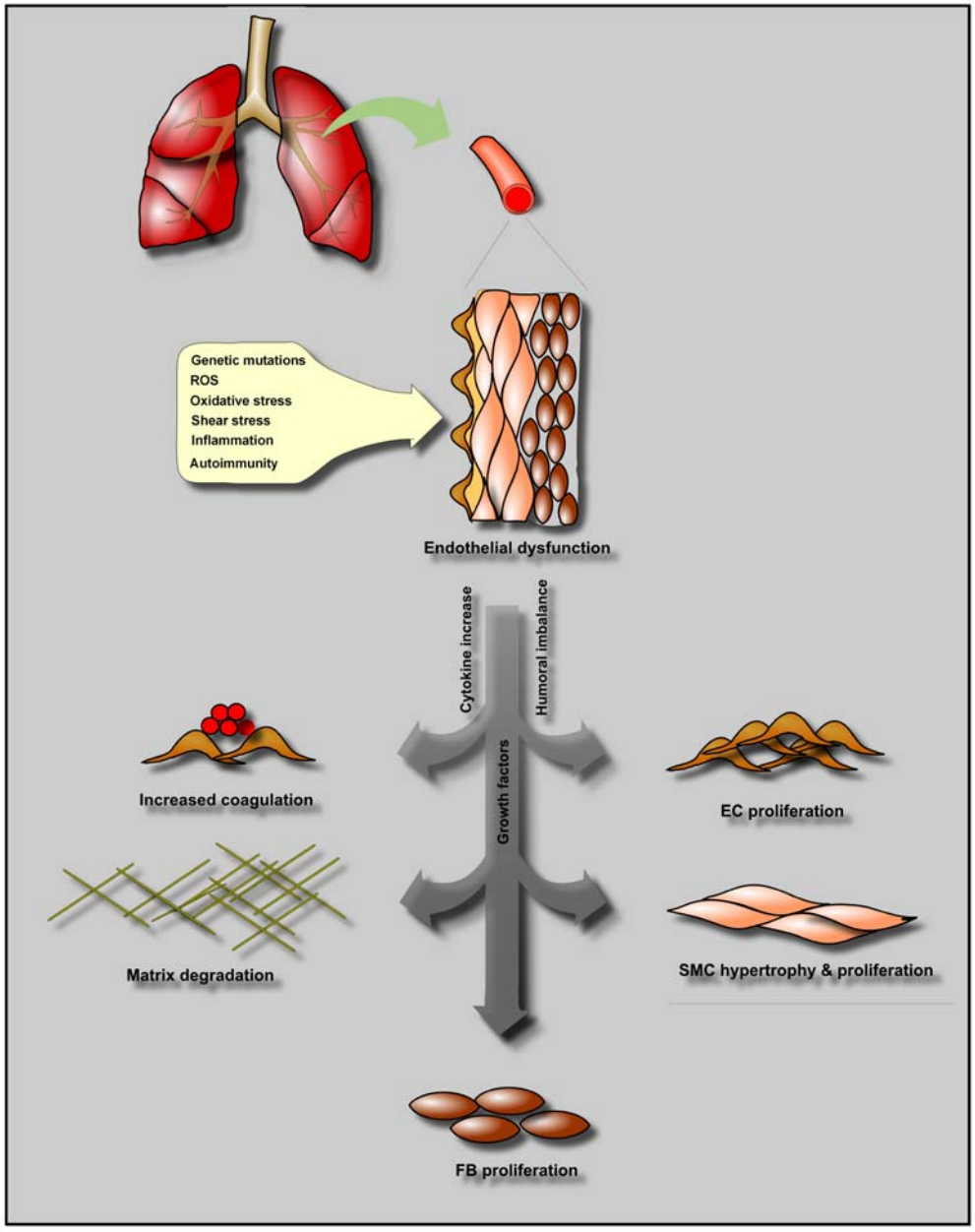

Figure 2: Pathogenesis of pulmonary arterial hypertension (PAH). Schematic outline of abnormalities seen in PAH that may contribute to its cause or progression. An injury and subsequent dysfunction of the endothelium by a variety of stimuli causes altered production of endothelial mediators and growth factors that thereby facilitates vasoconstriction and pulmonary arterial cell types (endothelial cells (EC), smooth muscle cells (SMC) and fibroblasts (FB)) proliferation and migration, leading to pulmonary vascular remodeling and in situ thrombosis. The reduction of cross-sectional area of the pulmonary microvasculature contributes to the increased pulmonary vascular resistance observed in this disease (Author's Slide).

1.3.1.3 Prostacyclin

The endothelium also produces prostacyclin (PGI_2) by cyclooxygenase metabolism of arachidonic acid. It possesses strong vasodilatory, anti-aggregatory, anti-inflammatory, and anti-proliferative properties (25). Prostacyclin synthesis is disturbed in endothelial cells of IPAH patients. Analysis of urinary metabolites of prostacyclin showed a decreased excretion of 6-ketoprostaglandin F1, a stable metabolite of prostacyclin in patients with IPAH (26). In addition, a decrease in prostacyclin synthase expression has been noted in pulmonary arteries of patients with severe IPAH, portopulmonary hypertension and HIV-associated PAH, further underscoring the role of endothelial dysfunction in the pathobiology of PAH (27).

1.3.2 Remodeling

In addition to pulmonary vasoconstriction that results from dysregulation of the local endothelial mediators as discussed above, pulmonary vascular remodeling seems to play a major role in the increased vascular resistance seen in IPAH (13). Pulmonary vascular remodeling is characterized by thickening of all three layers of the blood vessel wall, the adventitia, the media and the intima (28). The thickening is due to hypertrophy (cell growth) and/or hyperplasia (proliferation) of the predominant cell type within each of the layers and increased deposition of extracellular matrix components (e.g., collagen, elastin, and fibronectin) (29-32).
These cellular changes usually results from the anti-mitogenic and mitogenic substances (NO, PGI_2, and Endothelin) that are deranged by endothelial injury. In addition, other stimuli that derives from depolarized smooth muscle cells

(voltage-gated potassium channels (Kv)) and locally activated platelets (thromboxane A2 and serotonin) also plays a major role in the vascular cell proliferation.

1.3.2.1 Serotonin

Serotonin (5-hydroxytryptamine) is a vasoconstrictor that promotes smooth-muscle cell hypertrophy and hyperplasia. A role of 5-HT has been suggested in IPAH. Plasma serotonin levels are increased in IPAH patients compared with control subjects, and IPAH platelets have decreased serotonin concentrations (33). These increased levels were shown to be associated with mutations in the serotonin transporter (5-HTT), the 5-hydroxytryptamine 2b receptor (5-HT2B), or both that have been described in platelets and lung tissue from patients with IPAH (34). Most interestingly, various studies suggest that appetite suppressant, aminorex fumarate induced risk of IPAH is mainly mediated via its interaction with serotonin transporter, 5-HTT (6).

1.3.2.2 Potassium channels

Potassium channels are the transmembrane-spanning proteins that have a greater selectivity for K^+ ions. There are three major classes K+ channels channels: Kv channels (including Ca^{2+}-sensitive channels, KCa), the inward rectifier channels (Kir), and a family with a tandem, 2-pore motif (TASK). Among these, Kv channels have a voltage sensor and both respond to and contribute to determining membrane potential in PASMCs (35). However, patients with IPAH have low expression Kv1.5 channel that lead to membrane depolarization of PASMCs and to an increase in intracellular calcium resulting in both vasoconstriction and proliferation (36,37).

1.3.2.3 Thromboxane

Thromboxane, like prostacyclin, is an arachidonic acid metabolite and produced by endothelial cells and platelets. It is a potent vasoconstrictor, a smooth muscle mitogen, and an inducer of platelet aggregation (25). An increased production of

thromboxane A_2 metabolites is seen in IPAH (26). Furthermore, thromboxane-receptor density is increased in the right ventricle of patients with IPAH (38).

1.3.3 In situ thrombosis

The third major characteristic pathophysiologic abnormality in pulmonary hypertension is in situ thrombosis. It is believed to be initiated by abnormalities in the clotting cascade, the endothelial cells, or the platelets and thereby promoting the release of procoagulation mediators (39). Indeed, intravascular coagulation seems to be a continuous process in IPAH patients, characterized by increased blood thrombin activity and decreased thrombomodulin expression (40,41). In addition, PGI_2 and NO, both inhibitors of platelet aggregation, are decreased at the level of the injured endothelial cell, as discussed above.

Furthermore, circulating platelets in patients with IPAH seem to be in a continuous state of activation and contribute to the prothrombotic milieu by aggregating at the level of the injured endothelial cells (42). In most cases, however, it remains unclear whether thrombosis and platelet dysfunction are causes or consequences of the disease.

1.4 Diagnosis and evaluation

1.4.1 Clinical presentation

The onset of IPAH symptoms is usually insidious with several years elapsing before the diagnosis is actually made. Furthermore, pulmonary hypertension often presents with nonspecific symptoms. The most common initial clinical manifestation of IPAH is dyspnea, which is most apparent during effort or exercise (43). General fatigue and chest pain are common complaints as well. Other signs that are presented as the disease progresses include cyanosis, raised jugular venous pressure, right-ventricular heave, loud pulmonary component of the second heart sound, murmurs of tricuspid regurgitation, hepatomegaly, ascites and peripheral edema (44,45). An adaptation of the NYHA classification of functional capacity has proven useful in qualitatively assessing disease progression (Table 2, Page 4).

1.4.2 Diagnostic evaluation

A high index of suspicion, a meticulous history and a careful physical examination are paramount to the diagnosis of IPAH (45). Patients with above mentioned signs and symptoms were initially investigated with electrocardiogram, chest radiograph, and respiratory function tests. Once they were suspected of pulmonary hypertension, an extensive evaluation was performed to determine the etiology, severity and the responsiveness to vasodilator challenge. It includes echocardiography, serologic evaluations, ventilation-perfusion (V/Q) scanning, pulmonary function testing and an assessment of functional capacity (6 min walk test). The final step in this evaluation is right heart catheterization for the assessment of vasoresponsiveness (46).

1.5 Treatment

No cure for IPAH currently exists. However, medical therapies currently available can improve the functional status and quality of life of the patients. Treatment for IPAH patients begins with conventional therapies for the treatment include anticoagulants, inotropic agents, diuretics, and supplemental oxygen (47).

The anticoagulant agent warfarin is recommended for use by all patients with IPAH in order to prevent further formation of thrombotic lesions in the pulmonary arterioles. Retrospective and prospective studies have demonstrated that warfarin treatment is associated with increased survival. Diuretics are recommended if edema is present and adapted to prevent an excessive decrease in right ventricular preloads in the presence of tricuspid regurgitation. Further, as hypoxia is a potent stimulus to vasoconstriction, oxygen requirements should be assessed at rest and during exercise, and oxygen should be supplemented to achieve a saturation of >90% at all times (47,48).

Apart from the above, vasodilator therapy is considered to be the mainstay of treatment in patients with IPAH. Such therapy is used in an attempt to reduce pulmonary artery pressure and, thus, right-ventricular afterload. Before vasodilator therapy is initiated for IPAH, patients should be identified as

"responders" or "nonresponders" by measuring the change in pulmonary artery pressure and pulmonary vascular resistance in response to short-acting vasodilators such as inhaled nitric oxide, intravenous prostacyclin, or adenosine. Patients with a positive response (>20% reduction in mean pulmonary arterial pressure (mPAP) or pulmonary vascular resistance (mPVR)) are more likely to benefit from long term vasodilator therapy with calcium channel blockers (CCB) (49). During acute vasodilator testing, these patients showed significantly lower levels of both mPAP and mPVR, which reached near-normal values. Patients with a vasodilator response of this magnitude who are treated with a CCB have a reported survival of up to 94% at five years (compared with 38% in those who failed to respond and were not treated with a CCB). Nevertheless only a small subgroup of patients with PAH benefits from CCB therapy (<25%) (50).

As the medical therapies with pure vasodilators have provided little or no beneficial effects on survival in the vast majority of patients, the focus of the treatment in recent years has changed from vasodilators to anti-proliferative agents. Goals of specific IPAH therapy should include reduction of pulmonary pressure and pulmonary vascular resistance, inhibition or reversal of pulmonary vascular remodelling and improvement of right ventricular function.

1.5.1 Prostacyclin analogues

These agents act through an increase in cAMP, thereby mediating vasodilation and inhibition of platelet aggregation and PASMCs proliferation. Continuous intravenous infusion of epoprostenol (PGI_2 analogue) for 3 months, in a prospective, randomized, controlled trial in 81 patients with class III or IV IPAH demonstrated improved survival and exercise tolerance, increased cardiac output, and decreased pulmonary vascular resistance (51). Moreover, a significant improvement occurred in patients who do not respond acutely to pulmonary vasodilatation, indicating that long-term treatment may be influencing cellular proliferation, a crucial mechanism in pulmonary vascular remodeling (52). However, due to the lack of pulmonary selectivity, intravenous epoprostenol may induce hypotension and worsening of the ventilation-perfusion mismatch. In

addition, use of epoprostenol is complicated with short half-life, cost and complicated modes of delivery.

Nonetheless, the beneficial effects of continuous PGI_2 therapy have led to trials using more stable analogues and alternative routes of administration. These include iloprost, available for intravenous, oral and inhalation use; UT-15, which is administered subcutaneously; beraprost, an orally active analogue. Inhaled iloprost, a stable prostacyclin analogue holds great promise in the PAH treatment. A randomized double-blind placebo-controlled multicentre trial of three months duration conducted in Europe in 203 patients with severe PAH demonstrated improved exercise capacity, symptoms, hemodynamic and quality of life in actively treated patients compared to placebo (53).

1.5.2 Inhaled nitric oxide

Since pulmonary arterial hypertension is associated with a defect in the production of potent and pulmonary specific vasodilator - nitric oxide, supplementation with inhaled nitric oxide (iNO) has been proposed as a potential therapy (54). Short-term inhalation of nitric oxide has substantial pulmonary specific vasodilator effects and proved beneficial in the treatment of IPAH (55). However, this treatment modality suffers from two potential complications. First, iNO causes increase in the pulmonary artery wedge pressure subsequent to pulmonary edema formation. Second, sudden termination of iNO occasionally causes a potentially life threatening hypertensive rebound effect. In addition, long term iNO therapy in large number of IPAH patients is limited by its short half life and cost (55,56).

1.5.3 Endothelin antagonists

As described above, endothelin plays a significant pathogenetic role in the development and progression of IPAH. Bosentan, an orally active dual endothelin receptor ETA /ETB antagonist, has been evaluated in a large randomized 16-week trial with NYHA class III and IV IPAH and PAH related connective tissue disease. Bosentan improved pulmonary hemodynamics, exercise capacity,

functional status, and the clinical outcome (57,58). However, severe side effects such as abnormal hepatic function and anemia developed in a significant percentage of patients taking this drug.

1.5.4 Phosphodiesterase inhibitors

Cyclic nucleotide phosphodiesterases (PDEs) comprise a large and complex group of structurally related enzymes, which catalyze the hydrolysis of cAMP and cGMP, and thereby regulate intracellular concentrations of these important "second messengers". In this manner, PDEs can affect various biological processes including the effects mediated by nitric oxide and prostanoids. Till date, eleven different mammalian PDE gene families have been identified (59,60). Of these, PDE5 is shown to be largely responsible for cGMP metabolism in the lung (61). The development of potent and selective PDE5 inhibitors, such as sildenafil, E4021, and E4010, has provided an opportunity to examine the effects of PDE5 inhibition as a treatment for PAH.

Sildenafil, when used for the treatment, has been shown to have acute hemodynamic effects in IPAH (61,62). Its clinical use in patients with IPAH in several, nonrandomized trials has been associated with improvements in function and hemodynamics (63). Thereby, suggesting the therapeutic efficiency of PDE5 inhibitors in IPAH and drives towards exploiting other PDE subtypes and their inhibitors in this disease.

Though not described in detail, other potential therapies include vasoactive intestinal peptides and selective serotonin reuptake inhibitors.

1.6 Nitric Oxide

NO is a gaseous free radical with only a few seconds of biological half-life, and has been identified as critical player in a remarkable array of essential biological processes, ranging from neurotransmission, the control of vascular tone, apoptosis to inflammation (16,64).

A glance back in history of NO, early studies by Furchgott and Zawadski demonstrated that endothelial cells are able to release a labile factor upon

acetylcholine stimulation, named as endothelium derived relaxing factor (EDRF) that diffuses to the adjacent muscle layer and causes vasorelaxation (65). Finally, in 1987, Ignarro and colleagues pharmacologically and chemically proved EDRF to be NO (66). This discovery and subsequent findings related to its biological functions such as platelet aggregation, vasodilation and neurotransmission were honored by Nobel Prize in 1998.

1.6.1 Nitric oxide synthase

NO is synthesized from amino acid L-arginine by a family of enzymes termed nitric oxide synthases (NOS) (67). Nitric oxide synthases are dimeric heme containing enzymes composed of oxygenase and reductase domains which possess binding sites for flavine dinucleotide (FAD), flavine mononucleotide (FMN), calmodulin (CaM) and tetrahydrobiopterin (BH4). In its active form, NOS forms a tetramer where two NOS monomers associate with two calmodulins and catalyzes five-electron oxidation of the terminal guanidino nitrogen atoms of L-arginine to generate L-citrulline and NO (Figure 3, Page 17) (68).

To date, three distinct isoforms of NOS enzymes have been identified, they are neuronal (nNOS or NOS1), inducible (iNOS or NOS2) and endothelial (eNOS or NOS3) NOS, the genes for which are located on chromosomes 12, 17 and 7 respectively (17). NOS 1, 2 and 3 were originally purified from neurons, vascular endothelium and cytokine-induced macrophages although the three isoforms are now known to be distributed across a wide spectrum of cell types and tissues. Furthermore, a particular type of cell can express more than one isoform of NOS (69).

Despite distinctions, due to high degree of sequence homology at c-terminal reductase domain, all NOS isoforms share important biochemical features mainly in concern to catalysis. All are NADPH and calmodulin-dependent and contain consensus binding sites for FAD and FMN, BH4, and a heme complex and mediates a five-electron oxidation of L-arginine to form NO and L-citrulline (68).

Notably, for all three NOS isoforms, NO synthesis depends upon the enzyme's binding of the ubiquitous calcium regulatory protein calmodulin. For eNOS and

nNOS, increases in resting intracellular Ca^{2+} concentrations [Ca^{2+}_i] are required for their binding to calmodulin and, consequently, for their becoming fully activated. In contrast, iNOS appears able to bind calmodulin with extremely high affinity even at the low [Ca^{2+}_i] characteristic of resting cells. Thus, the intracellular activity of the eNOS and nNOS may be closely modulated by transient changes in [Ca^{2+}_i], and signaling molecules such as bradykinin, acetylcholine and glutamate that increases intracellular Ca^{2+} concentration through receptor associated mechanisms (17,18). On the contrary, iNOS activity is no longer temporally regulated by intracellular calcium transients. Its expression and activity can be induced by various cytokines and produces huge amounts of NO for long periods of time (70).

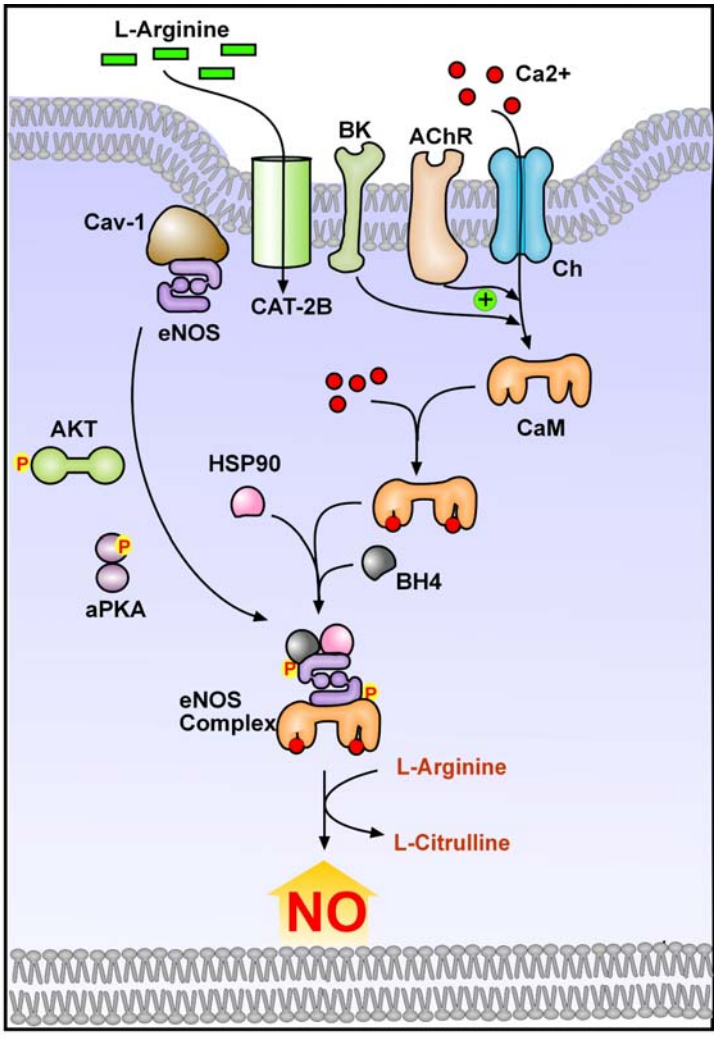

Figure 3: Nitric Oxide (NO) synthesis. NO is synthesized from the amino acid L-arginine by endothelial nitric oxide synthase (eNOS) in endothelial cells. Under basal conditions, eNOS is tethered to caveolin-1 (Cav-1) and inactive. However, with agonist (Bradykinin or fluid shear stress) induced increases in intracellular Ca^{2+}, calmodulin (CaM) binds to eNOS and displaces Cav-1 in a mechanism facilitated by cooperative binding of hsp-90, leading to activation of eNOS activity. In addition, kinases like Akt and PKA also facilitates CaM interaction with eNOS. The activated eNOS then translocates to the cytoplasm where it catalyzes NO generation from the amino acid L-arginine that was transported inside the cell via. a family of transporters called cationic amino acid transporter (CAT-2B). Tetrahydrobiopterin (BH4) is an important cofactor needed for NO generation (Author's Slide).

As discussed above, NO is a potent vasodilator known and plays an important role to maintain the stability of systemic and pulmonary hemodynamics. It causes relaxation by diffusing across the endothelial cell and stimulating soluble guanylate cyclase on the vascular smooth muscle cell. This converts GTP into cGMP. Relaxation results from an accumulation of cGMP, which then modifies several intracellular processes, lowers intracellular calcium and inhibits the contractile apparatus within the vascular smooth muscle cell (71). In addition, NO can also directly activate calcium-dependent potassium channels (72) leading to endothelium-dependent hyperpolarization of vascular smooth muscle cells, resulting in vasodilation.

The effects mediated NO are versatile and multifactorial. Beyond vasodilation, it also regulates leukocyte adhesion to the endothelium, inhibits vascular smooth muscle cell proliferation, apoptosis, platelet aggregation, and angiogenesis (Figure 4, Page 19). These intracellular processes are mediated not only by cGMP-dependent but also in a guanylate cyclase and cGMP - independent manner. cGMP independent biological functions involving high levels of NO, where NO reacts with superoxide anion (O^{2-}) to yield peroxynitrite anion ($ONOO^-$). Peroxynitrite by lipid peroxidation, direct deamination of DNA and inactivation of proteins mediates cytotoxic effects and tissue injury the effects that were mainly observed in iNOS induced inflammation. These controversial responses, cytoprotective and cytotoxic, can only be explained by direct and indirect interactions of nitric oxide (73).

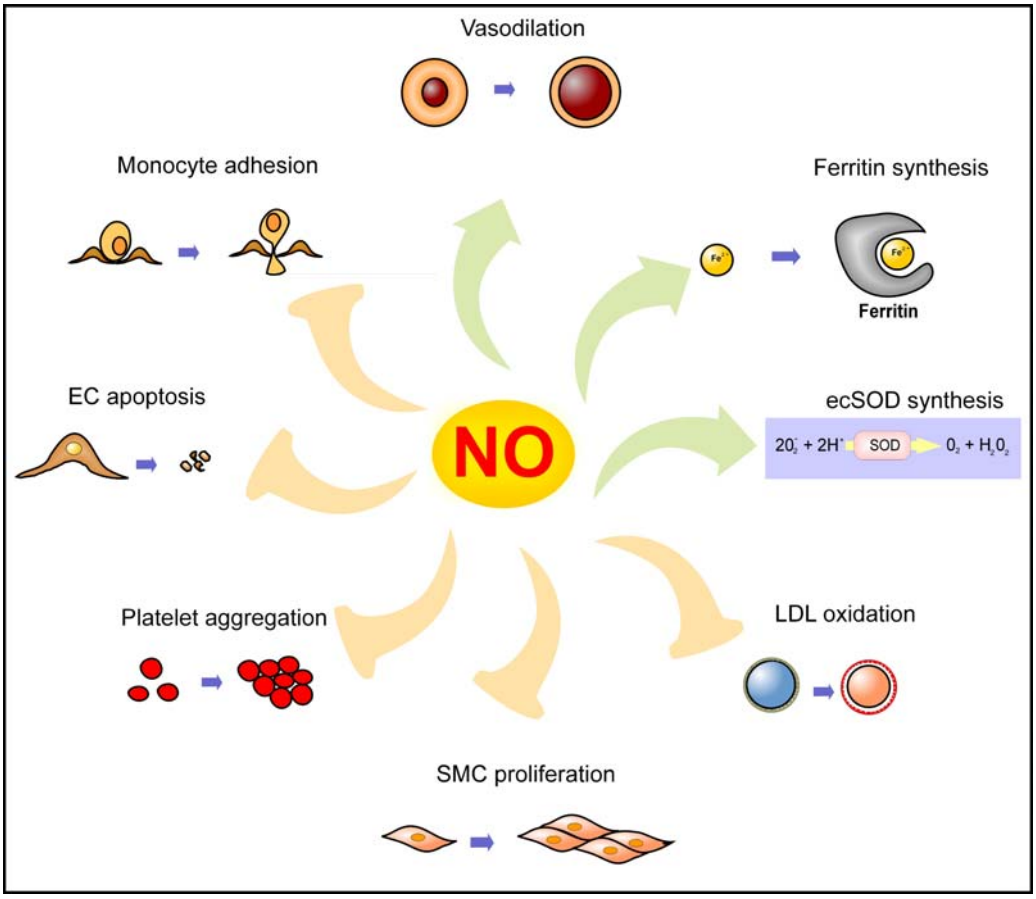

Figure 4: Pleotropic effects of Nitric oxide (NO). NO is an important molecular mediator of numerous physiologic processes in virtually every organ. In the vasculature, endothelium derived NO plays a crucial role in the regulation of vascular tone, inflammation, growth and the pro-thrombotic and anti-thrombotic properties (Author's Slide).

1.6.2 Regulation of NO

Due to short biological half-life and rapid diffusion rates, the cellular concentrations of NO are mainly dependent on rates of synthesis by the enzyme nitric oxide synthase (NOS). Therefore, cellular concentrations of NO depend on (i) NOS expression, (ii) NOS activity, (iii) substrate availability, (iv) NO half life, and (v) sensitivity to NO.

Although eNOS is constitutively expressed in vascular endothelial cells, both in vivo and vitro studies have demonstrated that basal expression and stability of

eNOS mRNA can be influenced by several stimuli. Shear stress, estrogen, lysophosphatidylcholine, and oxidized low density lipoprotein have been found to upregulate eNOS expression. On the other hand, tumor necrosis factor-α (TNF-α), erythropoietin, and high concentrations of oxidized LDL downregulates eNOS expression (74,75). Most interestingly, as a negative-feedback regulatory mechanism eNOS expression is modulated by NO itself via a cGMP-mediated process (76).

NO production in the endothelial cells can also be influenced by posttranslational modifications and subcellular targeting of eNOS. These post-translational modifications include phosphorylation, N-myristoylation and thiopalmitoylation. Studies from many research groups have indicated that the localization of eNOS within the cell, mainly at caveolae also determines its activity. In caveolae, caveolin-1 inhibits eNOS activity is by interfering with the calmodulin binding site. Factors like Ca^{2+}i increase, shear stress and certain post-translational modifications were shown to displace caveolin-1 and subsequently eNOS activation. Besides these, changes in substrate and cofactor availability, protein–protein interactions and phosphorylation state have gained importance as significant regulators of NOS activity (77).

The amino acid L-arginine is the only physiological substrate for NOS; hence regulation of its availability could be a major determinant in NO production. Interestingly, intracellular levels of L-arginine far exceed the Km of the NOS enzyme, so its availability is unlikely ever to be the rate-limiting step in the formation of NO by the endothelium in normal physiological conditions. However, a number of in vivo and in vitro experiments have shown that L-arginine availability can be rate-limiting for the generation of NO; especially in endothelial dysfunction associated pathophysiological conditions. This availability of L-arginine is not only influenced by its synthesis and degradation but also by its transcellular transport into the cell (77,78). A high-affinity carrier resembling the cationic amino acid transport (CAT) system y^+ is responsible for the transcellular transport of L-arginine and its analogues. The transporter activity is mediated by the CAT family that is composed of four isoforms, CAT-1, CAT-2A, CAT-2B, and

CAT-3. The recent finding that y^+ transporter co-locates with caveolin-bound eNOS suggest that activity of this transporter may be important to determine the local concentrations of L-arginine and their analogues (79). Various exogenous stimuli such as lipopolysaccharide (LPS), interleukin-1ß (IL-1ß), TNF-α, insulin, angiotensin II and bradykinin have been found to stimulate the L-arginine transport into endothelial cells by the y^+ system, which may increase NO production (80). Finally, an increasing number of reports in the literature indicate that endogenously produced inhibitors of NOS majorly regulate NO generation and may be responsible in numerous endothelial dysfunction associated disease states.

1.6.2.1 Endogenous NOS inhibitors

The guanidino-methylated L-arginine analogues N_G, monomethyl-L-arginine (L-NMMA), N_G,N_G-asymmetric dimethylarginines (ADMA); N_G,N_G-symmetric dimethylarginines (SDMA) have been shown to endogenously modulate L-arginine handling and / or NO synthesis in biological systems (Figure 5).

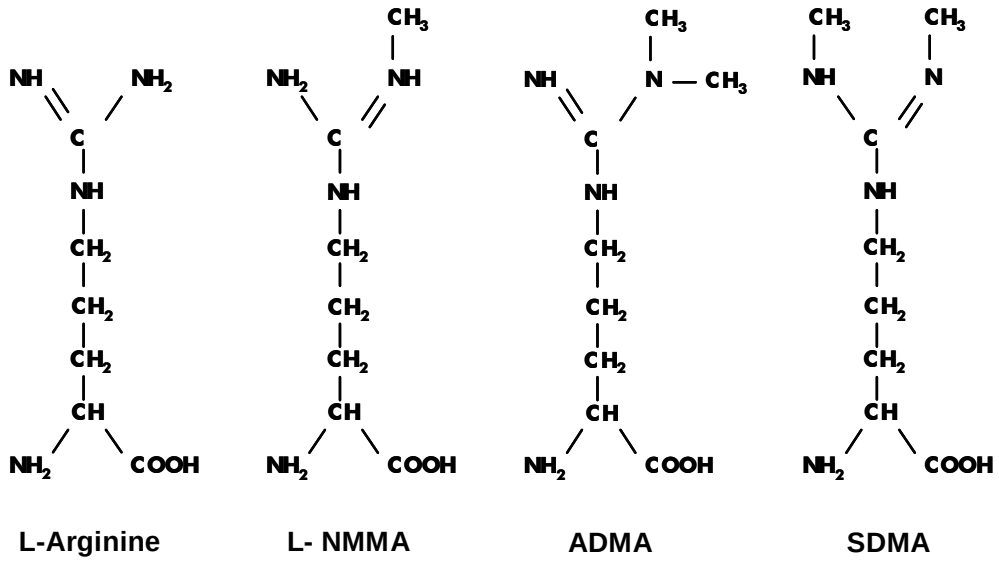

Figure 5: Chemical structures of methylarginines. (Masuda H, Nippon Yakurigaku Zasshi. 2002)

The biological significance of guanidino-methylated L-arginine analogues, mainly L-NMMA was first identified in 1986 as a compound that inhibits cytotoxic effects

of activated macrophages and prevents the release of nitrite and nitrate derived from L-arginine within these cells (81). Afterwards, L-NMMA became the standard nitric oxide synthase inhibitor used to evaluate the role of the L-arginine - nitric oxide pathway.

Methylarginines are endogenously derived from the proteolysis of methylated arginine residues on wide range of highly specialized nuclear proteins that are involved in RNA processing and transcriptional control (82). The methylation is carried out by a group of enzymes referred to as protein-arginine methyl transferases (PRMT) (83). Subsequent proteolysis of proteins containing methylarginine groups leads to the release of free methylarginine into the cytoplasm. However, the release of specific methylarginines depends on the subtypes of PRMT (PRMT I and PRMT II). PRMT type I catalyses the production of L-NMMA and ADMA in non-myelin basic protein substrates whereas PRMT type II catalyzes L-NMMA and SDMA in myelin basic protein substrates.

Plasma L-NMMA, ADMA and SDMA enter the cell through the y^+ cationic amino acid transporter and most interestingly they compete with each other as well as with L-arginine for transport (80). In addition, among these L-NMMA and ADMA effectively inhibits NOS but not SDMA. In cases of L-arginine limitation, they can even influence eNOS to generate even free oxygen radicals (84). However, ADMA seems to be more important as it is predominantly present (10 fold greater) than L-NMMA in plasma.

Once methyl arginines are released from methylated proteins by proteolysis, the two principal factors controlling levels are renal clearance and metabolism (85,86). SDMA is largely cleared by renal excretion. By contrast, the vast majority of ADMA and L-NMMA generated within the body is metabolized by the enzyme dimethylarginine dimethylaminohydrolase (DDAH) (Figure 6, Page 23).

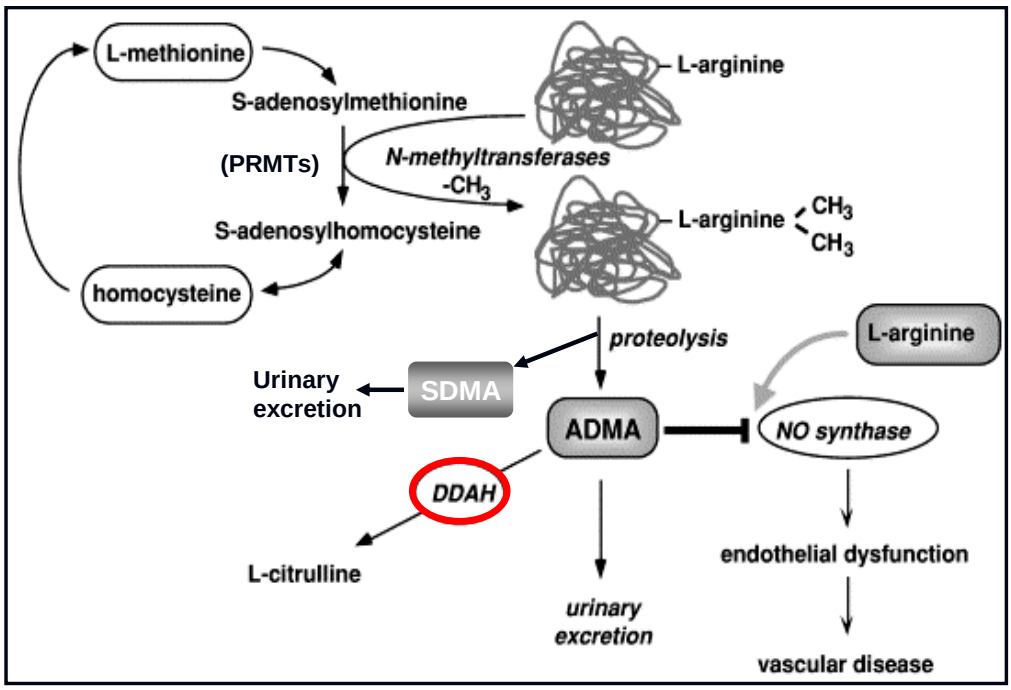

Figure 6: Schematic overview of the biochemical pathways related to methylarginines. Methylation of arginine residues within proteins or polypeptides occurs through *N*-methyltransferases (PRMTs), which utilize *S*-adenosylmethionine as a methyl group donor. After proteolytic breakdown of proteins, free ADMA, SDMA and L-NMMA are released into the cytoplasm. ADMA and L-NMMA acts as inhibitors of NOS by competing with the substrate of this enzyme, L-arginine, and causes endothelial dysfunction that was associated with various vascular diseases. SDMA can't inhibit NOS but it can efficiently compete with substrate L-arginine for its intracellular transport. ADMA is eliminated from the body via metabolism by the enzyme dimethylarginine dimethylaminohydrolase (DDAH) to citrulline and dimethylamine (Boger RH, Cardiovasc Res. 2003).

1.6.2.2 Dimethylarginine dimethylaminohydrolase (DDAH)

Identified and purified from rat kidney in 1987 by Ogawa and co-workers DDAH demonstrated specific hydrolysis of ADMA and L-NMMA to L-citrulline and either mono- or dimethylamine (86). Several studies suggest that DDAH is the key elimination route for ADMA as 83% of ADMA that was generated each day in humans is metabolized by DDAH and remaining small amount via renal system. Further, inhibition of DDAH activity with specific inhibitor, S-2-amino-4(3-methylguanidino) butanoic acid (4124W) on isolated vascular segments caused a

gradual vasoconstriction by elevating ADMA, evidenced for the first time the critical role of DDAH on ADMA levels and subsequent NO synthesis (87). This notion has been strengthened by recent observations made from a transgenic DDAH mouse model (88). The transgenic animals showed an increased DDAH activity and reduced ADMA levels. The reduction in plasma ADMA is associated with a significant increase in NOS activity and reduced systolic blood pressure, systemic vascular resistance, and cardiac stroke volume.

To date, two isoforms of DDAH have been described, DDAH1 and 2 located on chromosome 1p22 and 6p21.3, respectively (89). The overall amino acid sequence similarity between both isoforms is approximately 62% and is highly conserved among species. Isoforms, DDAH1 and 2 show different specific tissue distributions, DDAH1 is typically found in tissues expressing nNOS, whereas DDAH2 predominates in tissues containing the endothelial isoform of NOS (eNOS), supporting the hypothesis that intracellular ADMA concentration is actively regulated by DDAH in NO-generating cells.

Moreover, crystallographic analysis of DDAH have demonstrated that the active site of DDAH contains a free cysteine residue and one tightly bound non-catalytic zinc ion, which through reversible binding to the active site regulates the enzymatic activity (90), thus making it exquisitely sensitive to oxidative stress.

Human endothelial cells synthesize methylarginines and ADMA is produced in quantities that may affect NO synthesis. Chronic exposure of cultured endothelial cells with ADMA (2μM) has demonstrated intriguing changes in endothelial behavior (91). Yet under physiological conditions the production of ADMA and its effects are balanced by DDAH.

Intriguingly, plasma levels of ADMA were elevated and found to associate with endothelial vasodilator dysfunction in patients with coronary and peripheral arterial disease, and those with risk factors such as hypercholesterolemia, hyperhomocysteinemia, chronic renal failure and chronic heart failure (92-95). Specifically, each of these risk factors for cardiovascular disease is associated with endothelial oxidative stress and inactivation of DDAH (96,97). The impaired DDAH activity thereby allows ADMA to accumulate and to block NO synthesis.

Impaired DDAH activity in these disease conditions may be modulated by inflammatory cytokines, oxidized lipoproteins, glucose and homocysteine which are increased in endothelial oxidative stress.

1.6.3 NO and pulmonary hypertension

As described above, patients with IPAH have decreased levels of NO in their lungs, which may contribute to the development of pulmonary hypertension. Recently, patients with IPAH also illustrated reduced intrabronchial NO and NO biochemical reaction products compared with healthy individuals (19). Interestingly, the low levels of NO products correlated directly with the severity of disease. Even in experimental conditions, exposure to hypoxia impairs endothelium- dependent relaxation of isolated pulmonary vascular rings and the release of NO from cultured pulmonary endothelial cells (98). Thereby indicating the important role of NO axis in the regulation of vascular tone and remodeling of the hypertensive pulmonary circulation. The mechanisms of this impairment are undoubtedly multifactorial and may vary from patient to patient.

On the other hand, eNOS expression levels in IPAH patients remains controversial. Giaid and Saleh reported decreased eNOS expression (99) whereas Xue and Johns reported increased (100) and Tuder et al. unaltered eNOS immunostaining (27). While, eNOS expression is increased in the endothelium of the resistance pulmonary arteries in chronic hypoxia, Monocrotaline and the fawn hooded rats (101).

Thus, decreased levels of NO in the face of normal or increased NOS expression can be explained only by impaired NOS activity. Activity of arginase, an enzyme that metabolizes L-arginine was higher in PAH serum than in controls, suggesting that substrate availability affects NOS activity in the pathophysiology of PAH (102). If so, supplementary L-arginine could theoretically reverse this abnormality. Conversely, L-arginine infusion did not show any long term beneficial effects in these patients (103).

In experimental induced PAH, abnormal coupling with caveolin and HSP 90 were also shown to reduce NOS activation (104). Nevertheless, none of these entities

have shown to majorly influence the NO axis in pulmonary arterial hypertension. Despite the impact of endogenous NO-synthase inhibitors such as dimethylarginines (ADMA and SDMA) have come into the focus of attention for various endothelial dysfunction associated cardiovascular disorders. As current evidence strongly suggests a central role for endothelial dysfunction in the initiation and progression of IPAH, the plausible role of dimethylarginines is speculated in this disease. Hence forth, the present study was undertaken to investigate the potential role of dimethylarginines in the course of chronic pulmonary hypertension.

2. Aims of the study

Idiopathic pulmonary arterial hypertension (IPAH) is a progressive and life-limiting disorder which is associated with impaired bioactivity and/or synthesis of endogenous nitric oxide (NO). The mechanisms resulting in this impairment are multifactorial. Recently, the impact of endogenous NO-synthase inhibitors such as dimethylarginines (ADMA and SDMA) has come into the focus of attention in the pathogenesis and progression of various cardiovascular diseases associated with endothelial dysfunction. However, the potential role of dimethylarginines (ADMA and SDMA) in patients with IPAH has not yet been explored.

The aim of the present study was to evaluate dimethylarginines levels in IPAH patients and monocrotaline induced pulmonary hypertensive (MCT-PAH) rats. This was accompanied by assessing the precise mechanisms responsible for the altered dimethylarginines such as protein arginine methylation and/or alterations of the metabolizing enzyme DDAH in the course of chronic pulmonary hypertension were also investigated. This series of studies include the following aspects:

1. To evaluate plasma levels of L-arginine, ADMA and SDMA in IPAH patients and in MCT- PAH rats.

2. To evaluate biosynthesis of dimethylarginines in lung tissues from patients suffering from IPAH as well as on tissue from MCT- PAH rats.

3. To evaluate expression of DDAH isoforms in lung tissues from patients suffering from IPAH as well as on tissue from MCT-PAH rats.

4. To elucidate the molecular mechanisms responsible for DDAH alterations.

5. To test the therapeutic efficacy of selective phosphodiesterase inhibitors (PDEi) on the pulmonary hemodynamics and structural and molecular changes underlying MCT - PAH rats.

6. To test the hypothesis that selective PDEi may be effective in NO axis modulation and reversal of endothelial dysfunction associated with MCT-PAH.

7. To elucidate the effects of PDEi on dimethylarginines and subsequently on their biosynthesis and metabolism.

3. Materials and Methods

3.1. Materials

For RT-PCR analysis of human and rat tissue

huDDAH1 (NM_012137)

Forward	5’ GCAACTTTAGATGGCGGAGA 3´
Reverse	5’ TGGAAAGGCCCACAAAAA 3´

huDDAH2 (NM_013974)

Forward	5’ CTGTTGTGGCAGGCAGCAG 3´
Reverse	5’ GTCAGGGAGGCATATGGGTG 3´

huGAPDH (NM_002046)

Forward	5’ CGTCATGGGTGTGAACCATG 3´
Reverse	5’ GCTAAGCAGTTTGTGGTGCAG 3´

rDDAH1 (NM_022297)

Forward	5’ ATGGTGGGGACGTCCTATTC 3´
Reverse	5’ GCACCTCGTTGATTTGTCCT 3´

rDDAH2 (NM_212532)

Forward	5’ AGGGTCCAGAGAGGCGTAGG 3´
Reverse	5’ GGCTGGAAGCAGTGAGGCT 3´

rGAPDH (NM_017008)

Forward	5’ GTGATGGGTGTGAACCACGAG 3´
Reverse	5’ CCACGATGCCAAAGTTGTCA 3´

Antibodies

Antibodies used in the experiments are all commercially available. They were used for both western blot and immunohistochemistry until specifically specified.

Primary antibodies

Anti-DDAH1 (polyclonal) rabbit	Orbigen Inc, San Diego, USA
Anti-DDAH2 (polyclonal) goat	Calbiochem, BadSoden, Germany
Anti-GAPDH (monoclonal) mouse	Abcam,Cambridge, UK
Anti-ADMA (polyclonal) rabbit	Upstate, Hamburg, Germany
Anti-SDMA (polyclonal) rabbit	Upstate, Hamburg, Germany
Anti-alpha actin (monoclonal) mouse	Sigma,Steinheim,Germany
anti-von Willebrand factor (polyclonal) rabbit	Dako, Hamburg, Germany

HRP-conjugated secondary antibodies

Anti-mouse IgG rabbit	Sigma,Steinheim,Germany
Anti-rabbit IgG goat	Abcam,Cambridge, UK
Anti-goat IgG rabbit	Abcam,Cambridge, UK

Fluorophore-conjugated secondary antibodies

Anti-goat IgG rabbit Texas red conjugated	DakoCytomation, Hamburg, Germany
Anti-rabbit IgG goat FITC conjugated	Molecular Probes, Karlsruhe, Germany

Kits

Gibco, Eggenstein, Germany	
DNeasy Tissue kit	Qiagen, Hilden, Germany
Gel extraction kit	Qiagen, Hilden, Germany
qPCR™ Mastermix	Euro-genetec, Seraing, Belgium

Dye Reagent Concentrate	Bio-Rad, Muenchen, Germany
ECL detection kit	Amersham,Freiburg, Germany
Vectastain ABC kit	Vector, Burlingame, USA
Vector VIP substrate kit	Vector, Burlingame, USA
Vector DAB substrate kit	Vector, Burlingame, USA

Instruments

Cardiotherm 500-X	Harvard Apparatus GmbH, March-Hugstetten, Germany
ABI 7700 Sequence Detection System	Applied Biosystems, Darmstadt, Germany
Electrophoresis apparatus	Biometra, Gottingen, Germany
Small animal ventilator	IITC Life science, Woodland Hills, USA
Photodiode array detector,	Waters Corp, Milford, USA
cation-exchange extraction cartridges	Waters Corp, Milford, USA
BioDoc Analyzer	Biometra, Gottingen, Germany
Minigel-Twin	Biometra, Gottingen, Germany
Semi dry transfer unit	Biometra, Gottingen, Germany
Dot blot apparatus	Bio-Rad, Richmond, USA
Automatic vacuum tissue processor	Leica, Bensheim, Germany
Leica QWin Image Processing System	Leica, Bensheim, Germany
T3 Thermocycler	Biometra, Gottingen, Germany

Table 3: Buffer solutions

Buffer or solution	Components
1x Lysis buffer	50 µl 1M Tris (pH 7.6), 10 µl 1M $CaCl_2$,100µl 1.5 M NaCl, 100µl 0.6 M NaN_3, 10µl 10% Triton X, protease inhibitor cocktail made to 1 mL with DDH_2O
5x Laemmli buffer	1.8 mL DDH_2O, 2.5 mL 0.5 M Tris-HCl (pH 6.8), 2.5 mL Glycerol, 2 mL 20% SDS, 1mL ß-mercaptoethanol, 0.2 mL 1% (w/v) Bromophenol blue (in water)
1x Running buffer	3 g Tris base, 14.4 g Glycine, 10mL 10% SDS made to 1 Lit with DDH_2O
Transfer buffer	6 g Tris base, 3 g Glycine, 200 mL Methanol to 1 Lit with DDH_2O
1x PBS	7.2 g/L NaCl, 1.48 g/L Na_2HPO4, 0.43 g/L KH_2PO4, pH 7.4
1x TBST	2.24 g Tris base, 8.85 g NaCl, 1.8 g EDTA, 1 mL Tween 20 made to 1 Lit with DDH_2O
Blocking solution	5 g Skim milk powder in 100 mL 1x TBST
Stripping buffer	12.5 mL 0.5 M Tris-HCl (pH 6.8), 0.7 mL ß-Mercaptoethanol, 20 mL 10% SDS
Sodium phosphate Buffer (pH 7.2)	68.4 mL 1 M Na_2HPO4, 31.6 mL 1 M NaH_2PO4
5x TAE buffer	54 g Tris base, 11 mL Acetic acid, 20 mL of 0.5 M EDTA (pH 8.0) to1 Lit with DDH_2O

3.2 Methods

3.2.1 Patient characteristics and measurements

Eleven consecutive patients referred to our centre for diagnosis and treatment suffering from idiopathic pulmonary arterial hypertension (according to the classification of the Third World Symposium on Pulmonary Arterial Hypertension; (all NYHA class III or IV) gave written informed consent before inclusion into the study. Standard diagnostic procedures (imaging techniques, clinical chemistry, lung function testing, echocardiography, etc.) excluded other than the above mentioned causes for pulmonary hypertension. All patients were treated with chronic oral anticoagulation and diuretics before, but none received specific therapy for the pulmonary hypertension at timepoint of inclusion. Venous blood samples were drawn after a minimum resting period of two hours. Pulmonary and systemic hemodynamics were assessed by right heart catheterization following standard procedures of our Pulmonary Hypertension Centre. Exercise capacity was assessed by means of six minute walking test (according to the guidelines of the American Thoracic Society).

Human lung tissue was obtained from 7 donors and 7 IPAH patients undergoing lung transplantation. Patient lung tissue was snap frozen directly after explantation for mRNA and protein extraction or directly transferred into 4% buffered paraformaldehyde, fixed for 24 h at 4°C and embedded in paraffin. Hemodynamic measurements in 5 of the 7 IPAH patients who underwent transplantation revealed a mean pulmonary artery pressure of 68.4±8.5 mmHg. Tissue donation was regulated by the national ethical committee and national law. All patients enrolled in this study gave written informed consent.

3.2.2 Animal experiments

Adult male Sprague Dawley rats (300-350g body weight) were obtained from Charles River Laboratories (Sulzfeld, Germany). The experiments were performed in accordance with the National Institutes of Health Guidelines on the Use of Laboratory Animals. Both the University Animal Care Committee and the

Federal Authorities for Animal Research of the Regierungspräsidium Giessen (Hessen, Germany) approved the study protocol.

3.2.3 MCT treatment

The alkaloid Monocrotaline (MCT) (Sigma) was dissolved in 0.5 N HCl, and the pH was adjusted to 7.4 with 0.5 N NaOH. The MCT (20mg/ml) solution was given as a single subcutaneous injection (60 mg/kg) to male Sprague-Dawley rats. Control rats received an equal volume of isotonic saline.

3.2.4 Chronic dosing study

For chronic intervention studies, rats were randomized to receive either placebo or tolafentrine by osmotic minipumps. Tolafentrine (chemical name : (-)-cis-8,9-Dimethoxy-1,2,3,4,4a-10b-hexahydro-2-methyl-6-(4-p-toluenesulfonamido-phenyl)-benzo[c][1,6]naphthyridine) was synthesized in the laboratories of ALTANA Pharma, Konstanz, Germany. Tolafentrine, the active drug, is a mixed selective PDE3/4 inhibitor with PDE3 inhibitory activity with an IC_{50} of 60 nmol/L and inhibiting activity of PDE4 with an IC_{50} of 100 nmol/L. The dose of tolafentrine (625 ng/kg per minute) was chosen according to preceding pilot experiments, addressing long-term tolerability of this agent under investigation. Treatment was started 2 weeks after injection of MCT, during development of pulmonary hypertension, for duration of 2 weeks.

3.2.5 Surgical preparation and tissue preparation

Two weeks after a single MCT injection, rats were subjected to inhalation of tolafentrine or sham nebulization in an unrestrained, whole body aerosol exposure system as described. For assessment of chronic effects of inhaled saline or tolafentrine (dose deposited in the lungs ~ 120 µg/kg day), 15 min nebulization maneuvers using a jet nebulizer with a constant flow rate of 6 l/min (Pari LC Star, Pari, Starnberg, Germany) were repeated twelve times per day for 2 weeks (day 14 – 28).

At the end of the treatment protocol, the animals were anesthetized with intraperitoneal pentobarbital and tracheostomized. They were artificially ventilated with a small animal ventilator 10 ml per kg body weight (BW) and a frequency of 60 s^{-1}. Inspiratory oxygen (FIO_2) was set at 0.5, and a positive end-expiratory pressure of 1.5 cm H_2O was used throughout. Anesthesia was maintained by inhalation of isoflurane. The left carotid artery was cannulated for arterial pressure monitoring, and a right heart catheter (PE 50 tubing) was inserted through the right jugular vein for measurement of right ventricular systolic pressure (RVSP) with fluid-filled force transducers (zero referenced at the helium). Cardiac output (CO) was measured by thermodilution technique using Cardiotherm 500-X. Briefly, a thermistor (1.5F) was placed into the ascending thoracic aorta via the right carotid artery for measurement of transpulmonary thermodilution CO. A 0.15-mL bolus of room-temperature saline was injected into the right ventricle as the indicator. CO was averaged from three consecutive determinations and indexed to the weight of the animal to obtain cardiac index.

Arterial and mixed venous samples were collected (150 µl) and analyzed for PO_2, pH and PCO_2. Hemoglobin and oxygen saturation were measured using an OSM2 Hemoximeter. After exsanguination, the lungs were flushed with isotonic saline at a constant pressure of 22 cm H_2O via the pulmonary artery. The right lung was ligated at the hilus and shock frozen in liquid nitrogen, and stored at – 80 °C for molecular studies. The left lobe was perfused for 5 minutes with Zamboni's fixative at a pressure of 22 cm H_2O via the pulmonary artery. The tissue was fixed in Zamboni's fixative for 12 hours at 4°C and then transferred into 0.1 M phosphate buffer.

As an index of right ventricular hypertrophy, the ratio of the right ventricle weight to left ventricle plus septum weight (RV/LV+S) was calculated.

3.2.6 RNA isolation

Total RNA was isolated from exponentially growing cells and lung tissue homogenates using TRIzol® reagent. 100 mg of lung tissues were homogenized

in 1.0 ml TRIzol® reagent using polytron homogenizer and incubated for 10 min. Following 10 min incubation, 200 µl of chloroform was added to these samples. Samples were shaken vigorously by hand for 15 sec and incubated at 15-30°C for 2-3 min and centrifuged at 12,000 x g for 15 min at 4°C. The aqueous phase was removed after centrifugation and RNA was precipitated from the aqueous phase by adding 0.5 ml of isopropanol per 1 ml of TRIzol reagent used for initial homogenization. RNA was incubated for 10 min at room temperature and centrifuged at 12,000 x g for 10 min at 4°C. The RNA pellet was washed with 1 ml of 70% ethanol per 1 ml of TRIzol reagent used for initial homogenization. The sample was centrifuged at 7,500 x g for 5 min at 4°C. The RNA pellet was re-dissolved in DEPC treated water and stored at -70°C.

RNA concentration and purity were measured spectroscopically by its absorbance at 260 nm and 280 nm. For the estimation of RNA concentration the below formula was employed.

RNA concentration = A_{260} x dilution x 40 µg/ml

For the estimation of purity, A_{260}/A_{280} ratio is calculated. The Ratio between 1.7 to 2 is considered to be good RNA in our experiments.

3.2.7 cDNA Synthesis

Complementary DNA was synthesized from total RNA using Improm II Reverse Transcriptase (Promega, Germany). Two µg of total RNA was combined with 0.5 µg of Oligo$(dT)_{15}$ in nuclease-free water for a final volume of 5µl per RT reaction. This mixture was denatured at 70°C for 5 min followed by rapid cooling. After a short spin, the reverse transcription reaction mix was added and incubated at 25°C for 5 min followed by incubation at 42°C for 1 hr. After synthesis, cDNA samples were either used immediately for PCR, or stored at -20˚C.

Reverse Transcriptase reaction mix:

ImProm-II™ 5X Reaction Buffer	4.0µl
$MgCl_2$, 25mM	2.0µl
dNTP Mix, 10mM	1.0µl
RNasin® Ribonuclease Inhibitor	1.0µl
ImProm-II™ Reverse Transcriptase	1.0µl
Nuclease-Free Water	6.0µl
Final Volume	**15.0µl**

3.2.8 Polymerase chain reaction

To check for the presence of the gene of interest either in the animal tissue or primary cells, PCR was performed. PCR signal was amplified using the gene-specific primers (as described) designed from the sequence available in the Genbank. For standard PCR, 20-23 bp long primers were designed, AT and GC content was checked and the difference in the melting temperature (Tm) between the forward and reverse primers was kept not more then 2-4ºC. The primer sequence was checked using the NCBI BLAST search for probable similarity with unrelated genes. PCR reaction was done in 0.2 ml thin wall tubes in T3 Thermocycler. Negative control without template performed to check for self-annealing of primer pair. PCR for cDNA without reverse transcriptase was also done to check the genomic DNA contamination. Each primer pair was checked with several annealing temperatures depending on the Tm of the primer pair to get a single and specific PCR band.

3.2.9 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Semi quantitative RT-PCR was performed on cDNA samples by use of Taq polymerase (Promega, Germany). The reaction mixture included.

PCR master mix

Nuclease-Free Water	35.0µl
10X polymerase reaction buffer	5.0µl
$MgCl_2$, 25mM	3.0µl
PCR Nucleotide Mix, 10mM	1.5µl
Forward Primer, 10µM	1.0µl
Reverse Primer, 10µM	1.0µl
Taq DNA Polymerase (5.0 units)	0.5µl
Volume of RT reaction added	3.0µl
Total PCR Volume	**50.0µl**

The tubes were flicked to mix and microfuged briefly before taking to the PCR machine. The thermal cycler's program was as follows:

Activation of HotstarTaq	95°C	2 min
Denaturation	94°C	30 s
Annealing	variable	30 s
Extension	72°C	45 s
Final extension	72°C	10 min
Cycles	30-35	

For quantitative real-time RT-PCR analysis, 2 µl cDNA was placed into 50µl reaction volume containing SYBR Green PCR mix and sequence-specific oligonucleotide primers. In all cases forward and reverse primers were designed flanking an intron to make sure that the amplification signal comes from mRNA and not genomic DNA.

Real time PCR master mix:

Nuclease-Free Water	16.0µl
2x-Invitrogen Super Mix-Buffer	25.0µl
ROX Reference Dye 50x	1.0µl
$MgCl_2$, 50mM	2.0µl
Forward Primer, 10µM	1.0µl
Reverse Primer, 10µM	1.0µl
Volume of RT reaction added	1-3µl
Total PCR Volume	**50.0µl**

The thermal cycle conditions used for all reactions were as follows:

Activation of Taq	50°C	2 min
Denaturation	95°C	10 min
Denaturation II	95°C	10 s
Annealing	60°C	5 s
Cycles	40	

All real-time reactions were carried on ABI 7700 Sequence Detection System, and analysis was performed with the accompanying software. At the end of the PCR cycle, a dissociation curve was generated to ensure the amplification of a single product and the threshold cycle time (Ct values) for each gene was determined. Relative mRNA levels were calculated based on the Ct values and normalized to house keeping gene GAPDH.

3.2.10 Agarose gel electrophoresis and PCR product purification

PCR reactions were analyzed on 1% agarose gels in 1X TAE buffer, containing 1 µg/ml ethidium bromide. Samples were prepared in 6x loading buffer (containing bromophenol blue dye) (MBI Fermentas). Gel was run in 1X TAE buffer for 60 min at 80 V. Depending on the fragment size either GeneRuler 100bp DNA Ladder (1 kb) or GeneRuler DNA Ladder Mix (10 kb) (MBI Fermentas) used. DNA bands were visualised under UV-Transilluminator of Biometra system.

For Semi-quantitative analysis of the PCR product, 10 – 20 µl of each reaction was used for electrophoresis. PCR signals were quantified in arbitary units (A.U) from optical density x band area. PCR signals were normalized to the GAPDH signal of the corresponding RT product to get a semi-quantitative estimate of the gene expression.

The PCR product was purified with the QIAGEN gel extraction kit. Briefly, samples were loaded onto the gel. After gel run the corresponding bands were

excised and placed in 1.5 ml eppendorf tubes. Then add 300 µl of buffer QG for every 100 mg of gel. Incubated the gel slices in buffer at 50°C for minimum 15 min. Then add 100 µl of isopropanol and placed in a QIAquick column. Centrifuge for 1 min at maximum speed. Then add 500 µl of buffer QG to the column and centrifuge again. After add 700 µl of buffer PE (containing ethanol) to the column and incubated for 5 min at room temperature. After centrifugation, Place the column into a fresh 1.5 ml recovery tube and added 30 µl of prewarmed buffer EB directly to the center of the column. Incubated for 10 min at room temperature and then centrifuged for 2 min at 12,000 rpm. Discarded the column and stored the DNA at -20°C. Further DNA was sent for sequencing.

3.2.11 Western blotting

Lung tissues were homogenized in 1x lysis buffer including protease inhibitor cocktail using a polytron tissue homogenizer. Later, samples were centrifuged at 14,000 rpm in a pre-cooled centrifuge for 20 min. Supernatant was transferred into a fresh tube and measured for Protein concentration using Bradford assay with a bovine serum albumin standard. Lysates were aliquoted and stored at -80°C. In case of cells, confluent cells (6 well plates) were washed 1x with ice-cold PBS and lysed in 100 µl of 1x lysis buffer. The cell lysate was gently mixed on a rotator for 15 min at 4°C. Lysate was then sonificated 3x10 sec in Ultrasonificator on ice followed by centrifugation. Supernatant was measured for Protein content as described above.

When necessary, tissue and cell lysates containing equal amounts of protein were diluted with 5 x laemmli buffer (5:1 ratio of sample volume to buffer volume), boiled for 5 min at 95°C and subsequently pipette into the gel chambers for electrophoresis. The SDS PAGE was run at a constant voltage of 130 V for 1-1.5 hr in 1 x running buffer. Upon completion of electrophoresis, the gel was removed from glass plates and allowed to soak in transfer buffer for 15 min. Gels were blotted onto nitrocellulose membrane using a semi dry technique. From bottom to top, the following layers were put in a blotting apparatus: three layers of whatman filter paper soaked followed by the nitrocellulose membrane, the gel,

and three layers of whatman paper soaked in 1x transfer buffer. Special attention was paid to eliminate all air bubbles between the layers. Electroblotting occurred for 1-1.5 hr at 100 mA per two gels. Afterwards, membranes were incubated in blocking solution for 1 hr at room temperature, and then incubated with a polyclonal rabbit DDAH1 (1:10,000), polyclonal goat DDAH2 (1:5000) or a mouse monoclonal GAPDH (1:5000) antibody overnight at 4°C. Later the membranes were washed (3x10 min) with wash buffer, incubated with the respective HRP-conjugated polyclonal secondary antibodies for I hr at room temperature. Finally after three washing steps with wash buffer antibodies bound to the proteins on the membrane were detected using the "ECL Western Blotting Detection Reagent", according to the manufacturer's manual.

3.2.12 Dot Blotting

Samples were adjusted to 2 µg/L of protein and adsorbed on to a nitrocellulose membrane by using a dot blot apparatus. After non-specific blocking with 5% (w/v) lipid free milk, the membranes were incubated overnight with ADMA or SDMA antibody (1:1000) and then with HRP-conjugated goat anti-rabbit IgG (1:5000) for 1 hr at room temperature. Membranes were developed with ECL detection kit. The intensity of the dots were analyzed and quantified by Biometra image analysis software. Equal amount of protein loading was also confirmed by dot blotting membranes with an antibody against GAPDH.

3.2.13 Immunohistochemical staining

Paraffin-embedded lung tissue sectioned at a 3 µm thickness was deparaffinized in xylene and rehydrated in a graded ethanol series to phosphate-buffered saline (PBS, pH 7.2). Antigen retrieval was performed by pressure cooking in citrate buffer (pH 6.0) for 15 min. Immunohistochemical staining was performed using anti-ADMA, anti-SDMA, anti-DDAH1 and anti-DDAH2 antibodies in conjunction with an avidin-biotin-peroxidase kit. Briefly, the sections were pre-treated with 3% hydrogen peroxide to quench endogenous peroxidase activity. Then, the sections were incubated for 1h in 10% normal goat serum to block non-specific binding

sites prior to the application of primary antibodies. Sections were then incubated overnight at 4°C with the primary antibodies (Abs). Biotinylated anti-rabbit or anti-goat immunoglobulins (Ig) and avidin-biotinylated enzyme complex (ABC kit) were applied according to the manufacturer's instructions. After each incubation step, sections were washed briefly in PBS. Development of the reaction was carried out with VIP substrate for horseradish peroxidase for 10 min. Finally, sections were counterstained with methylgreen and dehydration using graded alcohol and xylene. The sections were then cover slipped and allowed to dry overnight.

3.2.14 Histological analysis

After dehydration in automatic vacuum tissue processor and paraffin embedding, 5µm sections were stained for Elastin-Nuclear Fast Red to assess the medial wall thickness. For quantitative analysis of the degree of muscularization of small pulmonary arteries a double staining for alpha smooth muscle actin and von Willebrand (vWF) factor was performed. For visualization of the alpha smooth muscle actin the Vector Vip substrate kit for horseradish peroxidase and for visualization of the anti-vWF antibody 3, 3′-diaminobenzidine (DAB) substrate was used. Nuclear counterstaining was done with methyl green.

3.2.15 Measurement of ADMA and SDMA

L-arginine, ADMA, and SDMA were simultaneously analyzed by high-performance liquid chromatography (HPLC). Briefly, plasma was mixed with internal standard and PBS and applied to Oasis MCX solid-phase extraction cartridges. After washing with Hydrochloric acid and methanol, amino acids were eluted with 1.0 ml of concentrated ammonia/water/methanol (10/40/50). The solvent was evaporated under a stream of nitrogen and the amino acids were derivatized with o-phthaldialdehyde reagent containing 3-mercaptopropionic acid. The derivatized amino acids were separated by isocratic reversed-phase chromatography on a C18 column at a column temperature of 30°C using a mobile phase consisting of potassium phosphate buffer (50 mmol/L ; pH 6.5),

containing 8.7% acetonitrile at a flow-rate of 0.3 ml/min. Fluorescence detection was performed at excitation and emission wavelengths of 340 and 455 nm, respectively. The stable derivatives were separated with near baseline resolution. Using a sample volume of 0.2 ml, linear calibration curves were obtained with limits of quantification of 0.08 µM for L-arginine and 0.01 µM for ADMA and SDMA.

3.2.16 DDAH activity assay

DDAH activity was assayed by determining L-citrulline formation in tissue homogenates by colorimetric method in 96-well microtiter plates.

Lung tissues were homogenized using polytron homogenizer and centrifuged at 13000 rpm to separate soluble material from insoluble cell debris. Aliquots of lysates were assayed for DDAH activity with a colorimetric assay in 96-well microtiter plates for citrulline production. For colorimetric assays, 55 µl of tissue lysate in 100 mM Na_2HPO_4, pH 6.5 was incubated at 37 °C for 60 min with 5 µl of containing 80 mM L-NMMA, ADMA, SDMA or L-arginine, or with buffer alone. After incubation, the reaction was stopped, and the activity was calculated after measuring the concentration of L-citrulline with a chromogenic reaction that specifically determines ureido groups. The calibration curve was obtained using L-Cit concentrations between 0 and 400 µM. One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 µmol/L L-citrulline per min at 37°C. As the assay blank, the homogenate was subjected to the same determination process of DDAH activity in the absence of ADMA to provide the background values. Genuine DDAH activity was obtained by experimental data subtracting the background values.

3.2.17 NOx measurements

Plasma samples were stored at –80°C for less than 2 weeks before analysis. At the time of NOx assay, plasma samples were ultrafiltered (30 kDa molecular weight cut-off) and centrifuged at 1000 *g* for 60 min in order to remove hemoglobin, which is known to interfere with spectrophotometric measurements.

NOx concentration in different dilutions of plasma ultrafiltrate was determined by using a reagent kit (Cayman, Ann Arbor, USA) based on the Griess reaction, which consists of three main steps: 1) enzymatic conversion of nitrate to nitrite by means of nitrate reductase in the presence of 5 mmol/l NADPH; 2) incubation with Griess reagent (0.1% *N*- (1-naphthyl) ethylenediamine dihydrochloride and 1% sulfanilamide in 2.5% H_3PO_4) for 10 min at 25°C to convert nitrite into a chromophore compound; 3) quantitative estimation of nitrite concentration by spectrophotometric measurement of the absorbance at 540 nm. Standards for calibration curves were prepared with nitrate and taken through the full assay procedure. The results were expressed as µmol/l of NOx.

3.2.18 Culture of human HUVEC cells

ECs were isolated from human umbilical veins (HUVEC) and grown in EGM-2 culture medium including supplements and growth factors by the vendor (Cambrex Bio Science, Walkersville, USA) at 37°C in a CO_2 incubator. For assessment of cytokine mediated DDAH expression, HUVECs at passage 3 or less were grown in 6-well plates and after 95% confluence incubated with 10 ng/mL interferon gamma (IFNγ) or 10 ng/mL tumor necrosis factor-α (TNF-α) or both. After 24 hrs cells were scrapped with protein lysis buffer.

3.2.19 Data analysis

All data are given as mean ± SEM. Differences between the groups were assessed by student's t test with a p value < 0.05 regarded to be significant.

4. Results

4.1 IPAH patients

4.1.2 IPAH patient characteristics

Patient characteristics are given in Table 4. Blood from eleven patients with IPAH (all NYHA class III and IV) was collected and L-arginine, ADMA and SDMA levels were determined. Mean pulmonary artery pressure was 52.7±3.2 mmHg and cardiac index 2.19±0.11 l/min m^2 with the pulmonary vascular resistance index (PVRI) calculated at 1360±104 dyne s cm^{-5} m^{-2}. The six-minute-walk distance was 318±28 m.

Patient	NYHA	SMW	HR	mSAP	mPAP	PAWP	CVP	SVRI	PVRI	CI	SaO_2	SvO_2
		[m]	beats/min	[mmHg]	[mmHg]	[mmHg]	[mmHg]	[dyne s cm^{-5} m^2]	[dyne s cm^{-5} m^2]	[l/min m^2]	[%]	[%]
1	3	432	90	97	60	5	5	1968	1176	2.17	99.1	69.3
2	3	401	73	112	64	10	7	1683	866	2.92	96.6	73.2
3	3	333	94	86	56	7	18	1397	1104	1.87	92.6	65.3
4	3	347	73	85	62	11	11	1691	1166	2.25	83.3	62.2
5	3	290	74	74	31	6	6	3425	1259	1.59	96.5	65.9
6	4	168	63	67	51	10	8	2517	1749	1.87	86.2	57.9
7	3	415	82	88	63	11	11	2898	1957	2.13	80.0	64.2
8	4	145	80	125	50	8	9	3811	1380	2.43	92.2	57.9
9	3	370	84	102	47	8	7	2831	1162	2.68	98.6	74.8
10	3	302	64	99	39	5	4	3613	1293	2.10	92.2	56.0
11	3	298	86	135	57	8	7	4854	1858	2.11	89.8	64.2
Mean		318	78	97.3	52.7	8.1	8.5	2789	1360	2.19	91.6	64.6
SEM		28	3	6.2	3.2	0.7	1.2	323	104	0.11	1.9	1.8

Table 4: IPAH patient characteristics. NYHA = New York Heart Association functional classes; SMW = six minute walk testing (according to the guidelines of the American Thoracic Society); HR = heart rate; mSAP = mean systemic arterial pressure; mPAP = mean pulmonary artery pressure; PAWP = pulmonary artery wedge pressure; CVP = central venous pressure; SVRI = systemic vascular resistance index; PVRI = pulmonary vascular resistance index; CI = cardiac index; SaO_2 = arterial oxygen saturation; SvO_2 = venous oxygen saturation.

4.1.2 ADMA and SDMA elaboration in IPAH patients

We have observed elevated plasma levels of ADMA and SDMA in patients with IPAH. As compared to healthy subjects, ADMA was increased from 0.48±0.04 to 1.06±0.06 µmol/l and SDMA from 0.53±0.07 to 1.46±0.24 µmol/l (Figure 7).We

also have found that plasma levels of L-arginine decrease significantly in IPAH patients compared to healthy subjects. Most impressively, L-arginine/ADMA ratio is also reduced in IPAH patients.

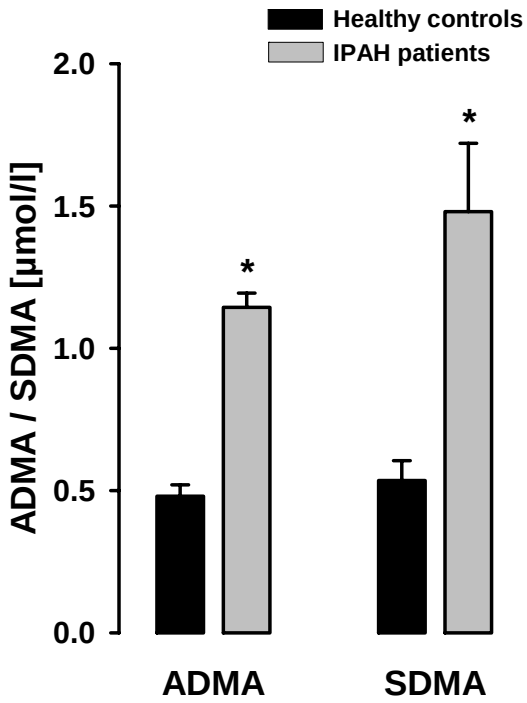

Figure 7: Measurement of ADMA and SDMA levels from plasma of healthy subjects and patients with IPAH. Free ADMA and SDMA were measured by reverse phase HPLC from plasma of healthy subjects and patients with IPAH. Values are means ± SEM and represent 11 patients and 8 healthy subjects. *, $p<0.05$ versus healthy subjects.

4.1.3 Localization of dimethylarginines in lungs from patients with IPAH

Immunohistochemical staining for asymmetric and symmetric dimethylated arginine proteins within human lung tissue is shown in Figure 8. In normal human lung, immunoreactivity to specific asymmetric and symmetric dimethylated arginine proteins was observed in the bronchiolar ciliated and terminal cuboidal epithelium, type I and type II alveolar epithelium, arterial and capillary endothelium, and alveolar macrophages with very little difference in their distribution throughout the lung. In comparison, the specific asymmetric and

symmetric dimethylated arginine proteins immunoreactivity was preferentially increased in the pulmonary endothelium of lung specimens from patients with IPAH. Immunostaining was more marked in those areas with more severe medial hypertrophy, as well as in plexiform lesions. In contrast, no staining difference was observed in alveolar or bronchiolar epithelium.

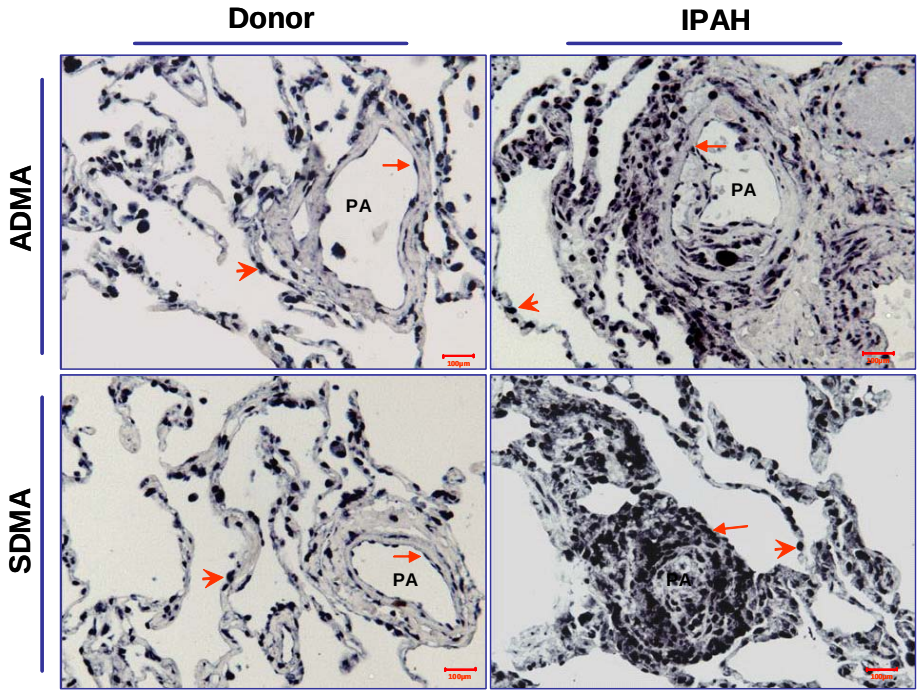

Figure 8: Immunostaining of dimethylated arginine proteins in lungs from healthy donors and patients with IPAH. Lung sections from a healthy donor and IPAH patient with anti-ADMA and anti-SDMA shows immunoreactivity of asymmetric and symmetric dimethylated arginine proteins in pulmonary arterial endothelial cells and in alveolar epithelial cells. →, indicates endothelium; ➤, indicates epithelium. Scale bar: 100µm; PA: pulmonary artery.

4.1.4 Increased biosynthesis of dimethylarginines in lungs from patients with IPAH

The tissue levels of asymmetric and symmetric dimethylated arginine proteins were quantified by dot-blotting. Significant higher degree of dimethylation was demonstrated in IPAH compared to healthy donors (Figure 9). As compared to donor lungs, asymmetric and symmetric dimethylated arginine proteins were upregulated 210 ±18 % and 240 ± 26% respectively, in IPAH lungs.

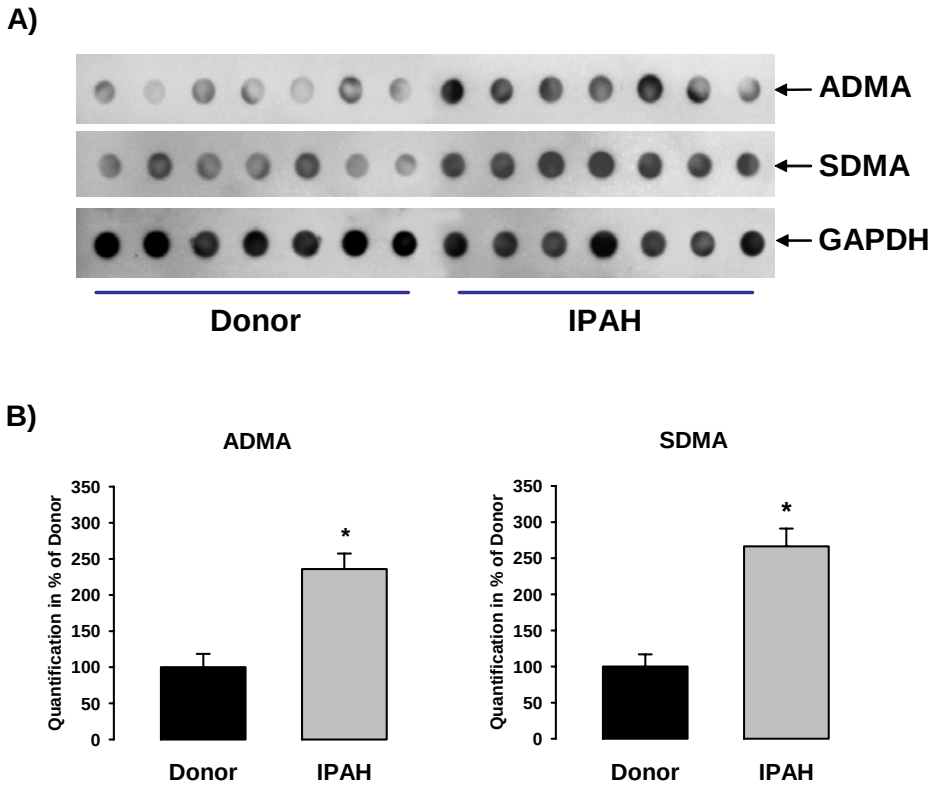

Figure 9: Dot blot analysis of dimethylated arginine proteins in lungs from healthy donors and patients with IPAH. (A) Dot blotting with anti-ADMA and anti-SDMA antibodies and subsequent **(B)** quantification of asymmetric and symmetric dimethylated arginine proteins in lung homogenates from healthy donors and patients with IPAH**.** Values (means ± SEM) are expressed as percentage of expression found in donor tissue (n=7). *, $p<0.05$ versus donor.

4.1.5 Localization of DDAH isoforms in lungs from patients with IPAH

Immunohistochemical localization of DDAH isoforms within human lung tissue is shown in Figure 10. Immunostaining was detected strongly in the endothelium of all generations of arteries, with a weak or no immunostaining for DDAH1 and DDAH2 expression in either vascular smooth muscle or adventitial cells. Additional immunostaining was detected in both alveolar (low level) and bronchiolar epithelium; specific and intense staining for DDAH1 was observed particularly on the apical surface of the bronchiolar epithelium, whereas DDAH2 dominated in basal cells of the bronchiolar epithelium. Neither airway smooth

muscle nor adventitial cells exhibited DDAH1 or DDAH2 immunostaining. When compared to controls, a marked reduction or even absence of DDAH2 immunoreactivity was observed in the endothelium of sections from patients with pulmonary arterial hypertension. In contrast, no significant difference was observed in DDAH1 immunostaining density between IPAH and healthy donor lungs. There was no immunostaining observed in the negative control sections.

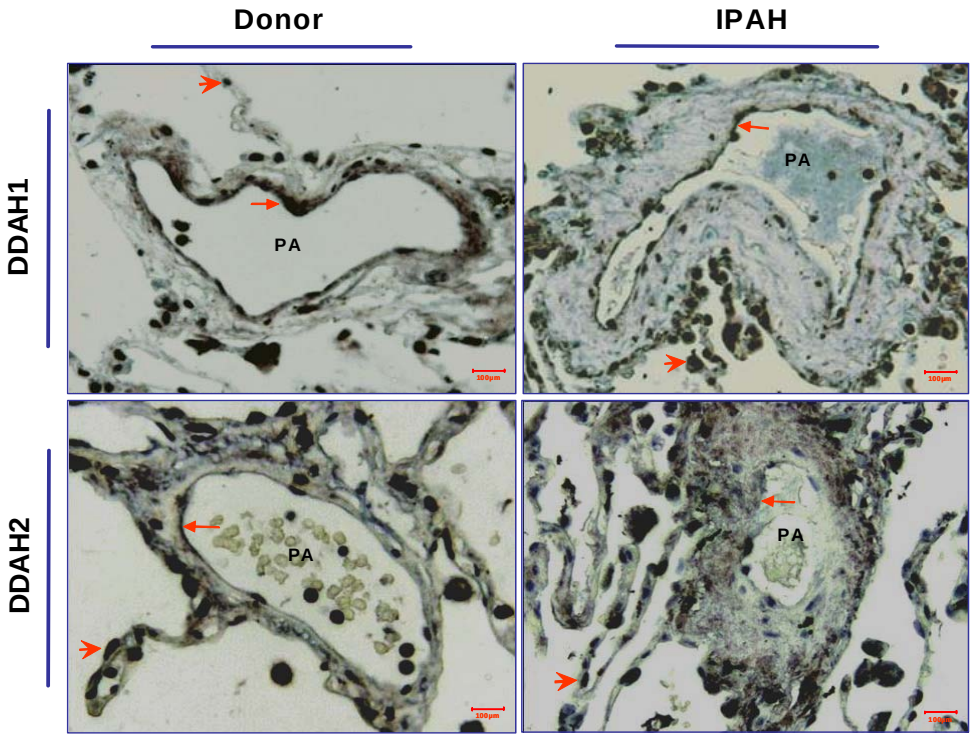

Figure 10: Immunostaining of DDAH isoforms in healthy donor and IPAH patient lungs. Lung sections from a healthy donor show immunoreactivity of DDAH1 and DDAH2 in pulmonary arterial endothelial cells and in alveolar epithelial cells. Lung sections from patients with IPAH even show strong immunoreactivity of DDAH1 with in pulmonary arterial endothelial cells and in alveolar epithelial cells but with no detectable immunostaining of DDAH2 in endothelial layer of pulmonary arteries. →, indicates endothelium; ➤, indicates epithelium Scale bar: 100µm; PA: pulmonary artery.

4.1.6 Decreased metabolism of dimethylarginines in lungs from patients with IPAH

4.1.6.1 mRNA expression of DDAH isoforms

DDAH1 mRNA levels were unchanged, while DDAH2 expression was significantly reduced, when comparing IPAH lungs to healthy donor lungs (Figure 11). DDAH2 was significantly downregulated by a factor of 2.12±0.38 fold in lungs from IPAH patients compared to healthy donors.

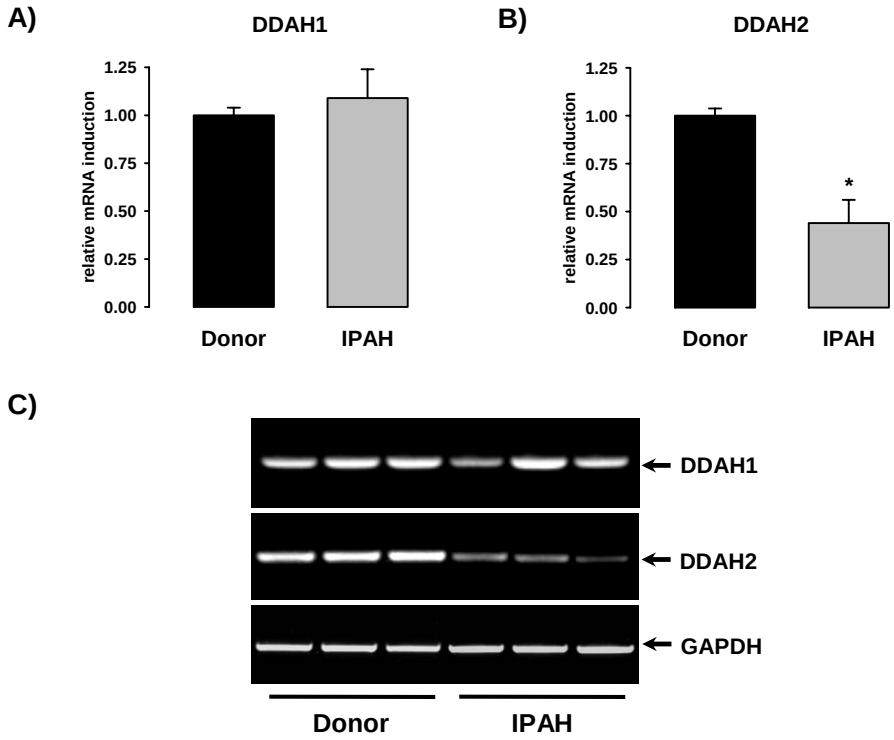

Figure 11: Real time RT-PCR analysis of DDAH isoforms from healthy donor and IPAH patient lungs. Relative quantification of mRNAs encoding for **(A)** DDAH1 and **(B)** DDAH2 related to the housekeeping gene GAPDH was undertaken by real-time RT-PCR. **(C)** Gel picture showing DDAH1 and DDAH2 expression in total RNA isolated from healthy donor and IPAH patient lung tissues**.** All values are given as mean ± SEM (n=3). *, p<0.05 versus donor.

4.1.6.2 Protein expression of DDAH isoforms

Western blot analysis of lung homogenates using a polyclonal DDAH1 antibody showed that the 34-kDa DDAH1 protein was strongly expressed in the donor

lungs and remains unaltered or change insignificantly in the IPAH patient lungs (Figure 12A, B). Densitometry revealed that the IPAH patient lungs expressed 10% less DDAH1 protein than those of donor lungs.

In contrast, Western blot analysis of lung homogenates using a polyclonal DDAH2 antibody revealed a band at 42-kDa DDAH2 protein and was significantly reduced in the IPAH patient lungs as compared to donor lungs. Densitometry revealed 77% reduction in DDAH2 expression compared to healthy donor lungs (Figure 12A, B).

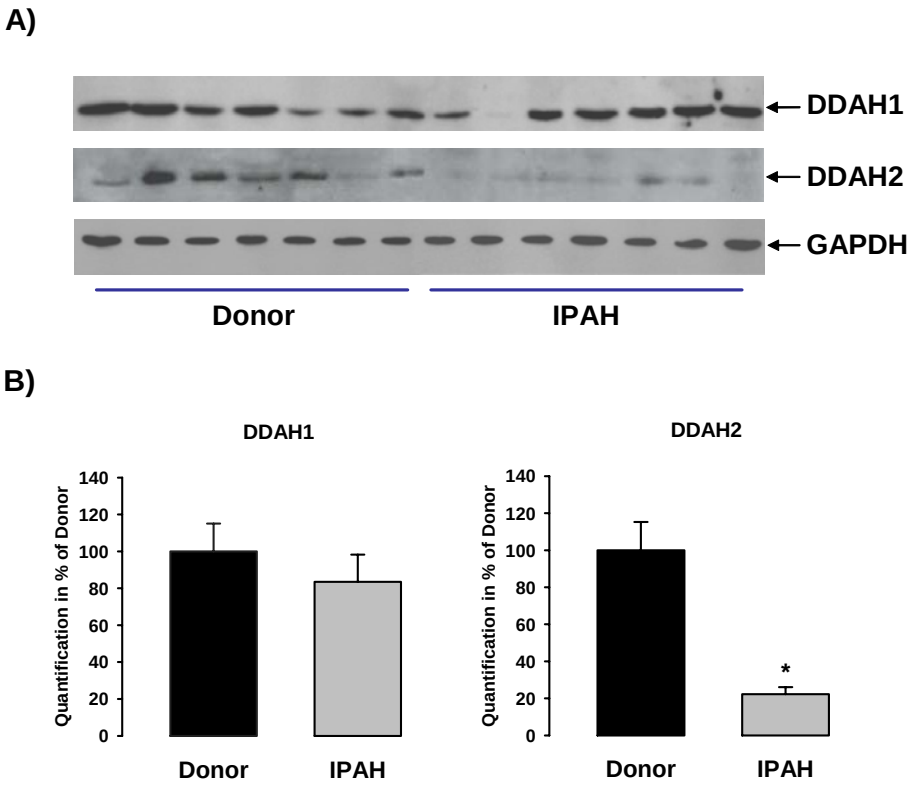

Figure 12: Western blot analysis of DDAH isoforms in lungs from healthy donors and patients with IPAH. (A) Western blot analysis was performed with anti-DDAH1 and anti-DDAH2 antibodies in lung homogenates from healthy donors and patients with IPAH. The specific antibodies recognize protein at a molecular weight of 34kDa and 42kDa. **(B)** Quantification of the DDAH1 and DDAH2 signal in each group. Values (means ± SEM) are expressed as percentage of expression found in donor tissue (n=7). *, $p<0.05$ versus donor.

In addition, a significant negative correlation between DDAH II expression (normalized to GAPDH) and mean pulmonary arterial pressure of the transplanted IPAH patients (Figure 13).

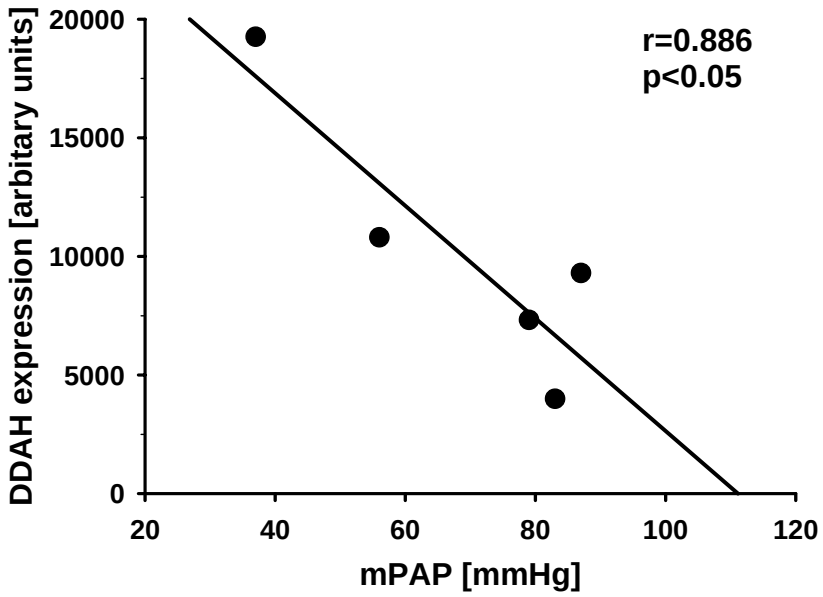

Figure 13: Correlation of DDAH2 expression to mean PAP of 5 transplanted IPAH patients. DDAH2 expression is given in arbitary units and normalized to GAPDH expression. Mean pulmonary arterial pressure (mPAP) is given in mmHg.

4.2 Monocrotaline (MCT) treated rats

4.2.1 Hemodynamics and right heart hypertrophy

Monocrotaline (MCT) treatment caused a marked increase in right ventricular systolic pressure (64.5±5.7 mm Hg) when compared to saline injected control animals (22.4±1.7 mm Hg) (Figure 14) without a significant difference in systemic arterial pressure. The ratio of right ventricular to left ventricular plus septal weight (RV/LV+S), an indirect index of severity of pulmonary hypertension, was significantly increased after monocrotaline administration (0.29±0.04 to 0.52±0.03). The MCT-treated groups had significantly lower BW than time-matched control animals. Eighty percent (8 of 10) animals survived after MCT treatment. The hemodynamic changes were accompanied by significant media

hypertrophy of pulmonary vessels, increased distal pulmonary artery muscularization and a subsequent reduction in the number of peripheral pulmonary arteries.

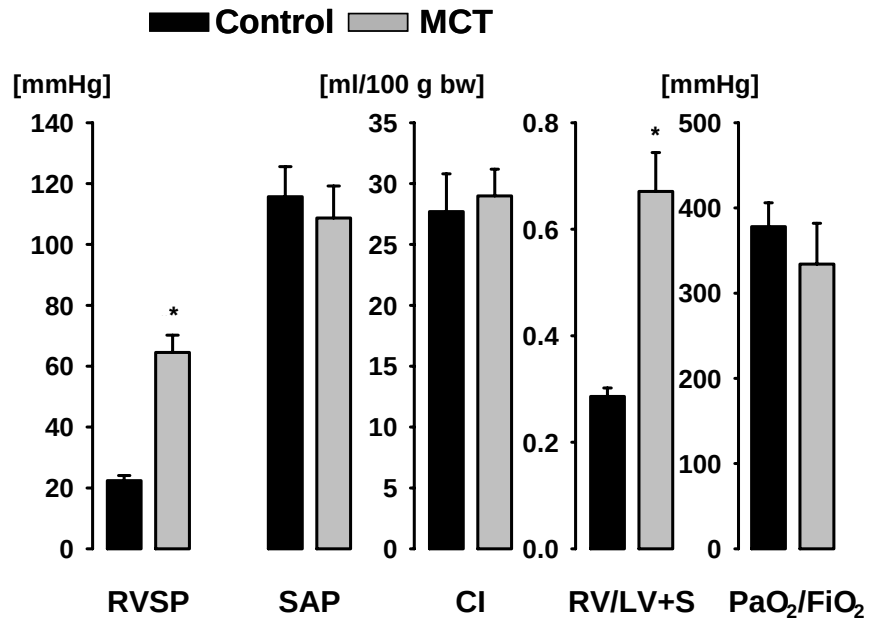

Figure 14: Hemodynamics, gas exchange and right heart hypertrophy in control and MCT-induced pulmonary hypertensive rats. Right ventricular systolic pressure (RVPsys, in mmHg), systemic arterial pressure (SAP, in mmHg), cardiac index (CI, in ml/min 100 g bodyweight), right to left ventricular weight ratio (RV/LV+S) and arterial oxygenation (PaO_2/FiO_2) are given (mean ± SEM of 8 independent experiments each). *, $p<0.05$ versus control.

4.2.3 Increased plasma levels of ADMA and SDMA in MCT-PAH rats

Plasma levels of ADMA were four-fold increased (0.37±0.08 versus 1.60±0.15 µmol/l), and SDMA levels seven fold increased from 0.41±0.10 to 2.74±0.21 µmol/l in MCT-treated rats compared to control rats (Figure 15).

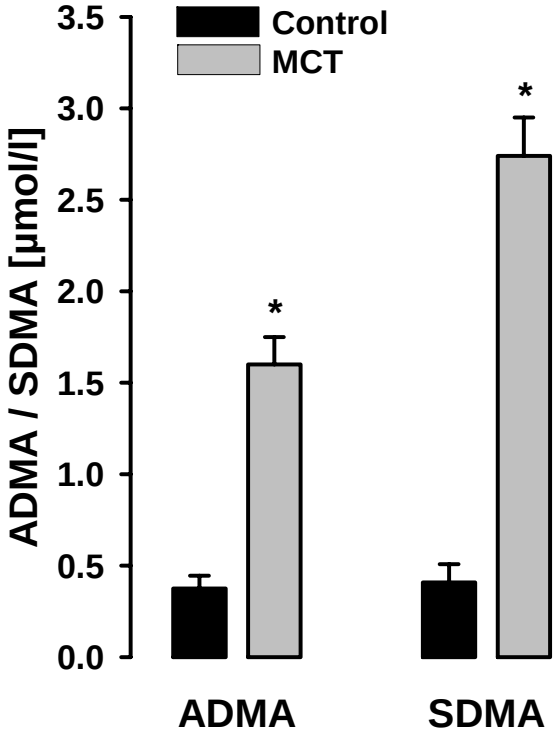

Figure 15: Plasma concentrations of ADMA and SDMA from control and MCT-PAH rats. Plasma ADMA and SDMA levels were measured by HPLC in saline treated (control) rats and rats 4 weeks after a single injection of monocrotaline (MCT). Values are given as mean ± SEM (n=8). *, $p<0.05$ versus control.

4.2.3 Localization of dimethylated arginine proteins in MCT-PAH rat lungs

Immunostaining of asymmetric and symmetric dimethylated arginine proteins in rat lung tissue results were well in line with those of the human lung tissue. In the control rat lungs, specific immunoreactivity of the asymmetric and symmetric dimethylated arginine proteins was observed in the bronchiolar ciliated and terminal cuboidal epithelium, type I and type II alveolar epithelium, arterial and capillary endothelium, and alveolar macrophages with very little difference in their distribution throughout the lung. However, MCT - exposure caused an increased immunostaining density of asymmetric and symmetric dimethylated arginine proteins in pulmonary arteries (Figure 16).

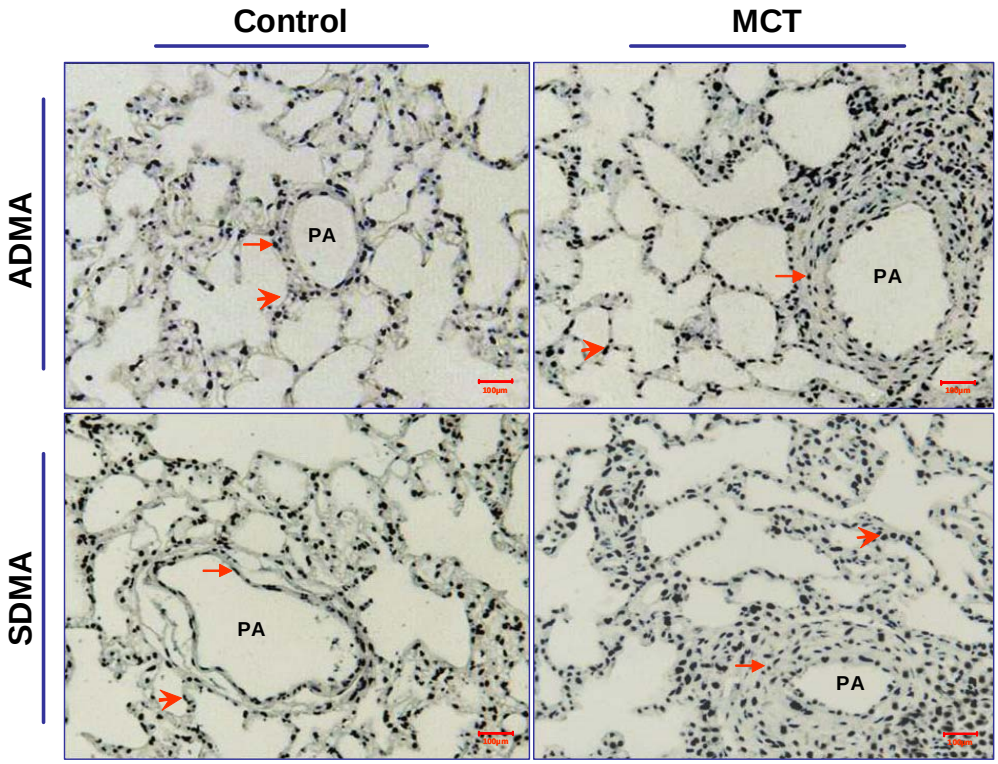

Figure 16: Immunoreactivity of dimethylarginines in lungs from control rats and rats with MCT-PAH. Immunostaining of control rat lung sections with anti-ADMA and anti-SDMA show a moderate immunoreactivity to asymmetric and symmetric dimethylated arginine proteins in pulmonary arterial endothelial cells and in alveolar epithelial cells. Distinctly, a much stronger immunoreactivity, especially in the endothelial layer of pulmonary arteries was illustrated in MCT-PAH rat lung sections. →, indicates endothelium; ➤, indicates epithelium. Scale bar: 100µm; PA: pulmonary artery

4.2.4 Augmentation of dimethylated arginine proteins in MCT-PAH rat lungs

The tissue levels of asymmetric and symmetric dimethylated arginine proteins were detected by dot-blot and a significant higher degree of dimethylation was demonstrated in MCT-treated rats (Figure 17A, B). As compared to control rat lungs, asymmetric and symmetric dimethylated arginine proteins were upregulated 218 ± 17 % and 268 ± 19% respectively, in MCT-PAH lungs.

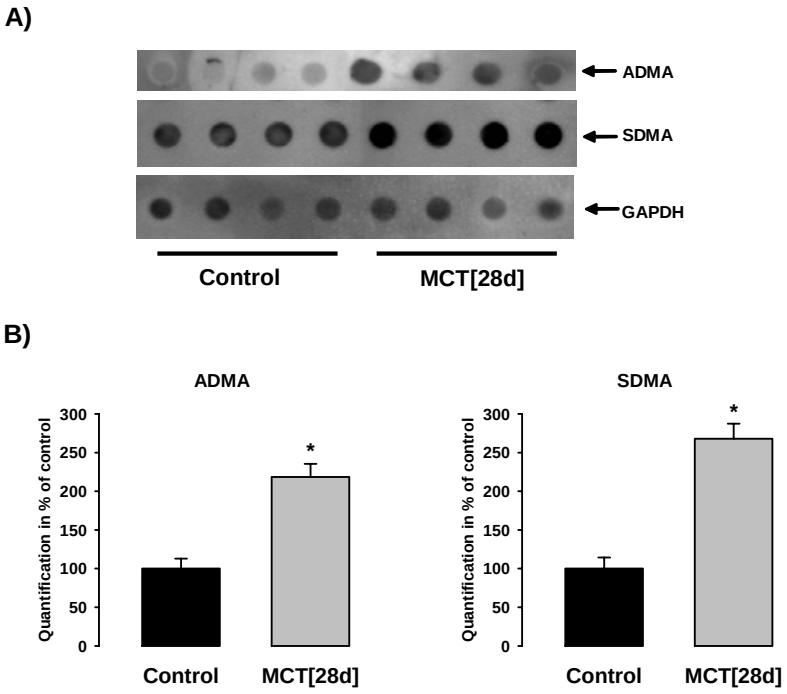

Figure 17: Dot blot analysis of dimethylated arginine proteins in lungs taken from control rats and rats with MCT-PAH. (A) Dot blotting with anti-ADMA and anti-SDMA antibodies and subsequent **(B)** quantification of asymmetric and symmetric dimethylated arginine proteins in lung homogenates from control rats and rats with MCT-PAH**.** Values (means ± SEM) are expressed as percentage of expression found in control tissue (n=4). *, $p<0.05$ versus control.

4.2.5 Localization of DDAH isoforms in MCT rat lungs

Similar to ADMA and SDMA, DDAH isoform (Figure 18) immunostaining of rat lung tissue results were well in line with those of the human lung tissue. As compared to humans, a greater degree of DDAH1 and DDAH2-like immunostaining was seen in vascular smooth muscle cells of the control rat lungs. Interestingly, a drastic reduction in DDAH2 immunoreactivity in pulmonary endothelium of MCT-PAH rat lungs was demonstrated when compared with control tissues. In contrast, DDAH1 immunoreactivity remained unaltered.

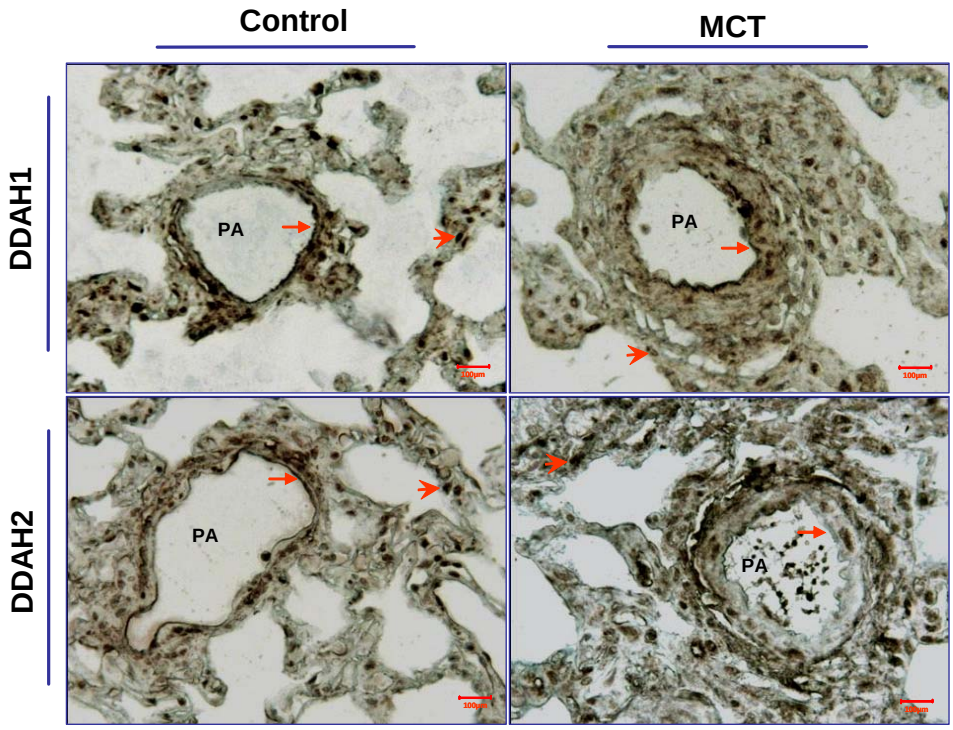

Figure 18: Immunoreactivity of DDAH isoforms in lungs from control rats and rats with MCT-PAH. Lung sections from a control rat demonstrate a moderate to strong DDAH1 and DDAH2 immunoreactivity in pulmonary arterial endothelial cells, smooth muscle cells and in alveolar epithelial cells. Lung sections from rats with MCT-PAH even show strong immunoreactivity of DDAH1 with in pulmonary arterial endothelial cells and in alveolar epithelial cells but with no detectable immunostaining of DDAH2 in endothelial layer of pulmonary arteries. →, indicates endothelium; ➤, indicates epithelium. Scale bar: 100µm; PA: pulmonary artery.

4.2.6 MCT inhibits expression of DDAH isoforms at mRNA level

The expression of DDAH1 at the mRNA level was unchanged, while DDAH2 was significantly decreased in MCT-treated rat lungs (Figure 19). As compared to control rat lungs DDAH2 expression was significantly decreased by a fold of 3.12±0.39 in MCT-treated rats.

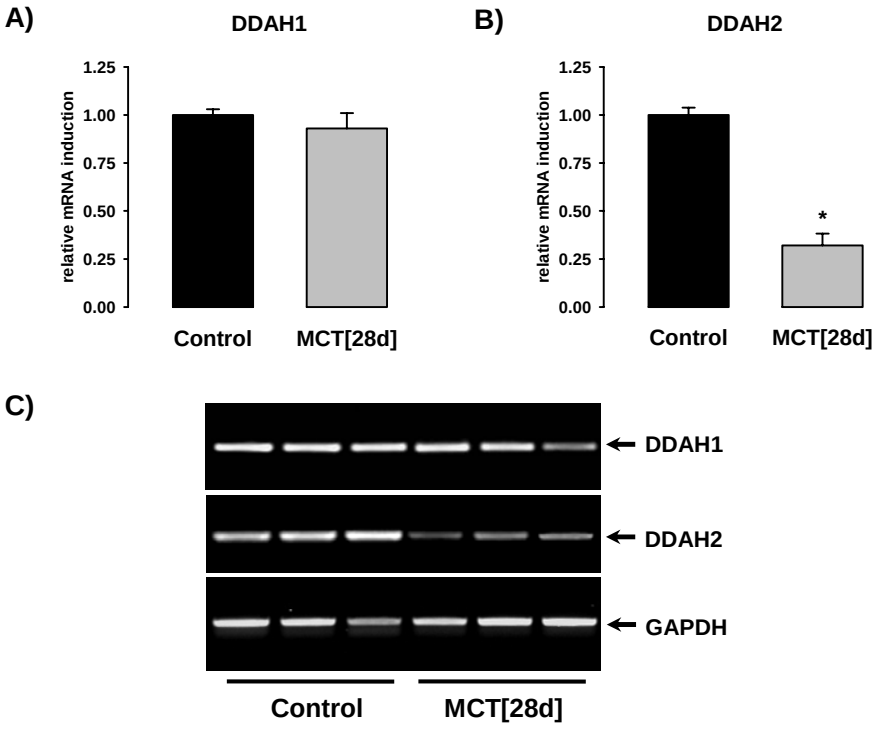

Figure 19: Real time RT-PCR analysis of DDAH isoforms from control and MCT-PAH rat lungs. Relative quantification of mRNAs encoding for **(A)** DDAH1 and **(B)** DDAH2 related to the housekeeping gene GAPDH was undertaken by real-time RT-PCR. **(C)** Gel picture showing DDAH1 and DDAH2 expression in total RNA isolated from control and MCT-PAH rat lung tissues**.** All values are given as mean ± SEM (n=3). *, $p<0.05$ versus control.

4.2.7 MCT inhibits expression of DDAH isoforms at protein and activity level

MCT-exposure caused significant changes in dimethylarginine metabolism. DDAH isoform expression and activity was significantly altered. DDAH1 protein expression was reduced by 12% (Figure 20A, B). In contrast, DDAH2 protein in lungs from monocrotaline-treated animals was reduced by 48% (Figure 20B, D). Total DDAH activity was reduced by 79% (Figure 21).

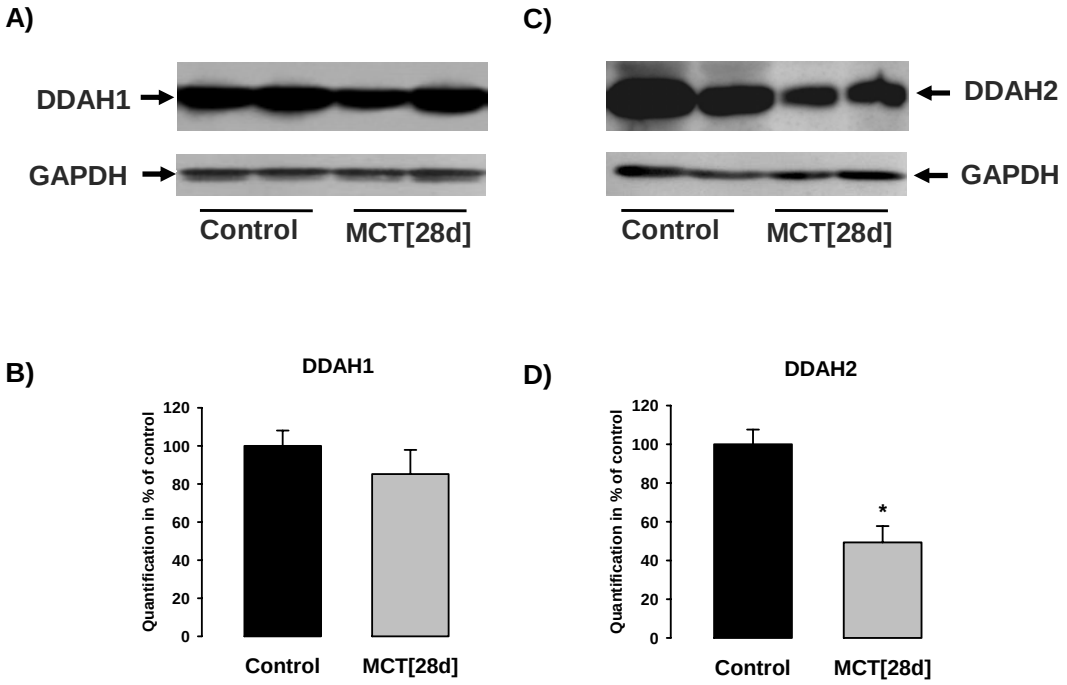

Figure 20: Expression of DDAH2 in lung homogenate from control and MCT-PAH rats. **(A,B)** Western blot analysis was performed with anti-DDAH1 and anti-DDAH2 antibodies in lung homogenates from control rats and rats with MCT-PAH. The specific antibodies recognize protein at a molecular weight of 34kDa and 45kDa. **(C,D)** Quantification of the DDAH1 and DDAH2 signal in each group. Values (means ± SEM) are expressed as percentage of expression found in control tissue (n=4). *, $p<0.05$ versus control.

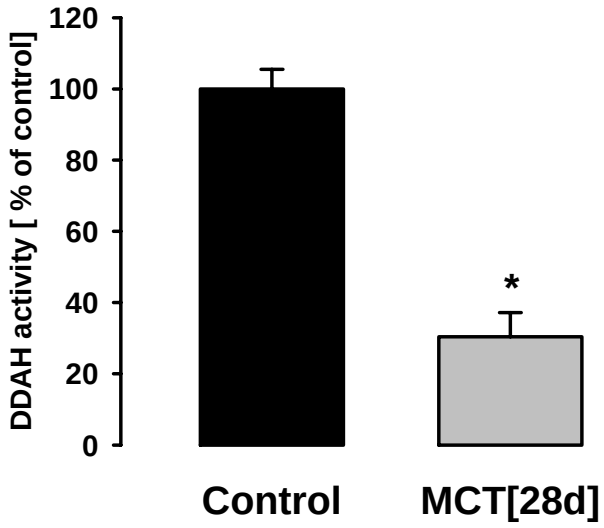

Figure 21: Activity of DDAH2 in lung homogenate from control and MCT-PAH rats. DDAH enzyme activity was determined by in vitro assay of crude lung homogenates from control and MCT-PAH rats. DDAH activity was assayed by the conversion of L-citrulline from ADMA. One unit of the enzyme was defined as the amount that catalyzed the formation of 1 mM L-citrulline from ADMA per minute at 37°C. Values (means ± SEM) are expressed as percentage of expression found in control tissue (n=4). *, p<0.05 versus control.

4.2.8 TNF-α and IFN-γ mediates DDAH dysregulation

HUVEC cells treatment with proinflammatory cytokines, TNF-α, IL-1β and IFN-γ dramatically modulated DDAH2 expression. TNF-α and IFN-γ treatment reduced DDAH2 expression by 53% and 41% respectively. In contrast IL-1β induced DDAH2 expression by 138 % (Figure 22)

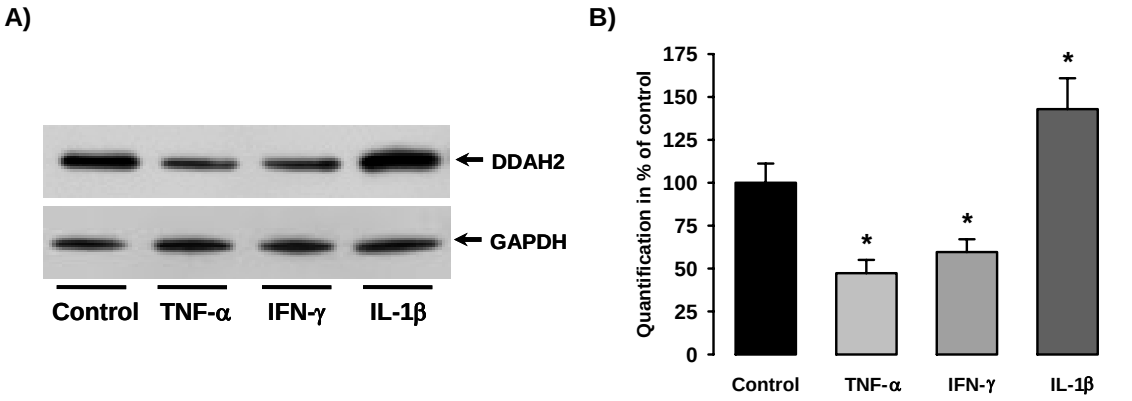

Figure 22: DDAH modulation by cytokines. (A) Western blot analysis of DDAH2 and GAPDH expression in HUVEC cells treated with TNF-α, IL-1β and IFN-γ (10 ng/ml) for 24 hrs. **(B)** Quantification of the DDAH2 signal related to the house keeping gene GAPDH in each group. Values (means ± SEM) are expressed as percentage of expression found in control cells (n=3). *, p<0.05 versus control.

4.3 Chronic effects of aerosolized tolafentrine in MCT treated rats

4.3.1 Acute vasodilatory effects

Aerosolized tolafentrine reduced right ventricular systolic pressure [RVSP] in $MCT_{[28d]}$ rats in a dose-dependent manner (Figure 23). As depicted, this pulmonary vasodilatation was accompanied by less pronounced decrease in systemic arterial pressure.

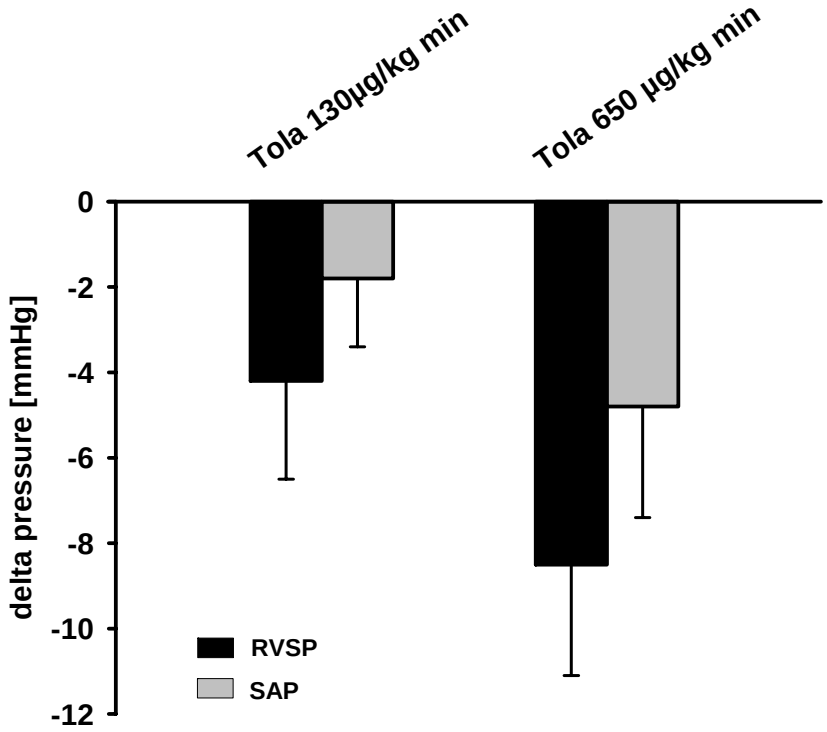

Figure 23: Immediate vasodilatory effects of inhaled tolafentrine in MCT-induced PAH. Monocrotaline ($MCT_{[28d]}$) treated animals received tolafentrine over an inhalation period of 5 min subsequent to catheterization. The decrease in right ventricular systolic pressure (RVSP, in mmHg) and systemic arterial pressure (SAP, in mmHg) in response to the vasodilatory treatment is given. All values are given as mean ± SEM (n=8).

4.3.2 Hemodynamics

After injection of monocrotaline, pulmonary hypertension developed (right ventricular systolic pressure on day 28 = 66.5±3.2 mm Hg (n=11), as compared to 25.9±4.0 mm Hg in the control animals (n=10)) (Figure 24). No significant changes in systemic arterial pressure occurred. As compared to control animals (36.5±3.5 ml/min 100 g body weight), cardiac index was slightly decreased on day 28 (31.8±1.3 ml/min/100 g body weight). Aerosolized tolafentrine treatment (625 ng/kg per minute) for 2 weeks significantly lowered right ventricular pressure to 43.4±2.1 mmHg.

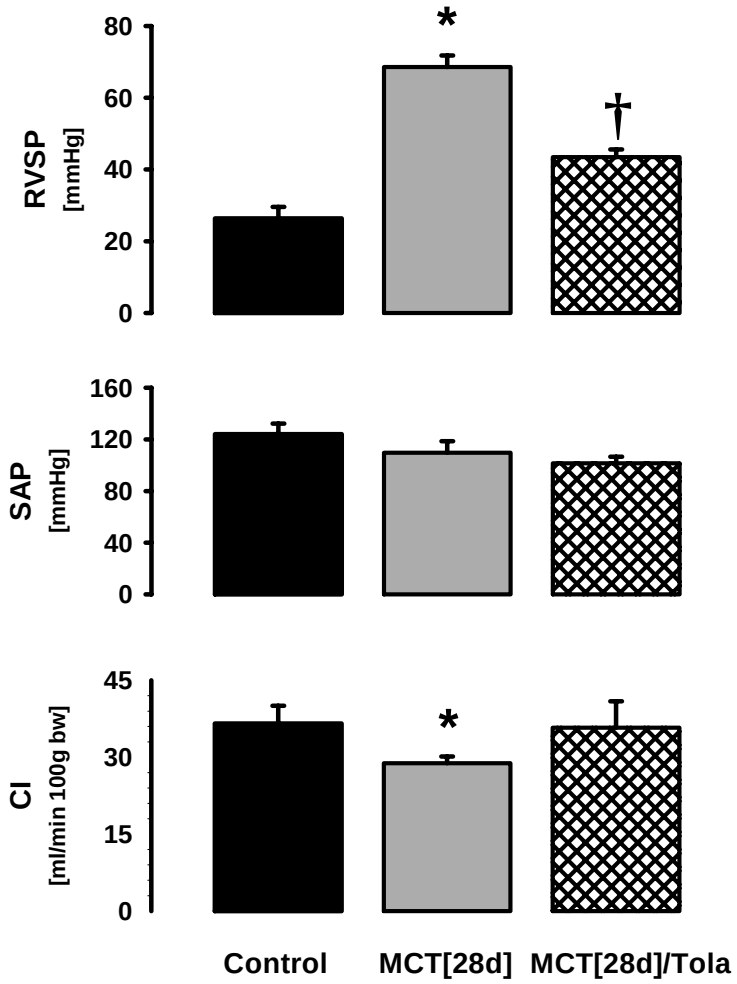

Figure 24: Influence of inhaled tolafentrine on hemodynamics in MCT–induced PAH. Right ventricular systolic pressure (RVSP, in mmHg), systemic arterial pressure (SAP, in mmHg), cardiac index (CI, in ml min^{-1} 100 g body $weight^{-1}$) and pulmonary vascular resistance index (PVRI, in mmHg min ml^{-1} 100 g body $weight^{-1}$) are given. Tolafentrine was applied by repetitive inhalations from day 14 to day 28. All values are given as mean ± SEM (n=9). *, $p<0.05$ versus control; †, $p<0.05$ versus $MCT_{[28d]}$.

Both arterial PO_2 and central venous oxygen saturation (SvO_2) decreased on MCT treatment. The MCT treated groups had significantly lower BW than time matched control animals. The decrease in arterial PO_2 and SvO_2 in response to

MCT treatment was normalized (~70%) in the tolafentrine-treated animals (Figure 25).

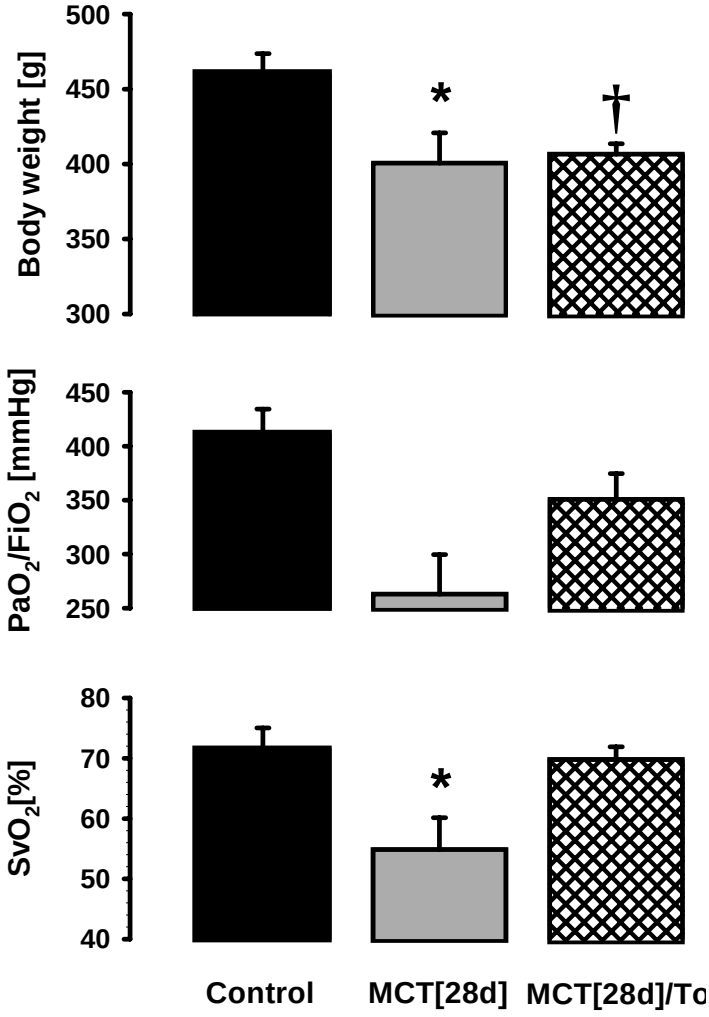

Figure 25: Influence of long-term treatment with inhaled tolafentrine (Tola) on BW and blood gases in MCT-induced PAH. Animals were investigated 28 days after MCT treatment ($MCT_{[28d]}$). BW (in g), PaO_2/ FIO_2 (in mm Hg), and SvO_2 (in %) are given. Tolafentrine was applied by repetitive inhalations from day 14 to day 28 ($MCT_{[28d]}$/Tola). Control animals received sham injection of saline. All values are given as mean ± SEM (n=9). *, $p<0.05$ versus control.

4.3.3 Right ventricular hypertrophy

Four weeks after injection of MCT, animals demonstrated significant right heart hypertrophy, as indicated by an increase in the right ventricular to left ventricular plus septum weight ratio (RV/LV+S) from 0.29±0.02 (control animals) to 0.53± 0.04. Inhaled tolafentrine prevented and slightly reversed established right ventricular hypertrophy ($MCT_{[28d]}$/Tola = 0.47±0.03) (Figure 26).

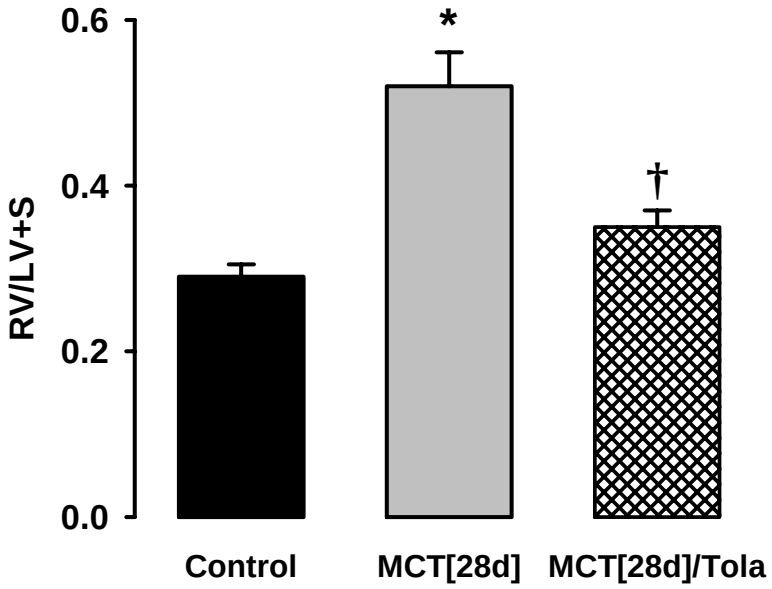

Figure 26: Influence of inhaled tolafentrine on right heart hypertrophy. Right to left ventricular plus septum ratio (RV/LV+S) of different treatment groups is given. Tolafentrine was applied by repetitive inhalations from day 14 to day 28. All values are given as mean ± SEM (n=9). *, $p<0.05$ versus control; †, $p<0.05$ versus $MCT_{[28d]}$.

4.3.4 Histopathology

Elastin staining and subsequent morphometric analysis of pulmonary arteries demonstrated a markedly increased medial wall thickness in both the $MCT_{[28d]}$ and the $MCT_{[28d]}$ groups, when compared with the saline-treated group. Most impressively, medial wall thickness was significantly reduced by long term treatment of aerosolized tolafentrine (Figure 27). The hemodynamic changes were accompanied by significant media hypertrophia of pulmonary vessels that was reversed by long term treatment of aerosolized tolafentrine (Figure 27).

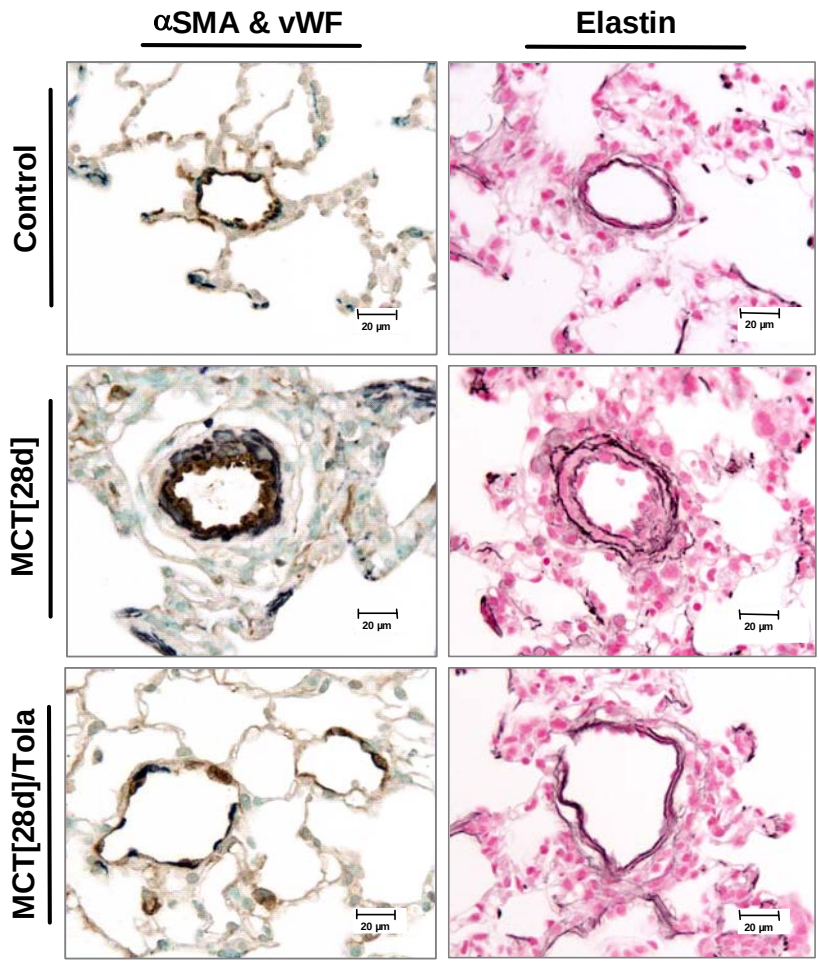

Figure 27: Effect of inhaled tolafentrine on the degree of muscularization and on the medial wall thickness of small pulmonary arteries. Immunohistochemical analysis of lung sections originating from saline (Control), monocrotaline ($MCT_{[28d]}$) and monocrotaline plus tolafentrine ($MCT_{[28d]}$/Tola) treated animals. Staining was undertaken for von willebrand-factor (brown; endothelial cells) and alpha smooth muscle actin (purple; smooth muscle cells) as well as elastin. Scale bar: 20 µm.

4.3.5 Effect on methylarginine production

Tolafentrine treatment of MCT rats for 2 weeks caused a substantial and significant decrease in the plasma ADMA and SDMA levels. Plasma ADMA levels in the tolafentrine lungs is decreased from 1.65±0.15 to 0.31±0.04 compared to MCT treated rats ($MCT_{[28d]}$) alone (Figure 28). In addition, the

absolute concentration of SDMA decreased from 2.75±0.35 to 0.37±0.07, suggesting decreased protein turnover in tolafentrine treated MCT lungs.

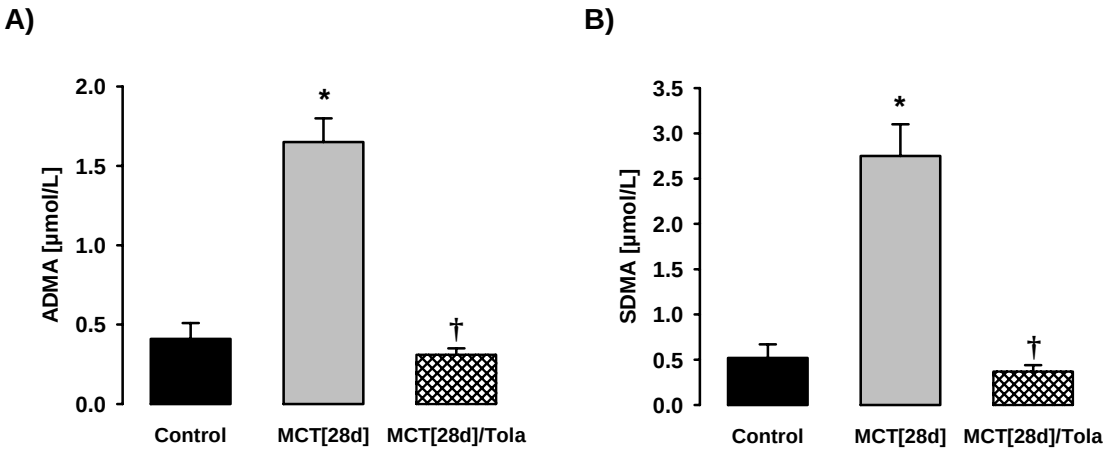

Figure 28: Effect of tolafentrine on plasma monocrotaline induced ADMA and SDMA levels. **(A)** Plasma ADMA and **(B)** SDMA levels of rats treated with saline (Control), monocrotaline ($MCT_{[28d]}$) and monocrotaline plus tolafentrine ($MCT_{[28d]}$/Tola) treated animals. All values are given as mean ± SEM (n=8). *, $p<0.05$ versus control; †, $p<0.05$ versus $MCT_{[28d]}$.

4.3.6 Effect of tolafentrine on DDAH expression

DDAH2 mRNA levels that were significantly reduced by MCT treatment were upregulated with 2 weeks treatment of tolafentrine (Figure 29). Further, western blot analysis confirmed that DDAH2 protein expression was also increased by tolafentrine treatment of MCT treated ($MCT_{[28d]}$) rats (Figure 30). Interestingly, the DDAH2 expression at both mRNA and protein level was near normalized to control rat lung DDAH2 expression.

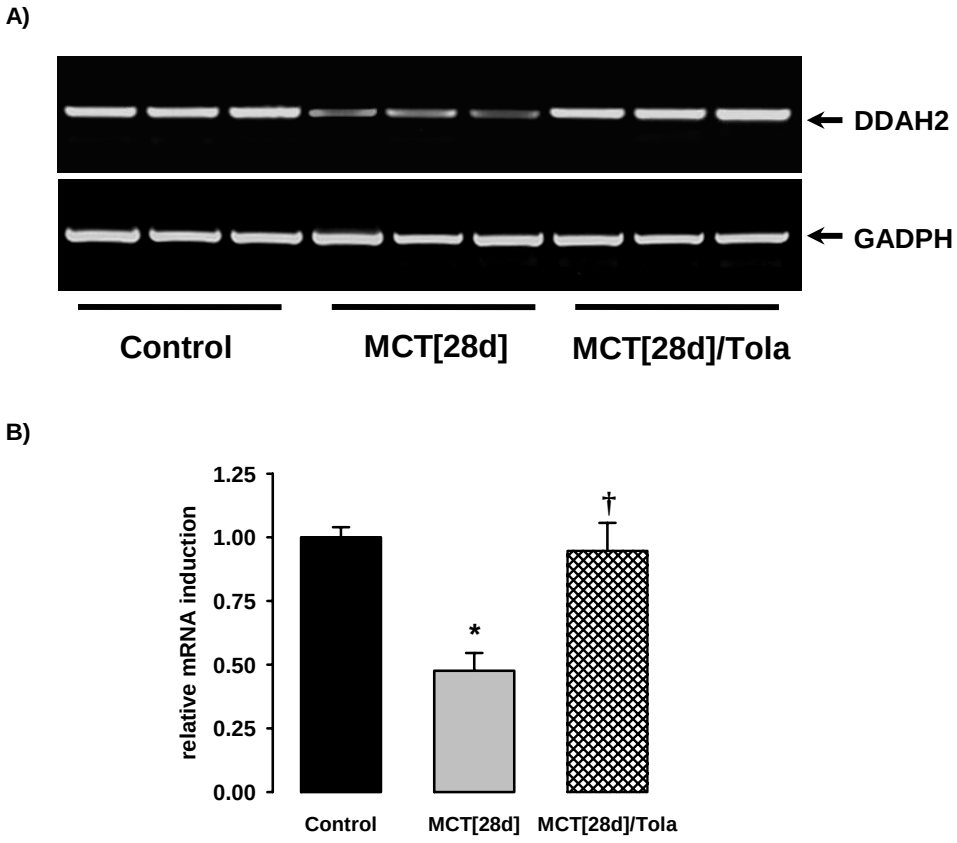

Figure 29: Effect of tolafentrine on DDAH2 mRNA expression. (A) mRNA expression and subsequent **(B)** quantification of DDAH2 in lung homogenates of rats treated with saline (Control), monocrotaline ($MCT_{[28d]}$) and monocrotaline plus tolafentrine ($MCT_{[28d]}$/Tola) treated animals. All values are given as mean ± SEM (n=3). *, p<0.05 versus control; †, p<0.05 versus $MCT_{[28d]}$.

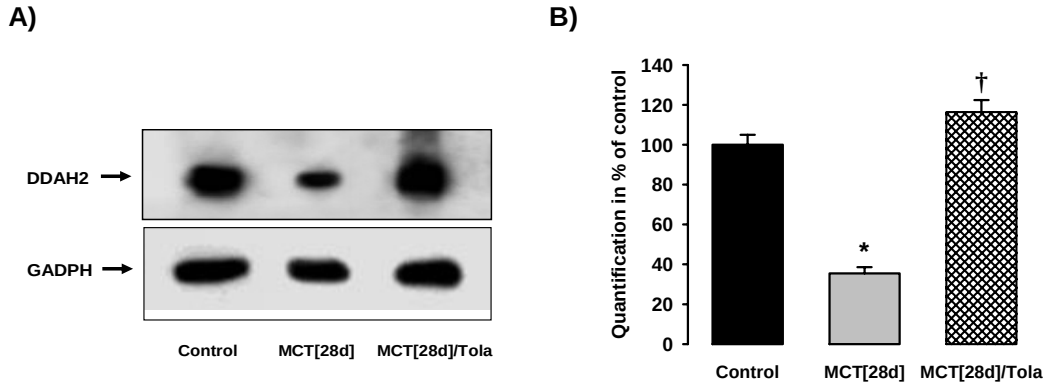

Figure 30: Effect of tolafentrine on DDAH2 protein expression. (A) Immunoblots and subsequent **(B)** densitometric quantification of DDAH2 in lung homogenates of rats treated with

saline (Control), monocrotaline ($MCT_{[28d]}$) and monocrotaline plus tolafentrine ($MCT_{[28d]}$/Tola) treated animals. All values are given as means ± SEM (n=4). *, p<0.05 versus control; †, p<0.05 versus $MCT_{[28d]}$.

4.3.7 Effect of tolafentrine on DDAH Activity

Figure 30 shows the effects of tolafentrine on DDAH activity of MCT lungs. As expected, DDAH activity was decreased by 4 fold after MCT treatment as compared to controls. 2 weeks treatment of MCT rats with tolafentrine restored DDAH activity nearly to a normal level compared with MCT ($MCT_{[28d]}$) group (Figure 31). But tolafentrine treatment of control lungs per se did not affect DDAH activity compared with control lungs.

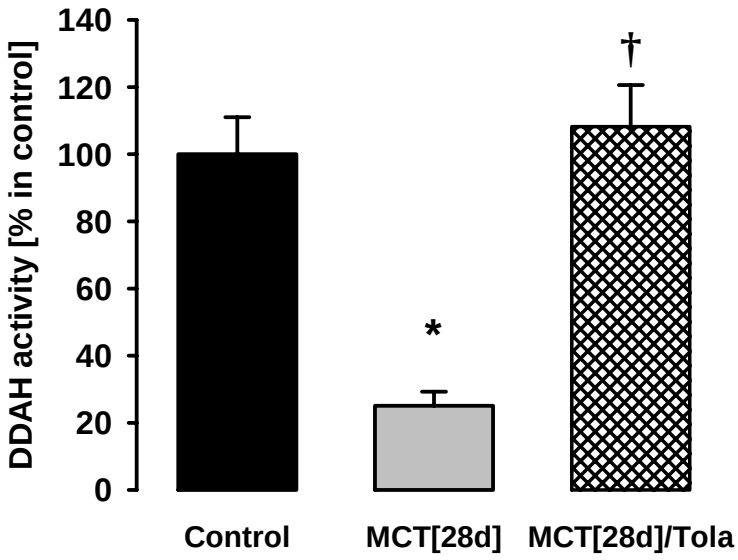

Figure 31: Effect of tolafentrine on DDAH activity. DDAH enzyme activity was determined by in vitro assay of crude lung homogenates from rats treated with saline (Control), monocrotaline ($MCT_{[28d]}$) and monocrotaline plus tolafentrine ($MCT_{[28d]}$/Tola) treated animals. All values are given as mean ± SEM (n=4). *, p<0.05 versus control; †, p<0.05 versus $MCT_{[28d]}$.

4.3.8 Effect of tolafentrine on NO synthesis

In parallel to the alterations in DDAH activity, tolafentrine also induced statistically significant increase in plasma nitrite/nitrate (NOx) levels (Figure 32). Four weeks MCT treated rats showed a remarkable decrease in NOx levels from

14.8±4.7 to 3±1.28 µmol/l in control rats. Most impressively, treatment of MCT rats with tolafentrine significantly elevated NOx levels from 3±1.28 to 12.9±3.4 µmol/l and nearly normalized to control plasma NOx values.

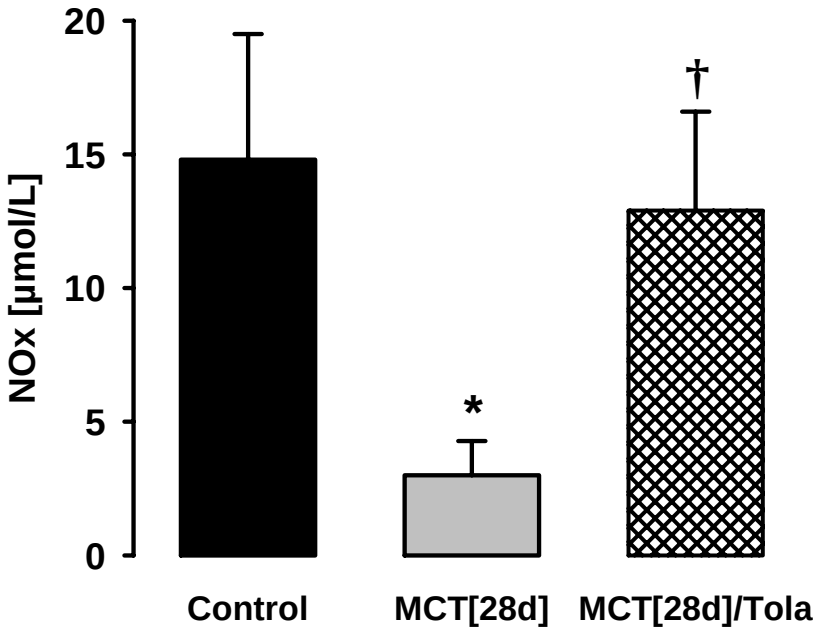

Figure 32: Effect of tolafentrine on nitrite/nitrate (NOx) levels. Plasma nitrite/nitrate levels was determined by griess reagent from rats treated with saline (Control), monocrotaline ($MCT_{[28d]}$) and monocrotaline plus tolafentrine ($MCT_{[28d]}$/Tola) treated animals. All values are given as mean ± SEM (n=8). *, $p<0.05$ versus control; †, $p<0.05$ versus $MCT_{[28d]}$.

5 Discussion

5.1 Increased levels and reduced catabolism of asymmetric and symmetric dimethylarginines in pulmonary hypertension

An increase in levels of ADMA, an endogenous inhibitor of nitric oxide synthases is known to contribute to the pathogenesis and progression of various cardiovascular diseases associated with endothelial dysfunction. Interestingly, Increased plasma concentrations of ADMA (1.06 µmol/L) were also observed in the currently studied IPAH patients as compared to levels in healthy subjects ranging between 0.30 to 0.42 µmol/L. This increase is comparable with values obtained from other conditions associated with endothelial dysfunction such as in patients with pulmonary hypertension associated with congenital heart disease (0.55µmol/L), hypercholesterolemia (0.60µmol/L), Type 2 diabetes mellitus (1.59µmol/L) and hyperhomocystinemia (1.6µmol/L) (92-95).

In the present investigation, we employed monocrotaline for induction of severe pulmonary hypertension in rats, as documented by a threefold increase in right ventricular systolic pressure and right heart hypertrophy (105). Similar to previous observations in hypoxia-induced experimental pulmonary hypertension (106), a marked increase in plasma ADMA levels was noted in the MCT treated animals. Such enhanced ADMA levels may well result in substantial NOS inhibition, and it is in line with this reasoning that reduced NO production is an underlying mechanism responsible for impaired endothelium-dependent relaxation, that was previously illustrated in response to MCT treatment (107,108).

Moreover, the main endothelial ADMA metabolizing enzyme, DDAH2, was found to be drastically reduced in response to MCT treatment, as shown on the mRNA, protein and functional level and by immunohistochemistry. This loss of DDAH2 activity may result in local (intracellular) ADMA levels even surpassing those in the blood compartment, thereby aggravating the suppression of endothelial NO

formation. This notion is in line with previous studies in hypoxic lungs, where reduced DDAH expression was also observed (106). The molecular mechanisms underlying DDAH suppression in hypoxia- and MCT-induced pulmonary hypertension remain to be elucidated, but may include the impact of oxidative stress (96), viral infection (97) and the nuclear receptor PPARγ on the transcriptional and translational control of DDAH (109). Recently, several polymorphisms within the DDAH2 promoter were identified which could be a risk factor for cardiovascular diseases (110). In our study, we found that protein levels of DDAH were affected by pro-inflammatory cytokines, TNF-α and IFNγ. DDAH expression of endothelial cells was decreased to almost 50% of baseline values by incubation with TNF-α and IFNγ. The study nicely corroborates with human IPAH patients that had an aggravated production of TNF-alpha as well as with MCT-induced PAH rats causing endothelial dysfunction.

Interestingly, the plasma levels of SDMA also markedly increased in MCT-treated rats. The role of SDMA in vascular dysfunction is not yet clear, however, since SDMA is an inhibitor of the human cationic amino acid transporter hCAT-2B (80), it may indirectly inhibit NO synthesis by interfering with arginine uptake. Thus, enhanced SDMA levels are to be expected to synergize with enhanced ADMA levels in causing endothelial NOS inhibition, along with a substantial amount of substrate depletion by transstimulation, which may well contribute to prolonged pulmonary vasoconstriction and lung vascular remodeling in the MCT model.

As demonstrated by dot-blot and immunohistochemistry, asymmetric and symmetric dimethylated arginines are highly increased in both human lung tissue of idiopathic pulmonary arterial hypertension and rat tissue of monocrotaline induced pulmonary hypertension. The source of asymmetric and symmetric dimethylated arginines are protein arginine *N*-methyltransferases (PRMTs) which were shown to be upregulated in response to oxidative stress in human endothelial cells (111). Since oxidative stress is involved in pulmonary hypertension (112,113), it is possible that both, an increase biosynthesis of

methylated arginines by upregulation of PRMTs and the reduction of metabolism by downregulation of DDAH are involved in the pathogenesis of pulmonary hypertension (Figure 33).

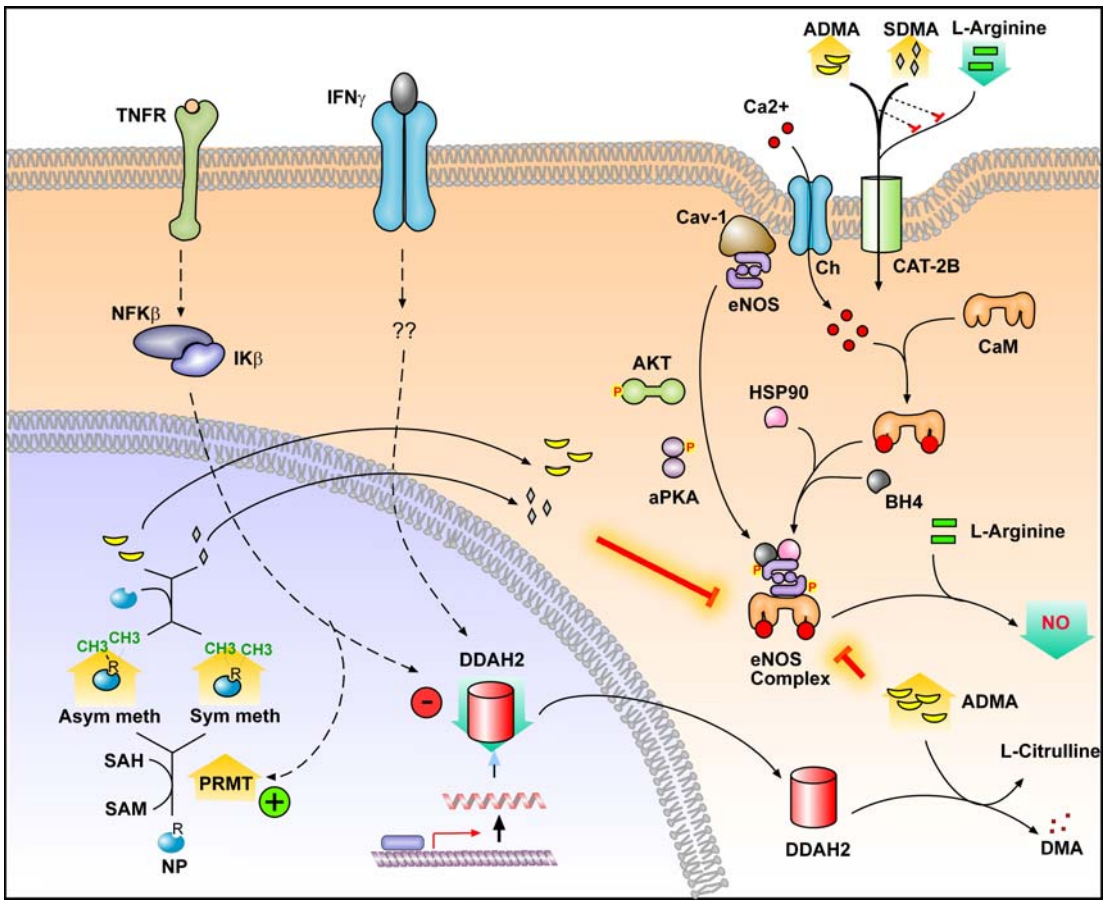

Figure 33: Central role of ADMA, SDMA and DDAH in the pathogenesis of pulmonary hypertension. Schematic depiction of methyl arginine (ADMA and SDMA) elevation and their contribution to impaired NOS activity and its substrate (L-arginine) depletion that subsequently leads to reduced NO production, an underlying mechanism responsible for the pathogenesis of PAH. This elevation as evidenced by our experiments is due to both increased dimethylation and decreased expression of ADMA metabolizing enzyme, DDAH. The molecular mechanisms underlying DDAH suppression may include pro-inflammatory cytokines, TNF-α and IFNγ (Author's Slide).

Most impressively, corresponding changes in dimethylarginines were also encountered in the human system: patients with IPAH displayed significantly

enhanced plasma and tissue ADMA and SDMA levels, and lung tissue DDAH2 was markedly reduced on both the mRNA and protein level, in line with immunohistochemical findings. This consistency of results between the experimental model and the human disease strongly suggests that changes in dimethylarginine regulation may be of major importance for endothelial dysfunction in pulmonary hypertension.

Besides the NO pathway, corresponding changes in dimetylarginines may influence various vasoactive mediator pathways, as suggested by previous findings. A positive correlation between plasma ADMA levels and endothelin (known to be a potent vasoconstrictor and mitogen), was found in various disorders of endothelial dysfunction. In denuded vessel endothelium an accumulation of endogenous NOS inhibitors (L-NMMA and ADMA) was accompanied by an increased endothelin-1 content within the vessel wall (114). Ohnishi et al demonstrated that ET-1 inhibits NO-mediated vasodilation in part via increased ADMA production in chronic heart failure (CHF). Furthermore, administration of an ET (A) receptor antagonist can prevent this increase in plasma ADMA levels, and also the increase in peripheral vascular resistance observed in these patients (115). Piatti et al. suggest that acute intravenous L-arginine infusion in patients with angina pectoris decreases endothelin-1 levels and improves endothelial function (116). However, the influence of non selective or selective ET receptor antagonist treatment on endogenous dimethyl arginines (ADMA, SDMA), in the context of PAH, has not yet been addressed in detail.

Future studies have to address the question whether enhanced supply with L-arginine may overcome increases in dimethylarginine levels. Studies with L-arginine supplementation for treatment of experimental or clinical pulmonary hypertension have provided controversial results. First, L-arginine analogues SDMA and ADMA, compete with L-arginine for intracellular uptake, but also drive out the intracellular L-arginine (80). Therefore, we believe that the huge increase of ADMA and SDMA, observed in our study (in both human and rat MCT model of PH) may competes with L-arginine uptake which results in a substantial

substrate depletion even when provided extracellularly. Second, increased levels of ADMA, the endogenous NOS inhibitor, may also “uncouple” endothelial NOS (84). In such conditions, molecular oxygen becomes the substrate for electron transfer rather than L-arginine, thereby blocking or inhibiting the access of intracellular L-arginine to NOS. Subsequently, endothelial NOS generates superoxide anion, increases oxidative stress, attenuates NO bioactivity, and induces additional endothelial dysfunction. Third, L-arginine may not be useful in later stages of pulmonary hypertension, in which cytokine- or lipid-induced instability and/or reduced transcription of NOS may decrease its expression. On the other hand, L-arginine supplementation showed beneficial effects on hemodynamics in patients with primary, secondary and precapillary pulmonary hypertension (56,103). Possible explanations for these short-term beneficial effects include nonenzymatic generation of NO from L-arginine, release of growth hormone or insulin, or effects at the level of the CAT proteins responsible for cellular uptake of L-arginine. Nevertheless, reversal of the effects of ADMA represents a persuasive mechanism. Another therapeutic strategy is manipulation of DDAH expression and function to foster dimethylarginine catabolism by DDAH overexpression or by existing therapies of pulmonary hypertension such as statins, ET receptor antagonist or prostacyclin analogues. This may represent new strategies for treatment of this enigmatic disease.

In conclusion, we were able to demonstrate that enhanced dimethylarginine levels contribute to vascular abnormalities in severe pulmonary hypertension. Furthermore, suppression of endothelial DDAH2 expression and function represents an important underlying mechanism in the course of the disease.

These findings are confirmed in a well-established animal model of pulmonary hypertension as well as in tissue and plasma of patients suffering from idiopathic pulmonary arterial hypertension. The current study might stimulate development of novel therapeutics that target the signaling pathway of endogenous NOS inhibitors and support the functional capacity of DDAH.

5.2 Tolafentrine increases nitric oxide synthesis in MCT induced pulmonary hypertension: a role for the induction of DDAH2

Astonishingly, in our screening tolafentrine was identified as novel therapeutic that can target the signaling pathway of endogenous NOS inhibitors. Daily repetitive inhalations of tolafentrine, a combined PDE 3/4 inhibitor, to MCT-PAH rats restored DDAH expression and activity. Besides, it also improved the impairment of endothelium-dependent relaxation and normalized nitrite levels in MCT-induced pulmonary hypertensive rats. Furthermore, the inhalative therapy commenced during the development of pulmonary hypertension, 2 weeks after application of monocrotaline, improved pulmonary hemodynamics and reversed structural and molecular changes underlying MCT induced PAH in rats.

The phosphodiesterases (PDEs) are a large family of intracellular enzymes that degrade cyclic nucleotides cAMP and cGMP (59,60). cAMP- PDEs, mainly PDE 3 and 4 isoenzymes have been demonstrated as essential players co-regulating cAMP catabolism in many organs, including the lung, and were shown to be upregulated in experimental PAH models (117-119). Moreover, experimental evidence has suggested that combined phosphodiesterase (PDE) 3/4 inhibitors increase cyclic AMP levels within cells greater than inhibition of either isoenzyme alone (120). Because of cAMP potential for altering a variety of cellular responses, cAMP-PDEs are appealing targets for the treatment of PAH (121). cAMP-PDEs may become a very useful tool for the treatment of PAH.

A recent study by our group showed that intravenous infusion of the combined selective PDE 3/4 inhibitor (tolafentrine) prevented the development of pulmonary hypertension and right ventricular hypertrophy in response to monocrotaline (122). However, the complexity and complications associated with the intravenous application of an agent exerting at the same time pulmonary and systemic vasodilation prompted us to evaluate the inhalative route of application in the present study. Moreover, the *therapeutic* potential of tolafentrine, and its

impact on molecular mechanisms closely linked with the structural wall changes were not addressed in the previous study.

For the inhalation therapy, aimed to achieve a selective pulmonary vasodilation, a 15 fold lower dose compared with the intravenous route of application was employed (120µg/kg day versus 2mg/kg day). Even with such low dose, tolafentrine treatment drastically improved MCT-induced hemodynamic abnormalities: RVSP values were markedly lower than those before onset of treatment, and cardiac index as well as pulmonary vascular resistance was fully normalized. Accordingly, the right heart hypertrophy was found to be largely decreased, as were the structural changes of the lung vasculature evoked by monocrotaline treatment. To our knowledge, we were the first to demonstrate the efficacy of combined selective PDE 3/4 inhibition in prevent the development of pulmonary hypertension both with respect to hemodynamics and the structural remodeling of the lung vasculature. These most impressive beneficial effects of tolafentrine may in part be explained by cAMP-mediated inhibition of proliferation, cell cycle progression of PASMC (123,124). In one of our previous studies we showed that cAMP-PDEi mainly combined PDE 3/4 inhibitors prevents experimental induced PAH by anti-migratory and matrix (MMP) regulation properties. Those were mainly mediated by downregulation of gelatinases, MMP2 and MMP9 (122).

Besides, we have shown for the first time that tolafentrine reverses endothelial dysfunction in chronic pulmonary hypertensive rats. This may be related to increased synthesis of endothelial NO, as evidenced by increased nitrite/nitrate levels. Consistent with the functional data, PDE3 and PDE4 were the major cAMP hydrolysis enzymes in intact endothelial cells and their role in lung inflammation and hyperpermeability of pulmonary endothelial monolayers, strongly suggests the protective role of combined PDE 3/4 inhibitors on vascular endothelial function (125-127).The mechanisms responsible for the cAMP enhancement mediated NO formation are not yet fully understood and may

include both eNOS expression and activity modulation. Nevertheless, to date, no data are available regarding effects of cAMP on endogenous NOS inhibitors.

Most interestingly, cAMP augmentation by PDE3/4 inhibition was shown to influence dimethylarginines levels. Tolafentrine treatment of MCT-PAH rats for 2 weeks drastically reduced both ADMA and SDMA levels that were upregulated during development of MCT-induced PAH. Decreased ADMA levels can be partly explained by restored DDAH2 expression and activity mediated by tolafentrine. Because DDAH is a key regulator of endogenous ADMA levels, increased DDAH expression and activity may accelerate the degradation of endogenous ADMA, thereby enhancing the activity of eNOS and eventually augmenting the synthesis of NO. Clearly evidenced by our experiments, nitrite levels were near normalized to control values in tolafentrine treated pulmonary hypertensive rats (Figure 34).

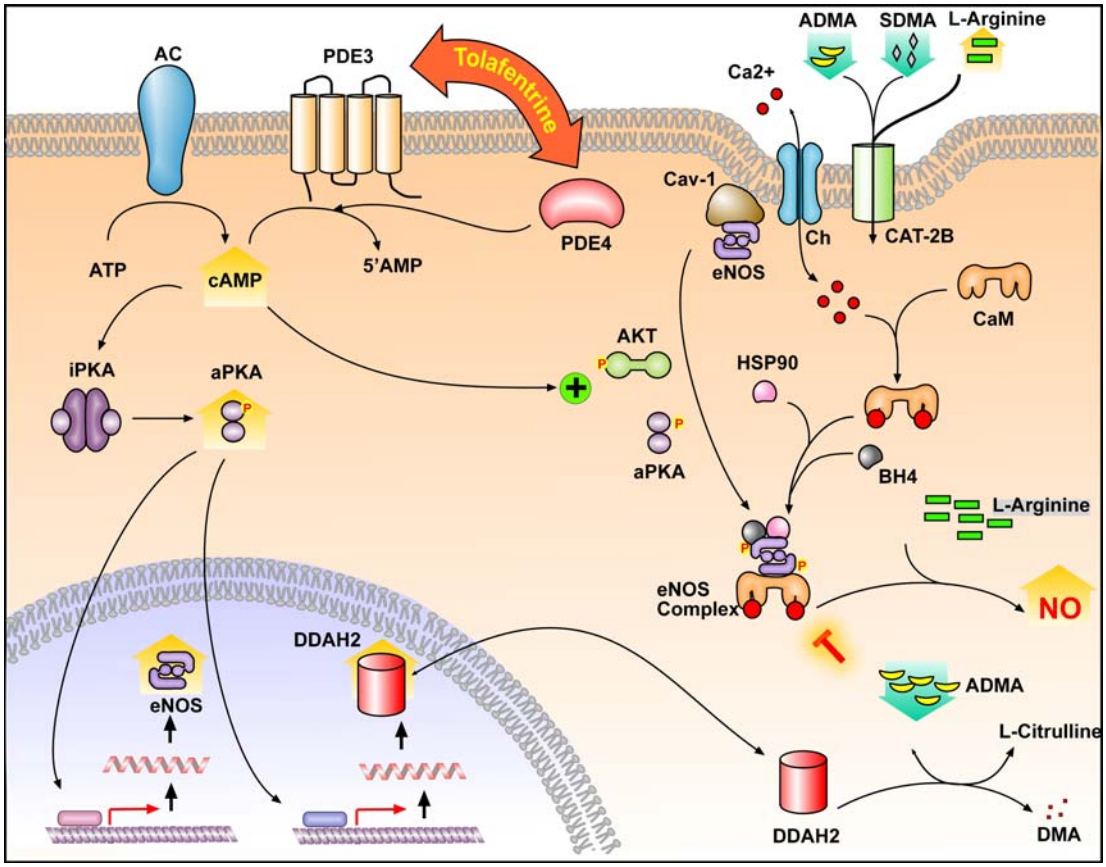

Figure 34: Tolafentrine: Augmentation of NO production in chronic pulmonary hypertensive rats. Schematic depiction of molecular mechanisms responsible for augmented

NO production by Tolafentrine. Tolafentrine, a combined PDE 3/4 inhibitor by increasing intracellular cAMP levels enhances NO production by modulating methyl arginine (ADMA and SDMA) concentrations, that were altered during progressive pulmonary hypertension. These modulatory effects as evidenced by our experiments are due to an upregulation of DDAH expression and activity (Author's Slide).

Although we did not address the effects of tolafentrine on eNOS expression and activity, previous reports suggest that a rise in intracellular cAMP may activate endothelial NOS (eNOS) either directly or indirectly and evoke NO cGMP mediated relaxation (128,129). Chen et al. reported that eNOS is phosphorylated at Ser-1177 by protein kinase A that can be phosphorylated and activated by cAMP (130). Importantly, two recent studies by Fulton et al and Dimmeler et al found that eNOS is an efficient substrate for protein kinase Akt (PKB). PKB via activation PI3 kinase phosphorylates eNOS directly and thereby increases its activity (131,132). Further, Niwano K et al. showed that additional regulatory mechanisms control eNOS expression via cAMP signaling in vitro (133). Even though, the effect of cAMP on eNOS transcription in vivo remained speculative.

Several anti-inflammatory molecules were already shown to reduce ADMA levels by reducing oxidative stress and subsequent enhancement of DDAH activity (134,135). But the results of two small studies of antioxidant vitamins have been negative or inconclusive, thereby cautioning to target DDAH enzyme expression. Our study identified combined PDE 3/4 inhibitor as one of the transcriptional modulator of DDAH2 other than all-trans-Retinoic Acid (109). In addition to transcriptional regulation, combined PDE 3/4 inhibitor by its potent anti-inflammatory properties directly influences DDAH activity. Therefore, these two pathways together enhance the bioavailability of NO, improving the impairment of endothelium-dependent relaxation induced by MCT.

In conclusion, combined PDE 3/4 inhibitors by increasing NO synthesis and bioactivity acutely reduces pulmonary pressures by its effects on vasoreactivity and chronically reduces the progression of disease-media hypertrophy, intimal hyperplasia, and thrombosis by elevating both NO and cAMP.

6. Summary

Idiopathic pulmonary arterial hypertension (IPAH) is a progressive and life-limiting disorder which is associated with impaired bioactivity and/or synthesis of endogenous nitric oxide (NO). The mechanisms resulting in this impairment are multifactorial. Recently, the impact of endogenous NO-synthase inhibitors such as dimethylarginines (ADMA and SDMA) has come into the focus of attention for various endothelial dysfunction associated cardiovascular disorders. As current evidence strongly suggests a central role for endothelial dysfunction in the initiation and progression of pulmonary arterial hypertension (PAH), the plausible role of dimethylarginines is speculated in this disease. Hence forth, the present study was undertaken to investigate the potential role of dimethylarginines in the course of chronic pulmonary hypertension. If this speculation was to be proven, further studies aimed to delineate the precise mechanisms responsible for these alteration including biosynthesis and metabolism of these mediators. These studies were performed mainly on plasma and lung tissues obtained from both IPAH patients and monocrotaline induced pulmonary hypertensive (MCT-PAH) rats.

Interestingly, in both MCT-PAH rats and patients suffering from IPAH (NYHA class III and IV), a marked increase in plasma ADMA and SDMA levels compared to their healthy counterparts was observed. These findings were nicely corroborated by increased biosynthesis as evidenced by high expression of asymmetric and symmetric dimethylated arginine proteins in lung tissues from patients suffering from PAH as well as from MCT-PAH rats. Moreover, the main endothelial ADMA metabolizing enzyme, DDAH2, was found to be drastically reduced in IPAH patients and MCT-PAH rat tissue, at both the mRNA and the protein level with no significant changes in DDAH1 expression. The consistency of results seen in human disease and in an established experimental animal model of pulmonary hypertension strongly suggests that changes in dimethylarginine regulation may contribute considerably to the course of the disease. The current study also suggests that novel therapeutics that target the

signaling pathway of endogenous NOS inhibitors and promote the functional capacity of DDAH2 would be beneficial for the treatment of IPAH.

Interestingly, we identified the phosphodiesterase 3/4 inhibitor, tolafentrine as the first transcriptional modulator of DDAH2 in chronic MCT-PAH rats. Daily repetitive inhalation of tolafentrine by augmenting intracellular cAMP levels restored DDAH expression, activity and nitrite levels that were decreased during the development of MCT-induced PAH. DDAH2 activity restoration was functionally evidenced by near normalized ADMA plasma levels in tolafentrine treated MCT-PAH rats. Furthermore, we also observed a decreased SDMA levels in tolafentrine treated animals, although the mechanisms responsible for this change is not yet clear. Finally, as a regulator of endogenous NOS inhibitors, tolafentrine treatment drastically improved MCT-induced hemodynamic abnormalities and reversed structural and molecular changes underlying MCT induced PAH in rats in the current study.

7. Zusammenfassung

Die chronische pulmonalarterielle Hypertonie (PAH) hat eine schlechte Prognose und betrifft ein großes und zudem wachsendes Patientenkollektiv. Als Auslöser dieser Erkrankung gelten Hypoxie, Entzündung, Thromboembolie und Hyperzirkulation. Ein wesentlicher Pathomechanismus ist die Verschiebung des komplexen Gleichgewichts vasokonstriktiver und vasodilatativer Mediatoren in Richtung der Vasokonstriktoren, verbunden mit verschiedenen strukturellen Veränderungen an den Pulmonalarterien. Der Mangel an Vasodilatatoren wie Stickstoffmonoxid und Prostazyklin und die Überexpression von Vasokonstriktoren, wie Endothelin und Thromboxan, tragen allerdings nicht nur zum erhöhten vaskulären Tonus bei, sondern sind auch maßgeblich am Verlust der physiologischen flussabhängigen Vasodilatation beteiligt. Insbesondere die Stickstoffmonoxidsynthase (NOS) spielt eine zentrale Rolle in der Pathophysiologie dieser pulmonalvaskulären Dysfunktion. Kürzlich konnte gezeigt werden, dass bei verschiedenen kardiovaskulären Erkrankungen erhöhte Konzentrationen endogener Hemmstoffe (symmetrisch dimethyliertes Arginin (SDMA) oder asymmetrisch dimethyliertes Arginin (ADMA)) der NOS messbar sind.

In der vorliegenden Arbeit wurden zunächst Untersuchungen an Plasma und Lungengewebe von Patienten mit idiopathischer pulmonalarterieller Hypertonie (IPAH) und Ratten mit experimenteller pulmonaler Hypertonie durch Injektion von Monocrotalin (MCT-PAH) untersucht. Dabei wurde erstbeschreibend gefunden, dass in IPAH Patienten (NYHA Klasse III und IV) und MCT-PAH Ratten die Plasmaspiegel der endogenen NOS-Hemmstoffe ADMA und SDMA im Vergleich zu Kontrollen drastisch erhöht sind. Die erhöhte Biosynthese von ADMA und SDMA wurde im Gewebe durch immunhistochemische und biochemische Untersuchungen nachgewiesen. Es konnte weiterhin gezeigt werden, dass das ADMA metabolisierende Enzym Dimethylarginin-Dimethylaminohydrolase (DDAH2) hochsignifikant bei klinischer und experimenteller pulmonaler

Hypertonie herabreguliert ist. Durch die verringerte Aktivität des Enzyms wird die gefäßengstellende Wirkung des ADMA verstärkt und verlängert.

Die transkriptorische Modulation der DDAH2 Expression gelang mit Hilfe des dual-selektiven Phosphodiesterase Inhibitors Tolafentrin, welcher nach chronischer Applikation bei experimenteller PAH eine Hochregulation der DDAH2 und dadurch Reduktion der Plasmaspiegel von ADMA induzierte. Durch Erhöhung der intrazellulären cAMP Spiegel wurde die DDAH2-Expression (gezeigt durch immunhistochemische und biochemische Methoden) sowie -Aktivität angehoben. Diese pharmakologische Hochregulation der DDAH2 führte neben der Reduktion der Plasmaspiegel von ADMA und SDMA auch zu Verbesserungen der Hämodynamik und zu einer deutlichen Verbesserung der strukturellen Gefäßveränderungen. Zusammenfassend konnte erstmalig gezeigt werden, dass endogene Hemmstoffe der NO-Synthase bei pulmonalarterieller Hypertonie erhöht sind, was durch eine erhöhte Synthese im Lungengewebe, aber auch reduzierten Abbau durch die im Gefäß herunterregulierte DDAH2 erklärt werden kann. Gleichzeitig gelang es in der vorliegenden Arbeit, durch eine pharmakologische Intervention dieses Schlüsselenzym im kardiopulmonalen Gefäßsystem hoch zu regulieren, was in dem experimentellen Modell des Lungenhochdrucks zu einer Normalisierung der hämodynamischen und histologischen Veränderungen führte.

8.References

1. Simonneau, G., N. Galie, L. J. Rubin, D. Langleben, W. Seeger, G. Domenighetti, S. Gibbs, D. Lebrec, R. Speich, M. Beghetti, S. Rich, and A. Fishman. 2004. Clinical classification of pulmonary hypertension. *J. Am. Coll. Cardiol.* 43:5S.

2. D'Alonzo, G. E., R. J. Barst, S. M. Ayres, E. H. Bergofsky, B. H. Brundage, K. M. Detre, A. P. Fishman, R. M. Goldring, B. M. Groves, J. T. Kernis, and . 1991. Survival in patients with primary pulmonary hypertension. Results from a national prospective registry. *Ann. Intern. Med.* 115:343.

3. Loyd, J. E., R. K. Primm, and J. H. Newman. 1984. Familial primary pulmonary hypertension: clinical patterns. *Am. Rev. Respir. Dis.* 129:194.

4. Loyd, J. E., M. G. Butler, T. M. Foroud, P. M. Conneally, J. A. Phillips, III, and J. H. Newman. 1995. Genetic anticipation and abnormal gender ratio at birth in familial primary pulmonary hypertension. *Am. J. Respir. Crit Care Med.* 152:93.

5. Dresdale, D. T., M. SCHULTZ, and R. J. MICHTOM. 1951. Primary pulmonary hypertension. I. Clinical and hemodynamic study. *Am. J. Med.* 11:686.

6. Gurtner, H. P. 1979. Pulmonary hypertension, "plexogenic pulmonary arteriopathy" and the appetite depressant drug aminorex: post or propter? *Bull. Eur. Physiopathol. Respir.* 15:897.

7. Rubin, L. J. 1988. Primary pulmonary hypertension. *Chest* 93:894.

8. Rich, S. 1998. Primary pulmonary hypertension: executive summary. Evian, France: World Health Organization.

9. Pietra, G. G., W. D. Edwards, J. M. Kay, S. Rich, J. Kernis, B. Schloo, S. M. Ayres, E. H. Bergofsky, B. H. Brundage, K. M. Detre, and . 1989. Histopathology of primary pulmonary hypertension. A qualitative and quantitative study of pulmonary blood vessels from 58 patients in the National Heart, Lung, and Blood Institute, Primary Pulmonary Hypertension Registry. *Circulation* 80:1198.

10. Palevsky, H. I., B. L. Schloo, G. G. Pietra, K. T. Weber, J. S. Janicki, E. Rubin, and A. P. Fishman. 1989. Primary pulmonary hypertension. Vascular structure, morphometry, and responsiveness to vasodilator agents. *Circulation* 80:1207.

11. Smith, P., D. Heath, M. Yacoub, B. Madden, A. Caslin, and J. Gosney. 1990. The ultrastructure of plexogenic pulmonary arteriopathy. *J. Pathol.* 160:111.

12. Tuder, R. M., C. D. Cool, M. Yeager, L. Taraseviciene-Stewart, T. M. Bull, and N. F. Voelkel. 2001. The pathobiology of pulmonary hypertension. Endothelium. *Clin. Chest Med.* 22:405.

13. Humbert, M., N. W. Morrell, S. L. Archer, K. R. Stenmark, M. R. MacLean, I. M. Lang, B. W. Christman, E. K. Weir, O. Eickelberg, N. F. Voelkel, and M. Rabinovitch. 2004. Cellular and molecular pathobiology of pulmonary arterial hypertension. *J. Am. Coll. Cardiol.* 43:13S.

14. Budhiraja, R., R. M. Tuder, and P. M. Hassoun. 2004. Endothelial dysfunction in pulmonary hypertension. *Circulation* 109:159.

15. Lopes, A. A., N. Y. Maeda, R. C. Goncalves, and S. P. Bydlowski. 2000. Endothelial cell dysfunction correlates differentially with survival in primary and secondary pulmonary hypertension. *Am. Heart J.* 139:618.

16. Bredt, D. S., and S. H. Snyder. 1994. Nitric oxide: a physiologic messenger molecule. *Annu. Rev. Biochem.* 63:175.

17. Wang, Y., and P. A. Marsden. 1995. Nitric oxide synthases: gene structure and regulation. *Adv. Pharmacol.* 34:71.

18. Forstermann, U., J. P. Boissel, and H. Kleinert. 1998. Expressional control of the 'constitutive' isoforms of nitric oxide synthase (NOS I and NOS III) 56. *FASEB J.* 12:773.

19. Kaneko, F. T., A. C. Arroliga, R. A. Dweik, S. A. Comhair, D. Laskowski, R. Oppedisano, M. J. Thomassen, and S. C. Erzurum. 1998. Biochemical reaction products of nitric oxide as quantitative markers of primary pulmonary hypertension. *Am. J. Respir. Crit Care Med.* 158:917.

20. Giaid, A., and D. Saleh. 1995. Reduced expression of endothelial nitric oxide synthase in the lungs of patients with pulmonary hypertension. *N. Engl. J. Med.* 333:214.

21. McQuillan, L. P., G. K. Leung, P. A. Marsden, S. K. Kostyk, and S. Kourembanas. 1994. Hypoxia inhibits expression of eNOS via transcriptional and posttranscriptional mechanisms. *Am. J. Physiol* 267:H1921-H1927.

22. Yanagisawa, M., H. Kurihara, S. Kimura, Y. Tomobe, M. Kobayashi, Y. Mitsui, Y. Yazaki, K. Goto, and T. Masaki. 1988. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 332:411.

23. Cacoub, P., R. Dorent, P. Nataf, A. Carayon, M. Riquet, E. Noe, J. C. Piette, P. Godeau, and I. Gandjbakhch. 1997. Endothelin-1 in the lungs of patients with pulmonary hypertension. *Cardiovasc. Res.* 33:196.

24. Giaid, A., M. Yanagisawa, D. Langleben, R. P. Michel, R. Levy, H. Shennib, S. Kimura, T. Masaki, W. P. Duguid, and D. J. Stewart. 1993. Expression of endothelin-1 in the lungs of patients with pulmonary hypertension. *N. Engl. J. Med.* 328:1732.

25. Moncada, S., and J. R. Vane. 1978. Pharmacology and endogenous roles of prostaglandin endoperoxides, thromboxane A2, and prostacyclin. *Pharmacol. Rev.* 30:293.

26. Christman, B. W., C. D. McPherson, J. H. Newman, G. A. King, G. R. Bernard, B. M. Groves, and J. E. Loyd. 1992. An imbalance between the excretion of thromboxane and prostacyclin metabolites in pulmonary hypertension 39. *N. Engl. J. Med.* 327:70.

27. Tuder, R. M., C. D. Cool, M. W. Geraci, J. Wang, S. H. Abman, L. Wright, D. Badesch, and N. F. Voelkel. 1999. Prostacyclin synthase expression is decreased in lungs from patients with severe pulmonary hypertension 61. *Am. J. Respir. Crit Care Med.* 159:1925.

28. Stenmark, K. R., and R. P. Mecham. 1997. Cellular and molecular mechanisms of pulmonary vascular remodeling. *Annu. Rev. Physiol* 59:89.

29. Chazova, I., J. E. Loyd, V. S. Zhdanov, J. H. Newman, Y. Belenkov, and B. Meyrick. 1995. Pulmonary artery adventitial changes and venous involvement in primary pulmonary hypertension. *Am. J. Pathol.* 146:389.

30. Rabinovitch, M. 2001. Pathobiology of pulmonary hypertension. Extracellular matrix. *Clin. Chest Med.* 22:433, viii.

31. Tuder, R. M., B. Groves, D. B. Badesch, and N. F. Voelkel. 1994. Exuberant endothelial cell growth and elements of inflammation are

present in plexiform lesions of pulmonary hypertension. *Am. J. Pathol.* 144:275.

32. Voelkel, N. F., and R. M. Tuder. 1997. Cellular and molecular biology of vascular smooth muscle cells in pulmonary hypertension. *Pulm. Pharmacol. Ther.* 10:231.

33. Herve, P., J. M. Launay, M. L. Scrobohaci, F. Brenot, G. Simonneau, P. Petitpretz, P. Poubeau, J. Cerrina, P. Duroux, and L. Drouet. 1995. Increased plasma serotonin in primary pulmonary hypertension. *Am. J. Med.* 99:249.

34. Eddahibi, S., M. Humbert, E. Fadel, B. Raffestin, M. Darmon, F. Capron, G. Simonneau, P. Dartevelle, M. Hamon, and S. Adnot. 2001. Serotonin transporter overexpression is responsible for pulmonary artery smooth muscle hyperplasia in primary pulmonary hypertension. *J. Clin. Invest* 108:1141.

35. Coppock, E. A., J. R. Martens, and M. M. Tamkun. 2001. Molecular basis of hypoxia-induced pulmonary vasoconstriction: role of voltage-gated K+ channels. *Am. J. Physiol Lung Cell Mol. Physiol* 281:L1.

36. Yuan, J. X., A. M. Aldinger, M. Juhaszova, J. Wang, J. V. Conte, Jr., S. P. Gaine, J. B. Orens, and L. J. Rubin. 1998. Dysfunctional voltage-gated K+ channels in pulmonary artery smooth muscle cells of patients with primary pulmonary hypertension. *Circulation* 98:1400.

37. Yuan, X. J., J. Wang, M. Juhaszova, S. P. Gaine, and L. J. Rubin. 1998. Attenuated K+ channel gene transcription in primary pulmonary hypertension. *Lancet* 351:726.

38. Katugampola, S. D., and A. P. Davenport. 2001. Thromboxane receptor density is increased in human cardiovascular disease with evidence for inhibition at therapeutic concentrations by the AT(1) receptor antagonist losartan. *Br. J. Pharmacol.* 134:1385.

39. Fuster, V., P. M. Steele, W. D. Edwards, B. J. Gersh, M. D. McGoon, and R. L. Frye. 1984. Primary pulmonary hypertension: natural history and the importance of thrombosis. *Circulation* 70:580.

40. Chaouat, A., E. Weitzenblum, and T. Higenbottam. 1996. The role of thrombosis in severe pulmonary hypertension. *Eur. Respir. J.* 9:356.

41. Welsh, C. H., K. L. Hassell, D. B. Badesch, D. C. Kressin, and R. A. Marlar. 1996. Coagulation and fibrinolytic profiles in patients with severe pulmonary hypertension 13. *Chest* 110:710.

42. Lopes, A. A., N. Y. Maeda, A. Almeida, R. Jaeger, M. Ebaid, and D. F. Chamone. 1993. Circulating platelet aggregates indicative of in vivo platelet activation in pulmonary hypertension. *Angiology* 44:701.

43. Rich, S., D. R. Dantzker, S. M. Ayres, E. H. Bergofsky, B. H. Brundage, K. M. Detre, A. P. Fishman, R. M. Goldring, B. M. Groves, S. K. Koerner, and . 1987. Primary pulmonary hypertension. A national prospective study. *Ann. Intern. Med.* 107:216.

44. Mikhail, G. W., J. S. Gibbs, and M. H. Yacoub. 2001. Pulmonary and systemic arterial pressure changes during syncope in primary pulmonary hypertension 8. *Circulation* 104:1326.

45. McGoon, M., D. Gutterman, V. Steen, R. Barst, D. C. McCrory, T. A. Fortin, and J. E. Loyd. 2004. Screening, early detection, and diagnosis of

pulmonary arterial hypertension: ACCP evidence-based clinical practice guidelines 2. *Chest* 126:14S.

46. Budev, M. M., A. C. Arroliga, and C. A. Jennings. 2003. Diagnosis and evaluation of pulmonary hypertension. *Cleve. Clin. J. Med.* 70 Suppl 1:S9.

47. Badesch, D. B., S. H. Abman, G. S. Ahearn, R. J. Barst, D. C. McCrory, G. Simonneau, and V. V. McLaughlin. 2004. Medical therapy for pulmonary arterial hypertension: ACCP evidence-based clinical practice guidelines 2. *Chest* 126:35S.

48. Rich, S., E. Kaufmann, and P. S. Levy. 1992. The effect of high doses of calcium-channel blockers on survival in primary pulmonary hypertension. *N. Engl. J. Med.* 327:76.

49. Sitbon, O., M. Humbert, J. L. Jagot, O. Taravella, M. Fartoukh, F. Parent, P. Herve, and G. Simonneau. 1998. Inhaled nitric oxide as a screening agent for safely identifying responders to oral calcium-channel blockers in primary pulmonary hypertension. *Eur. Respir. J.* 12:265.

50. Sitbon, O., M. Humbert, X. Jais, V. Ioos, A. M. Hamid, S. Provencher, G. Garcia, F. Parent, P. Herve, and G. Simonneau. 2005. Long-term response to calcium channel blockers in idiopathic pulmonary arterial hypertension 3. *Circulation* 111:3105.

51. Rubin, L. J., J. Mendoza, M. Hood, M. McGoon, R. Barst, W. B. Williams, J. H. Diehl, J. Crow, and W. Long. 1990. Treatment of primary pulmonary hypertension with continuous intravenous prostacyclin (epoprostenol). Results of a randomized trial. *Ann. Intern. Med.* 112:485.

52. Higenbottam, T., D. Wheeldon, F. Wells, and J. Wallwork. 1984. Long-term treatment of primary pulmonary hypertension with continuous intravenous epoprostenol (prostacyclin). *Lancet* 1:1046.

53. Olschewski, H., G. Simonneau, N. Galie, T. Higenbottam, R. Naeije, L. J. Rubin, S. Nikkho, R. Speich, M. M. Hoeper, J. Behr, J. Winkler, O. Sitbon, W. Popov, H. A. Ghofrani, A. Manes, D. G. Kiely, R. Ewert, A. Meyer, P. A. Corris, M. Delcroix, M. Gomez-Sanchez, H. Siedentop, and W. Seeger. 2002. Inhaled iloprost for severe pulmonary hypertension. *N. Engl. J. Med.* 347:322.

54. Pepke-Zaba, J., T. W. Higenbottam, A. T. nh-Xuan, D. Stone, and J. Wallwork. 1991. Inhaled nitric oxide as a cause of selective pulmonary vasodilatation in pulmonary hypertension. *Lancet* 338:1173.

55. Hasuda, T., T. Satoh, A. Shimouchi, F. Sakamaki, S. Kyotani, T. Matsumoto, Y. Goto, and N. Nakanishi. 2000. Improvement in exercise capacity with nitric oxide inhalation in patients with precapillary pulmonary hypertension. *Circulation* 101:2066.

56. Nagaya, N., M. Uematsu, H. Oya, N. Sato, F. Sakamaki, S. Kyotani, K. Ueno, N. Nakanishi, M. Yamagishi, and K. Miyatake. 2001. Short-term oral administration of L-arginine improves hemodynamics and exercise capacity in patients with precapillary pulmonary hypertension. *Am. J. Respir. Crit Care Med.* 163:887.

57. Channick, R. N., G. Simonneau, O. Sitbon, I. M. Robbins, A. Frost, V. F. Tapson, D. B. Badesch, S. Roux, M. Rainisio, F. Bodin, and L. J. Rubin. 2001. Effects of the dual endothelin-receptor antagonist bosentan in patients with pulmonary hypertension: a randomised placebo-controlled study 13. *Lancet* 358:1119.

58. Sitbon, O., D. B. Badesch, R. N. Channick, A. Frost, I. M. Robbins, G. Simonneau, V. F. Tapson, and L. J. Rubin. 2003. Effects of the dual endothelin receptor antagonist bosentan in patients with pulmonary arterial hypertension: a 1-year follow-up study. *Chest* 124:247.

59. Beavo, J. A. 1995. Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms. *Physiol Rev.* 75:725.

60. Manganiello, V. C., T. Murata, M. Taira, P. Belfrage, and E. Degerman. 1995. Diversity in cyclic nucleotide phosphodiesterase isoenzyme families 35. *Arch. Biochem. Biophys.* 322:1.

61. Ahn, H. S., M. Foster, M. Cable, B. J. Pitts, and E. J. Sybertz. 1991. Ca/CaM-stimulated and cGMP-specific phosphodiesterases in vascular and non-vascular tissues. *Adv. Exp. Med. Biol.* 308:191.

62. Michelakis, E., W. Tymchak, D. Lien, L. Webster, K. Hashimoto, and S. Archer. 2002. Oral sildenafil is an effective and specific pulmonary vasodilator in patients with pulmonary arterial hypertension: comparison with inhaled nitric oxide. *Circulation* 105:2398.

63. Ghofrani, H. A., R. Wiedemann, F. Rose, R. T. Schermuly, H. Olschewski, N. Weissmann, A. Gunther, D. Walmrath, W. Seeger, and F. Grimminger. 2002. Sildenafil for treatment of lung fibrosis and pulmonary hypertension: a randomised controlled trial 27. *Lancet* 360:895.

64. Gross, S. S., and M. S. Wolin. 1995. Nitric oxide: pathophysiological mechanisms 52. *Annu. Rev. Physiol* 57:737.

65. Furchgott, R. F., and J. V. Zawadzki. 1980. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 288:373.

66. Ignarro, L. J., G. M. Buga, K. S. Wood, R. E. Byrns, and G. Chaudhuri. 1987. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc. Natl. Acad. Sci. U. S. A* 84:9265.

67. Moncada, S., and A. Higgs. 1993. The L-arginine-nitric oxide pathway. *N. Engl. J. Med.* 329:2002.

68. Griffith, O. W., and D. J. Stuehr. 1995. Nitric oxide synthases: properties and catalytic mechanism. *Annu. Rev. Physiol* 57:707.

69. Nathan, C., and Q. W. Xie. 1994. Nitric oxide synthases: roles, tolls, and controls. *Cell* 78:915.

70. Nathan, C. 1997. Inducible nitric oxide synthase: what difference does it make? *J. Clin. Invest* 100:2417.

71. Bredt, D. S., and S. H. Snyder. 1994. Nitric oxide: a physiologic messenger molecule 124. *Annu. Rev. Biochem.* 63:175.

72. Bolotina, V. M., S. Najibi, J. J. Palacino, P. J. Pagano, and R. A. Cohen. 1994. Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature* 368:850.

73. Dimmeler, S., and A. M. Zeiher. 1997. Nitric oxide and apoptosis: another paradigm for the double-edged role of nitric oxide. *Nitric. Oxide.* 1:275.

74. Yoshizumi, M., M. A. Perrella, J. C. Burnett, Jr., and M. E. Lee. 1993. Tumor necrosis factor downregulates an endothelial nitric oxide synthase mRNA by shortening its half-life. *Circ. Res.* 73:205.

75. Noris, M., M. Morigi, R. Donadelli, S. Aiello, M. Foppolo, M. Todeschini, S. Orisio, G. Remuzzi, and A. Remuzzi. 1995. Nitric oxide synthesis by cultured endothelial cells is modulated by flow conditions. *Circ. Res.* 76:536.

76. Vaziri, N. D., and X. Q. Wang. 1999. cGMP-mediated negative-feedback regulation of endothelial nitric oxide synthase expression by nitric oxide. *Hypertension* 34:1237.

77. Govers, R., and T. J. Rabelink. 2001. Cellular regulation of endothelial nitric oxide synthase. *Am. J. Physiol Renal Physiol* 280:F193-F206.

78. Mori, M., and T. Gotoh. 2000. Regulation of nitric oxide production by arginine metabolic enzymes. *Biochem. Biophys. Res. Commun.* 275:715.

79. McDonald, K. K., S. Zharikov, E. R. Block, and M. S. Kilberg. 1997. A caveolar complex between the cationic amino acid transporter 1 and endothelial nitric-oxide synthase may explain the "arginine paradox". *J. Biol. Chem.* 272:31213.

80. Closs, E. I., F. Z. Basha, A. Habermeier, and U. Forstermann. 1997. Interference of L-arginine analogues with L-arginine transport mediated by the y+ carrier hCAT-2B 24. *Nitric. Oxide.* 1:65.

81. Drapier, J. C., and J. B. Hibbs, Jr. 1986. Murine cytotoxic activated macrophages inhibit aconitase in tumor cells. Inhibition involves the iron-sulfur prosthetic group and is reversible. *J. Clin. Invest* 78:790.

82. Najbauer, J., B. A. Johnson, A. L. Young, and D. W. Aswad. 1993. Peptides with sequences similar to glycine, arginine-rich motifs in proteins interacting with RNA are efficiently recognized by methyltransferase(s) modifying arginine in numerous proteins 8. *J. Biol. Chem.* 268:10501.

83. Ghosh, S. K., W. K. Paik, and S. Kim. 1988. Purification and molecular identification of two protein methylases I from calf brain. Myelin basic protein- and histone-specific enzyme. *J. Biol. Chem.* 263:19024.

84. Sydow, K., and T. Munzel. 2003. ADMA and oxidative stress. *Atheroscler. Suppl* 4:41.

85. Kakimoto, Y., and S. Akazawa. 1970. Isolation and identification of N-G,N-G- and N-G,N'-G-dimethyl-arginine, N-epsilon-mono-, di-, and trimethyllysine, and glucosylgalactosyl- and galactosyl-delta-hydroxylysine from human urine. *J. Biol. Chem.* 245:5751.

86. Ogawa, T., M. Kimoto, and K. Sasaoka. 1987. Occurrence of a new enzyme catalyzing the direct conversion of NG,NG-dimethyl-L-arginine to L-citrulline in rats. *Biochem. Biophys. Res. Commun.* 148:671.

87. Macallister, R. J., H. Parry, M. Kimoto, T. Ogawa, R. J. Russell, H. Hodson, G. S. Whitley, and P. Vallance. 1996. Regulation of nitric oxide synthesis by dimethylarginine dimethylaminohydrolase. *Br. J. Pharmacol.* 119:1533.

88. Dayoub, H., V. Achan, S. Adimoolam, J. Jacobi, M. C. Stuehlinger, B. Y. Wang, P. S. Tsao, M. Kimoto, P. Vallance, A. J. Patterson, and J. P. Cooke. 2003. Dimethylarginine dimethylaminohydrolase regulates nitric oxide synthesis: genetic and physiological evidence 2. *Circulation* 108:3042.

89. Leiper, J. M., M. J. Santa, A. Chubb, R. J. Macallister, I. G. Charles, G. S. Whitley, and P. Vallance. 1999. Identification of two human dimethylarginine dimethylaminohydrolases with distinct tissue distributions and homology with microbial arginine deiminases 11. *Biochem. J.* 343 Pt 1:209.

90. Murray-Rust, J., J. Leiper, M. McAlister, J. Phelan, S. Tilley, M. J. Santa, P. Vallance, and N. McDonald. 2001. Structural insights into the hydrolysis of cellular nitric oxide synthase inhibitors by dimethylarginine dimethylaminohydrolase. *Nat. Struct. Biol.* 8:679.

91. Macallister, R. J., S. A. Fickling, G. S. Whitley, and P. Vallance. 1994. Metabolism of methylarginines by human vasculature; implications for the regulation of nitric oxide synthesis 37. *Br. J. Pharmacol.* 112:43.

92. Abbasi, F., T. Asagmi, J. P. Cooke, C. Lamendola, T. McLaughlin, G. M. Reaven, M. Stuehlinger, and P. S. Tsao. 2001. Plasma concentrations of asymmetric dimethylarginine are increased in patients with type 2 diabetes mellitus. *Am. J. Cardiol.* 88:1201.

93. Gorenflo, M., C. Zheng, E. Werle, W. Fiehn, and H. E. Ulmer. 2001. Plasma levels of asymmetrical dimethyl-L-arginine in patients with congenital heart disease and pulmonary hypertension. *J. Cardiovasc. Pharmacol.* 37:489.

94. Lu, T. M., Y. A. Ding, H. B. Leu, W. H. Yin, W. H. Sheu, and K. M. Chu. 2004. Effect of rosuvastatin on plasma levels of asymmetric dimethylarginine in patients with hypercholesterolemia 4. *Am. J. Cardiol.* 94:157.

95. Stuhlinger, M. C., R. K. Oka, E. E. Graf, I. Schmolzer, B. M. Upson, O. Kapoor, A. Szuba, M. R. Malinow, T. C. Wascher, O. Pachinger, and J. P.

Cooke. 2003. Endothelial dysfunction induced by hyperhomocyst(e)inemia: role of asymmetric dimethylarginine 3. *Circulation* 108:933.

96. Ito, A., P. S. Tsao, S. Adimoolam, M. Kimoto, T. Ogawa, and J. P. Cooke. 1999. Novel mechanism for endothelial dysfunction: dysregulation of dimethylarginine dimethylaminohydrolase 8. *Circulation* 99:3092.

97. Weis, M., T. N. Kledal, K. Y. Lin, S. N. Panchal, S. Z. Gao, H. A. Valantine, E. S. Mocarski, and J. P. Cooke. 2004. Cytomegalovirus infection impairs the nitric oxide synthase pathway: role of asymmetric dimethylarginine in transplant arteriosclerosis 17. *Circulation* 109:500.

98. McQuillan, L. P., G. K. Leung, P. A. Marsden, S. K. Kostyk, and S. Kourembanas. 1994. Hypoxia inhibits expression of eNOS via transcriptional and posttranscriptional mechanisms. *Am. J. Physiol* 267:H1921-H1927.

99. Giaid, A., and D. Saleh. 1995. Reduced expression of endothelial nitric oxide synthase in the lungs of patients with pulmonary hypertension. *N. Engl. J. Med.* 333:214.

100. Xue, C., and R. A. Johns. 1995. Endothelial nitric oxide synthase in the lungs of patients with pulmonary hypertension. *N. Engl. J. Med.* 333:1642.

101. Tyler, R. C., M. Muramatsu, S. H. Abman, T. J. Stelzner, D. M. Rodman, K. D. Bloch, and I. F. McMurtry. 1999. Variable expression of endothelial NO synthase in three forms of rat pulmonary hypertension 11. *Am. J. Physiol* 276:L297-L303.

102. Xu, W., F. T. Kaneko, S. Zheng, S. A. Comhair, A. J. Janocha, T. Goggans, F. B. Thunnissen, C. Farver, S. L. Hazen, C. Jennings, R. A.

Dweik, A. C. Arroliga, and S. C. Erzurum. 2004. Increased arginase II and decreased NO synthesis in endothelial cells of patients with pulmonary arterial hypertension 2. *FASEB J.* 18:1746.

103. Mehta, S., D. J. Stewart, D. Langleben, and R. D. Levy. 1995. Short-term pulmonary vasodilation with L-arginine in pulmonary hypertension. *Circulation* 92:1539.

104. Konduri, G. G., J. Ou, Y. Shi, and K. A. Pritchard, Jr. 2003. Decreased association of HSP90 impairs endothelial nitric oxide synthase in fetal lambs with persistent pulmonary hypertension. *Am. J. Physiol Heart Circ. Physiol* 285:H204-H211.

105. Schermuly, R. T., K. P. Kreisselmeier, H. A. Ghofrani, H. Yilmaz, G. Butrous, L. Ermert, M. Ermert, N. Weissmann, F. Rose, A. Guenther, D. Walmrath, W. Seeger, and F. Grimminger. 2004. Chronic sildenafil treatment inhibits monocrotaline-induced pulmonary hypertension in rats 13. *Am. J. Respir. Crit Care Med.* 169:39.

106. Arrigoni, F. I., P. Vallance, S. G. Haworth, and J. M. Leiper. 2003. Metabolism of asymmetric dimethylarginines is regulated in the lung developmentally and with pulmonary hypertension induced by hypobaric hypoxia 2. *Circulation* 107:1195.

107. Molteni, A., W. F. Ward, C. H. Ts'ao, C. D. Port, and N. H. Solliday. 1984. Monocrotaline-induced pulmonary endothelial dysfunction in rats. *Proc. Soc. Exp. Biol. Med.* 176:88.

108. Nakazawa, H., M. Hori, H. Ozaki, and H. Karaki. 1999. Mechanisms underlying the impairment of endothelium-dependent relaxation in the pulmonary artery of monocrotaline-induced pulmonary hypertensive rats. *Br. J. Pharmacol.* 128:1098.

109. Achan, V., C. T. Tran, F. Arrigoni, G. S. Whitley, J. M. Leiper, and P. Vallance. 2002. all-trans-Retinoic acid increases nitric oxide synthesis by endothelial cells: a role for the induction of dimethylarginine dimethylaminohydrolase 5. *Circ. Res.* 90:764.

110. Jones, L. C., C. T. Tran, J. M. Leiper, A. D. Hingorani, and P. Vallance. 2003. Common genetic variation in a basal promoter element alters DDAH2 expression in endothelial cells10. *Biochem. Biophys. Res. Commun.* 310:836.

111. Boger, R. H., K. Sydow, J. Borlak, T. Thum, H. Lenzen, B. Schubert, D. Tsikas, and S. M. Bode-Boger. 2000. LDL cholesterol upregulates synthesis of asymmetrical dimethylarginine in human endothelial cells: involvement of S-adenosylmethionine-dependent methyltransferases 52. *Circ. Res.* 87:99.

112. Bowers, R., C. Cool, R. C. Murphy, R. M. Tuder, M. W. Hopken, S. C. Flores, and N. F. Voelkel. 2004. Oxidative stress in severe pulmonary hypertension 16. *Am. J. Respir. Crit Care Med.* 169:764.

113. Cracowski, J. L., C. Cracowski, G. Bessard, J. L. Pepin, J. Bessard, C. Schwebel, F. Stanke-Labesque, and C. Pison. 2001. Increased lipid peroxidation in patients with pulmonary hypertension 32. *Am. J. Respir. Crit Care Med.* 164:1038.

114. Azuma, H. 1999. [Role of L-arginine--endogenous NOS inhibitors--endothelin-1 pathway for the vascular remodelling] 133. *Nippon Yakurigaku Zasshi* 114 Suppl 1:33P.

115. Ohnishi, M., A. Wada, T. Tsutamoto, M. Fujii, T. Matsumoto, T. Yamamoto, T. Takayama, X. Wang, and M. Kinoshita. 2002. Endothelin stimulates an endogenous nitric oxide synthase inhibitor, asymmetric

dimethylarginine, in experimental heart failure. *Clin. Sci. (Lond)* 103 Suppl 48:241S.

116. Piatti, P., G. Fragasso, L. D. Monti, E. Setola, P. Lucotti, I. Fermo, R. Paroni, E. Galluccio, G. Pozza, S. Chierchia, and A. Margonato. 2003. Acute intravenous L-arginine infusion decreases endothelin-1 levels and improves endothelial function in patients with angina pectoris and normal coronary arteriograms: correlation with asymmetric dimethylarginine levels 12. *Circulation* 107:429.

117. MacLean, M. R., E. D. Johnston, K. M. McCulloch, L. Pooley, M. D. Houslay, and G. Sweeney. 1997. Phosphodiesterase isoforms in the pulmonary arterial circulation of the rat: changes in pulmonary hypertension. *J. Pharmacol. Exp. Ther.* 283:619.

118. Murray, F., M. R. MacLean, and N. J. Pyne. 2002. Increased expression of the cGMP-inhibited cAMP-specific (PDE3) and cGMP binding cGMP-specific (PDE5) phosphodiesterases in models of pulmonary hypertension 8. *Br. J. Pharmacol.* 137:1187.

119. Wagner, R. S., C. J. Smith, A. M. Taylor, and R. A. Rhoades. 1997. Phosphodiesterase inhibition improves agonist-induced relaxation of hypertensive pulmonary arteries24. *J. Pharmacol. Exp. Ther.* 282:1650.

120. Torphy, T. J. 1998. Phosphodiesterase isozymes: molecular targets for novel antiasthma agents13. *Am. J. Respir. Crit Care Med.* 157:351.

121. Ghofrani, H. A., F. Rose, R. T. Schermuly, H. Olschewski, R. Wiedemann, N. Weissmann, C. Schudt, H. Tenor, W. Seeger, and F. Grimminger. 2002. Amplification of the pulmonary vasodilatory response to inhaled iloprost by subthreshold phosphodiesterase types 3 and 4 inhibition in severe pulmonary hypertension25. *Crit Care Med.* 30:2489.

122. Schermuly, R. T., K. P. Kreisselmeier, H. A. Ghofrani, A. Samidurai, S. Pullamsetti, N. Weissmann, C. Schudt, L. Ermert, W. Seeger, and F. Grimminger. 2004. Antiremodeling effects of iloprost and the dual-selective phosphodiesterase 3/4 inhibitor tolafentrine in chronic experimental pulmonary hypertension15. *Circ. Res.* 94:1101.

123. Wharton, J., N. Davie, P. D. Upton, M. H. Yacoub, J. M. Polak, and N. W. Morrell. 2000. Prostacyclin analogues differentially inhibit growth of distal and proximal human pulmonary artery smooth muscle cells 46. *Circulation* 102:3130.

124. Phillips, P. G., L. Long, M. R. Wilkins, and N. W. Morrell. 2005. cAMP phosphodiesterase inhibitors potentiate effects of prostacyclin analogs in hypoxic pulmonary vascular remodeling. *Am. J. Physiol Lung Cell Mol. Physiol* 288:L103-L115.

125. Blease, K., A. Burke-Gaffney, and P. G. Hellewell. 1998. Modulation of cell adhesion molecule expression and function on human lung microvascular endothelial cells by inhibition of phosphodiesterases 3 and 4 25. *Br. J. Pharmacol.* 124:229.

126. Suttorp, N., P. Ehreiser, S. Hippenstiel, M. Fuhrmann, M. Krull, H. Tenor, and C. Schudt. 1996. Hyperpermeability of pulmonary endothelial monolayer: protective role of phosphodiesterase isoenzymes 3 and 4 63. *Lung* 174:181.

127. Thompson, W. J., T. Ashikaga, J. J. Kelly, L. Liu, B. Zhu, L. Vemavarapu, and S. J. Strada. 2002. Regulation of cyclic AMP in rat pulmonary microvascular endothelial cells by rolipram-sensitive cyclic AMP phosphodiesterase (PDE4). *Biochem. Pharmacol.* 63:797.

128. Gray, D. W., and I. Marshall. 1992. Novel signal transduction pathway mediating endothelium-dependent beta-adrenoceptor vasorelaxation in rat thoracic aorta. *Br. J. Pharmacol.* 107:684.

129. Zhang, X., and T. H. Hintze. 2001. cAMP signal transduction cascade, a novel pathway for the regulation of endothelial nitric oxide production in coronary blood vessels40. *Arterioscler. Thromb. Vasc. Biol.* 21:797.

130. Chen, Z. P., K. I. Mitchelhill, B. J. Michell, D. Stapleton, I. Rodriguez-Crespo, L. A. Witters, D. A. Power, P. R. Ortiz de Montellano, and B. E. Kemp. 1999. AMP-activated protein kinase phosphorylation of endothelial NO synthase. *FEBS Lett.* 443:285.

131. Dimmeler, S., I. Fleming, B. Fisslthaler, C. Hermann, R. Busse, and A. M. Zeiher. 1999. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* 399:601.

132. Fulton, D., J. P. Gratton, T. J. McCabe, J. Fontana, Y. Fujio, K. Walsh, T. F. Franke, A. Papapetropoulos, and W. C. Sessa. 1999. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt 84. *Nature* 399:597.

133. Niwano, K., M. Arai, K. Tomaru, T. Uchiyama, Y. Ohyama, and M. Kurabayashi. 2003. Transcriptional stimulation of the eNOS gene by the stable prostacyclin analogue beraprost is mediated through cAMP-responsive element in vascular endothelial cells: close link between PGI2 signal and NO pathways2. *Circ. Res.* 93:523.

134. Lin, K. Y., A. Ito, T. Asagami, P. S. Tsao, S. Adimoolam, M. Kimoto, H. Tsuji, G. M. Reaven, and J. P. Cooke. 2002. Impaired nitric oxide synthase pathway in diabetes mellitus: role of asymmetric

dimethylarginine and dimethylarginine dimethylaminohydrolase 25. *Circulation* 106:987.

135. Teixeira, M. M., R. W. Gristwood, N. Cooper, and P. G. Hellewell. 1997. Phosphodiesterase (PDE)4 inhibitors: anti-inflammatory drugs of the future? *Trends Pharmacol. Sci.* 18:164.

9. Erklärung

“Ich erkläre: Ich habe die vorgelegte Dissertation selbständig, ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertaion angegeben habe. Alle Textstellen, die wörtlich oder sinngemäβ 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 Gieβen zur Sicherung guter wissenschaftlicher Praxis” niedergelegt sind, eingehalten.“

10 Acknowledgments

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11. Curriculum Vitae

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Year February, 2002 to July, 2005

Degree	:	**Ph.D Student**
Thesis topic	:	Role of Dimethylarginine Dimethylamino-hydrolases (DDAH) in Pulmonary Arterial Hypertension
University	:	Justus-Liebig-Universität Giessen.

Year 1997 to May 1999

Degree	:	**Master of Science [Biotechnology]**
Course-outline	:	Advanced Biochemistry, Molecular Biology, Immunology, Microbiology and Biotechnology.
University	:	Department of Biotechnology, G.G University, Chattisgarh, India.
Percentage	:	76%

Year 1994 to April 1997

Degree	:	**Bachelor of Science [Microbiology]**
Course-outline	:	Fundamentals in Chemistry, Zoology, Microbiology and Parasitology.
University	:	Osmania University.
Percentage	:	78%

Scientific presentations

1. **S Pullamsetti,** L Kiss, H.A. Ghofrani, N. Weissmann, F. Rose, N Hallal, W. Seeger F. Grimminger, R.T. Schermuly. Increased levels and reduced catabolism of asymmetric and symmetric dimethylarginines in pulmonary hypertension. **ATS 2005 – San Diego, 101th International Conference.**

2. **S Pullamsetti,** L Kiss, H.A. Ghofrani, N. Weissmann, F. Rose, N Hallal, W. Seeger F. Grimminger, R.T. Schermuly. Dysregulation of DDAH II in pulmonary arterial hypertension **Vascular NO: From Bench to Bedside - An International Symposium, Hannover , Germany.**

3. R. T. Schermuly, H. Yilmaz, H. A. Ghofrani, **S. Pullamsetti**, A. Schulz, N. Weissmann, F. Grimminger, W. Seeger. Inhaled iloprost reverses chronic experimental pulmonary hypertension. **ERS Programme, Glasgow, United Kingdom.**

4. R.T. Schermuly, K.P. Kreisselmeier, H. Yilmaz, G. Butrous, A. Samidurai, **S. Pullamsetti,** L. Ermert, N. Weissmann, F. Rose, D. Walmrath, W. Seeger, H.A. Ghofrani, F. Grimminger. Chronic Sildenafil Treatment Inhibits Monocrotaline-Induced Pulmonary Hypertension and Right Heart Hypertrophy. **ATS 2003 - Seattle, 99th International Conference.**

5. R.T. Schermuly, K.P. Kreisselmeier, H. Yilmaz, A. Samidurai, **S. Pullamsetti**, N. Weissmann, F. Rose, C. Schudt, L. Ermert, D. Walmrath, W. Seeger, H.A. Ghofrani, F. Grimminger. Anti-Remodeling Effects of Iloprost and the Dualselective Phosphodiesterase 3/4 Inhibitor Tolafentrine in Chronic Experimental Pulmonary Hypertension **ATS 2003 - Seattle, 99th International Conference.**

Achievements:

- Won the Rene-Baumgart Foundation Research Award, 2005
- One of the topper in the first year exams conducted by International graduate program (MBML)
- Gold medalist at post graduation (Biotechnology)
- Gold medalist at graduation (Zoology)
- Gold medalist at graduation (Chemistry)
- Received National Merit Scholarship throughout the academic career by the Indian government.

12. Publications

Published:

1. **Pullamsetti S**, Kiss L, Ghofrani HA, Voswinckel R, Haredza P, Klepetko W, Aigner C, Fink L, Muyal JP, Weissmann N, Grimminger F, Seeger W, Schermuly RT. Increased levels and reduced catabolism of asymmetric and symmetric dimethylarginines in pulmonary hypertension. ***FASEB J.*** 2005 Jul;19(9):1175-7. Epub 2005 Apr 12.

2. Schermuly R, Dony E, Ghofrani HA, **Pullamsetti S**, Savai R, Roth M, Sydykov A, Lai YJ, Weissmann N, Seeger W, and Grimminger F. Reversal of pulmonary hypertension in rats by platelet derived growth factor inhibition ***J Clin Invest.*** 2005 Oct;115(10):2811-21.

3. Schermuly RT, Kreisselmeier KP, Ghofrani HA, Samidurai A, **Pullamsetti S,** Weissmann N, Schudt C, Ermert L, Seeger W, Grimminger F. Antiremodeling effects of iloprost and the dual-selective phosphodiesterase 3/4 inhibitor tolafentrine in chronic experimental pulmonary hypertension. ***Circ Res***. 2004 Apr 30;94(8):1101-8. Epub 2004 Mar 18.

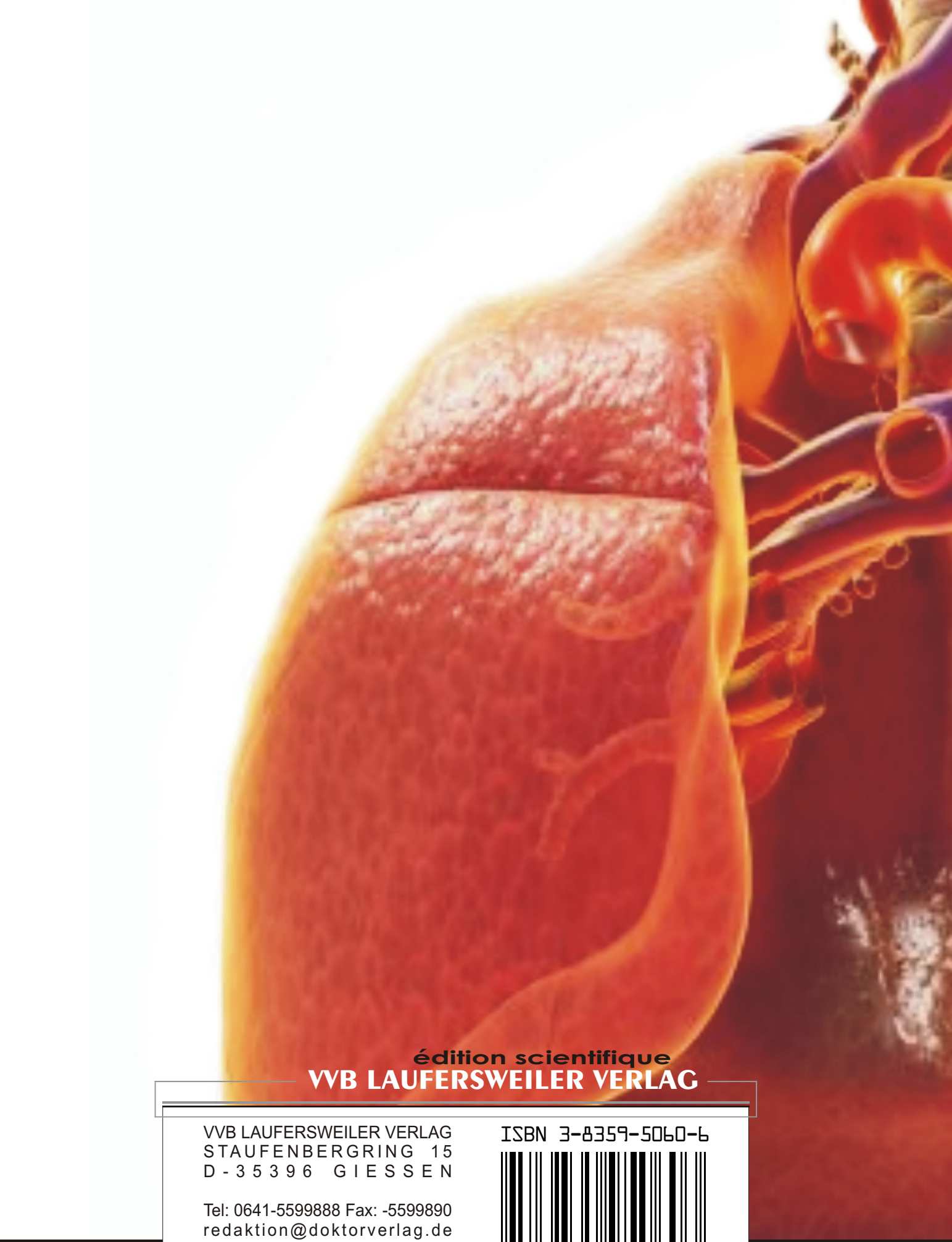
4. Schermuly RT, Yilmaz H, Ghofrani HA, Woyda K, **Pullamsetti S,** Schulz A, Gessler T, Dumitrascu R, Weissmann N, Grimminger F, Seeger W. Inhaled Iloprost Reverses Vascular Remodeling in Chronic Experimental Pulmonary Hypertension. ***Am J Respir Crit Care Med***. 2005 Aug 1;172(3):358-63. Epub 2005 May 5.

5. Grandel U, Hopf M, Buerke M, Hattar K, Heep M, Fink L, Bohle RM, Morath S, Hartung T, **Pullamsetti S**, Schermuly RT, Seeger W, Grimminger F, Sibelius U. Mechanisms of cardiac depression caused by lipoteichoic acids from Staphylococcus aureus in isolated rat hearts. ***Circulation***. 2005 Aug 2;112(5):691-8. Epub 2005 Jul 25.

6. Savai R, Schermuly R, Schneider M, **Pullamsetti SS**, Rose F, Grimminger F and Banat GA . Hybrid-primed lymphocytes and hybrid vaccination prevent tumor growth of Lewis lung carcinoma in mice. ***J Immunother***. 2006 Mar-Apr;29(2):175-87

7. **Pullamsetti S**, Krick S, Yilmaz H, Ghofrani HA, Schudt C, Weissmann N, Fuchs B, Seeger W, Grimminger F, and Schermuly RT. Inhaled tolafentrine reverses pulmonary vascular remodeling via inhibition of smooth muscle cell migration ***Respir Res***. 2005 Nov 1;6:128.

8. **Pullamsetti SS**, Ghofrani HA, Maring D, Mayer K, Weissmann N, Rosengarten B, Schudt C, Boer R, Grimminger F, Seeger W, and Schermuly RT. Effect of NOS inhibition on macro- and microcirculation in a model of rat endotoxic shock ***Thromb Haemost.*** 2006 Apr;95(4):720-7.



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