Asymmetric dimethylarginine metabolism and its involvement in the pathogenesis of pulmonary arterial hypertension

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ADMA	Asymmetric ω-N^G, N^G-dimethylarginine
Adox	Periodate-oxidized adenosine
ALK	Activin receptor-like kinase
ANOVA	Analysis of variance
APAH	Associated pulmonary arterial hypertension
APS	Ammonium persulfate
BAL	Broncho-alveolar lavage
BALF	Broncho-alveolar lavage fluid
BH4	Tetrahydrobiopterin
BMPR-II	BMP receptor II
BMP	Bone morphogenetic protein
BTG	B-cell translocation gene
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CARM1	Coactivator-associated arginine methyltransferase 1
Cal	Calmodulin
CBP	CREB binding protein
cDNA	Complementary deoxyribonucleic acid
CE	Capillary electrophoresis
cGMP	Cyclic guanosine monophosphate
CHR	Chronic heart failure
DAB	Diaminobenzidine
DDAH	N^G, N^G–dimmethylarginine dimethylaminohydrolase
DMA	Dimethylamine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECE	Endothelin-converting enzyme

EDTA	Ethylendinitrilo-N, N, *N´, N´,* -tetra-acetic-acid
EGTA	Ethylene glycol-bis (2-amino-ethylether)-N,N,*N'*,*N'*-tetraacetic-acid
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial NOS
ER	Estrogen receptor
ET	Endothelin
ET_A	ETA receptor
ET_B	ETB receptor
FAD	Flavin adenine dinucleotide
FCS	Fetal calf serum
FMN	Flavin mononucleotide
FPAH	Familial pulmonary arterial hypertension
GAR	Glycine and arginine-rich
H	Histone
HEPES	2-(-4-2-hydroxyethyl)-piperazinyl-1-ethansulfonate
HIV	Human immunodeficiency virus
HHT	Hereditary hemorhagic telangiectasia
HMG	High-mobility group
hnRNP	Heterogenous ribonucleoprotein
HPLC	High performance liquid chromatography
hPASMC	Human primary pulmonary arterial smooth muscle cells
HRP	Horseradish peroxidase
5-HT	5-hydroxytryptamin
IB	Immunoblotting
IFNAR1	Interferon α, β, receptor1
IHCH	Immunohistochemistry
ILF3	Interferon-like factor 3
iNOS	Inducible NOS
IPAH	Idiopathic pulmonary arterial hypertension
L-Arg	L-arginine
LC/MS	Liquid chromatography/mass spectrometry

LDL	Low density lipoprotein
L-H Arg	ω-N^G-homoarginine
L-NMMA	ω-N^G-monomethylarginine
Lsm4	Sm-like protein4
MBP	Myelin basic protein
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor-κB
nNOS	Neural NOS
NO	Nitric oxide
OPA	*Ortho*-phthaldialdehyde
PABP1	Poly(A)-binding protein 1
PAGE	Polyacrylamide gel electrophoresis
PAH	Pulmonary arterial hypertension
PAP	Pulmonary arterial pressure
PASMC	Pulmonary arterial smooth muscle cell
PBGD	Porphobilinogen deaminase
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PGI_2	Prostaglandin I_2
PR	Progesterone receptor
PRMT	Protein arginine methyltransferase
PVDF	Polyvinylidene difluoride
RACK1	Receptor for activated protein kinase C
rel.	Relative
RNA	Ribonucleic acid
RP-HPLC	Reverse phase-HPLC
RT-PCR	Reverse transcriptase PCR
SAM	S-adenosyl methionine
SAH	S-adenosyl homocysteine
Sam68	Src-associated in mitosis of 68 kDa
SH3	Src homology 3

SD	Standard deviation
SDMA	ω-N^G, N'^G-dimethylarginine
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS Polyacrylamide gel electrophoresis
SEM	Standard error of the mean
siRNA	Silencing RNA
SMA	Smooth muscle actin
SPE	Solid phase extraction
SPT	Streptomycin phosphotransferase
TAE	Tris-acetate-EDTA
TAT	Transactivator
TEMED	*N*,*N*,*N'*,*N'*-tetramethyl-ethane-1,2-diamine
TCA	Trichloroacetic acid
TGF	Transforming growth factor
Th	T helper
TOB	Transducer of erbB2
TR	Thyroid hormone receptor
TNF-α	Tumor necrosis factor-α
X	Any amino acid
y+	Amino acid transporter
WHO	World Health Organization

V Summary

Protein arginine methylation represents a posttranslational modification undertaken by protein arginine methyltransferases (PRMT) that results in production of protein-incorporated ω-N^G-monomethylarginine (L-NMMA), asymmetric ω-N^G, N^G-dimethylarginine (ADMA), or ω-N^G, N'^G-dimethylarginine (SDMA). Free cellular L-NMMA, ADMA and SDMA can be generated via the proteolytic cleavage of intracellular proteins, thereby also affecting methylarginine content in the plasma. Free methylarginines can be cleared from the body by renal excretion. L-NMMA and ADMA, but not SDMA, can be degraded via enzymes called N^G, N^G–dimmethylarginine dimethylaminohydrolase (DDAH).

ADMA is an endogenous inhibitor of nitric oxide synthases (NOS) and a marker of endothelial dysfunction. Increased plasma ADMA levels have been reported in patients with cardiovascular disorders including pulmonary arterial hypertension (PAH), a fatal disease characterized by elevated blood pressure in the pulmonary circulation, due to increased resistance of pulmonary arterioles. The major pathophysiologic hallmark of PAH is pulmonary arterial smooth muscle cell (PASMC) hypertrophy and proliferation, leading to the occlusion of pulmonary arterioles. The interplay between methylarginine synthesis and degradation *in vivo*, as well as specific alterations to intrapulmonary ADMA levels or distorted generation of ADMA in PAH, however, remains to be elucidated.

In the current study, we hypothesized that methylarginine production and degradation is tissue-specific and that the lung has a significant impact on serum/plasma ADMA levels, possibly leading to endothelial dysfunction observed in PAH. To this end, we sought to address the following specific aims: 1) to develop a novel, HPLC-based method to assess protein-incorporated and free cellular methylarginine content in biological samples, 2) to analyze the tissue-specific methylarginine metabolism in normal subjects, and 3) to analyze the methylarginine content in the lungs of patients with PAH compared with healthy donors.

First, to analyze tissue-specific methylarginine metabolism in the normal physiological state, we performed high performance liquid chromatography (HPLC)-driven assessment of protein-incorporated and free cellular methylarginine levels, together with Western blot analyses of PRMT and DDAH expression, in organs of the cardiovascular system. Our results revealed that pulmonary expression of type I PRMT was correlated with enhanced protein arginine methylation in the lung. Moreover, our studies also revealed that the kidney and the liver provide complementary routes for clearance and metabolic conversion of circulating ADMA.

To address the impact of intrapulmonary ADMA metabolism in pathogenic conditions, we next analyzed lung homogenates of PAH patients. HPLC analysis revealed significantly lower levels of protein-incorporated ADMA in the lungs of PAH patients (n=12), compared with controls (n=10, transplant donors). Western Blot analyses confirmed a significantly decreased content of asymmetrically dimethylated proteins in PAH lungs. The expression of PRMT, in particular PRMT1, was decreased in PAH. Immunohistochemical staining of IPAH and control lungs localized PRMT1 to pulmonary arterial vascular smooth muscle cells (PASMC). Moreover, PRMT1 knockdown in primary PASMC by siRNA technology significantly increased PASMC proliferation.

Our results demonstrate that, in the normal physiological state, methylarginine metabolism by the pulmonary system significantly contributes to circulating methylarginine levels. In pathogenic conditions, protein-incorporated ADMA concentrations do not reflect free cellular levels of ADMA in the lung. This may be explained by the alterations of DDAH activity in the lung, which, consequently, regulate ADMA content in the serum of IPAH patients. In addition, our studies demonstrated a novel regulatory role of PRMT1 in progression of PAH, by the alteration of PASMC proliferation, a major characteristic of PAH. This led to conclusions that protein arginine methylation plays a pivotal role in the pathogenesis of PAH.

VI Zusammenfassung

Posttranslationale Protein-Arginin Methylierung erfolgt durch eine Gruppe spezifischer Protein-Arginin Methyltransferasen (PRMTs), die neben der Bildung von asymmetrischem Dimethylarginin (ADMA) auch für die Synthese von Monomethylarginin (L-NMMA) und symmetrischem Dimethylarginin (SDMA) verantwortlich sind. Die Freisetzung von Methylarginine in das Blutplasma erfolgt nach heutigem Wissensstand über die Proteolyse zellulärer, methylierter Proteine. Alle Methylargininformen werden über renale Exkretion aus dem Körper eliminiert. Neuere Studien heben die Metabolisierung von ADMA und L-NMMA durch das Enzym Dimethylarginin-Dimethylaminohydrolase (DDAH) als Hauptabbauweg hervor.

ADMA ist ein endogener Inhibitor der NO-Synthase und ein Marker für endotheliale Fehlfunktion. Eine erhöhte ADMA Konzentration im Blut wird bei verschiedenen kardiovaskulären Erkrankungen, so auch bei pulmonal-arterieller Hypertonie (PAH), für einen Mangel an biologisch verfügbarem NO verantwortlich gemacht. Die pulmonal-arterielle Hypertonie ist durch eine pathologische Hypertrophie und Proliferation pulmonalarterieller glatter Muskelzellen (PASMC) gekennzeichnet, die eine Okklusion pulmonaler Arteriolen zur Folge hat. Ob ein Zusammenhang zwischen Arginin- und Dimethylargininstoffwechsel und den bei PAH zu beobachtenden Symptomen vorliegt, wurde bislang nicht untersucht.

Deshalb sollte in der vorliegenden Studie geprüft werden, ob der Methylarginin-metabolismus der Lunge signifikant zur ADMA Konzentration im Blut beiträgt und somit an der Ausbildung endothelialer Fehlfunktionen beteiligt sein könnte. Im Konkreten sollten hierfür folgende Vorhaben realisiert werden: (1) Entwicklung einer auf Hochdruckflüssigkeitschromatographie-basierenden Methode zur Quantifizierung von protein-inkorporiertem und freiem Methylarginin in biologischen Proben, (2) Analyse des gewebespezifischen Methylargininmetabolismus und (3) Bestimmung des pulmonalen Methylargininingehaltes von PAH Patienten und gesunden Organspendern.

Zur Beschreibung des Methylargininmetabolismus unter normalen physiologischen Bedingungen wurden protein-inkorporiertes und freies Methylarginin in Organen des

kardiovaskulären Systems bestimmt. Zudem wurde vergleichend Proteinexpression und Aktivität der PRMTs und DDAHs ermittelt. Unsere Untersuchungen ergaben eine klare Korrelation zwischen pulmonaler Typ I PRMT Proteinexpression und erhöhter Protein-Arginin Methylierung. Zudem konnte gezeigt werden, dass Niere und Leber komplementär an der Eliminierung und Metabolisierung von ADMA und L-NMMA beteiligt sind.

Zur Beurteilung der Frage, ob bei PAH ein geänderter Dimethylargininstoffwechsel zu beobachten ist, wurde Lungenhomogenat mittels HPLC untersucht. Die Analyse bei PAH Patienten (n=12) und gesunden Organspendern (n=10) ergab eine signifikante Abnahme an protein-inkorporiertem ADMA bei PAH Patienten. Zudem konnte über Western-Blot Analyse ein reduzierter Gehalt an asymmetrisch dimethylierten Proteinen nachgewiesen werden. Bei PAH Patienten zeigte sich auch eine signifikant reduzierte Expression jener Protein-Arginin-Methyltransferasen, insbesondere PRMT 1, die für eine asymmetrische Dimethylierung von Zielproteinen verantwortlich sind. Immunohistochemische Untersuchungen führten zu dem Ergebnis, dass PRMT 1 überwiegend in PASMCs lokalisiert ist. Zudem resultierte die Reduktion der PRMT 1 Expression mittels siRNA Technologie in einer Zunahme der PASMC Proliferation.

Aus den vorliegenden Ergebnissen lässt sich somit schlussfolgern, dass der pulmonale Dimethylargininmetabolismus maßgeblich zum Plasma ADMA-Spiegel beiträgt. Bei PAH Patienten konnte keine Korrelation zwischen protein-inkorporiertem ADMA und freiem Methylarginin nachgewiesen werden. Dieses Ergebnis deutet auf eine Änderung der pulmonalen DDAH Aktivität und Plasma ADMA-Werte bei PAH Patienten hin. Des Weiteren konnte eindeutig demonstriert werden, dass PRMT 1 an der Regulation der PASMC Proliferation beteiligt ist. Zusammenfassend lässt sich somit feststellen, dass Protein-Arginin Methylierung an der Entwicklung und am Fortschreiten von PAH beteiligt sein könnte.

1 Introduction

1.1 ADMA metabolism

1.1.1 ADMA synthesis

Asymmetric dimethylarginine (ADMA) is an amino acid derivative, circulates in the plasma, is excreted in the urine, and found in broncho-alveolar lavage fluid (BALF). During the last 30 years, the occurrence of ADMA has been extensively studied in the cell and in tissues (McDermott 1976; Vallance et al. 1992; Cooke 2000). The ADMA is synthesized during methylation of protein L-arginine residues by the action of specific enzymes called protein arginine methyltransferases (PRMT) (Clarke 1993; McBride and Silver 2001; Boisvert et al. 2003).

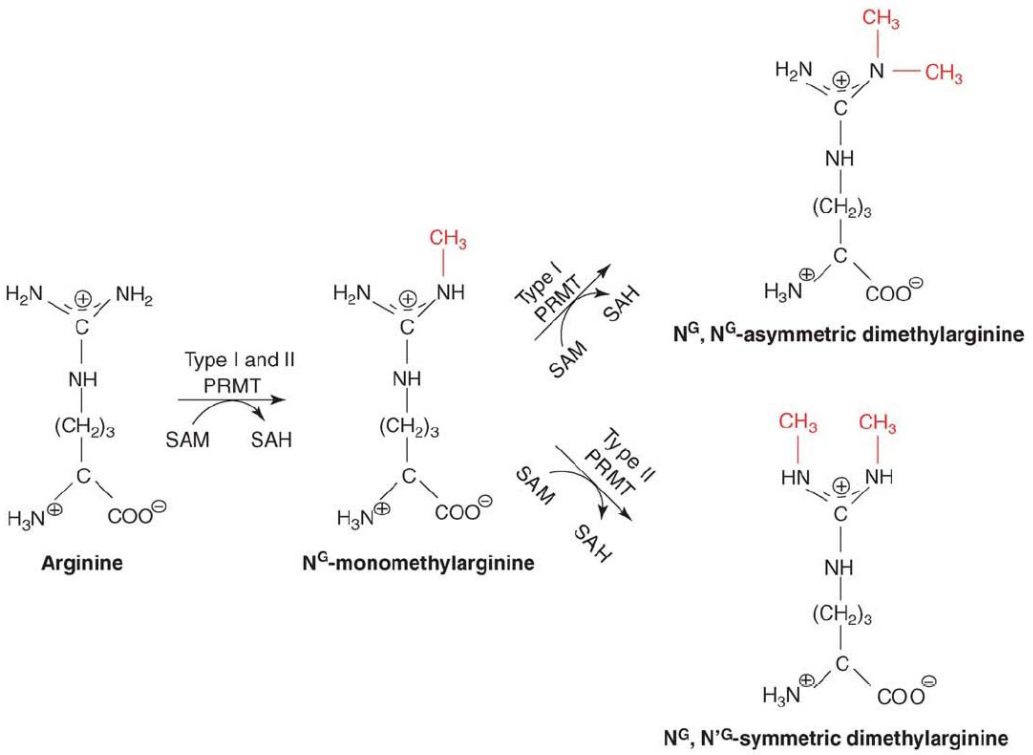

Figure 1.1 Methylarginines are generated during methylation of protein L-arginine residues. Type I PRMTs catalyze the formation of L-NMMA and ADMA, while type II PRMTs are responsible for generation of L-NMMA and SDMA. S-adenosylmethionine (SAM) is a substrate for this reaction and converts to S-adenosylhomocysteine (SAH) (Blanchet et al. 2006).

This posttranslational protein modification results in the addition of one or two methyl groups to the guanidino nitrogen atoms of L-arginine (Gary and Clarke 1998). Three main forms of methylated L-arginine have been identified in eukaryotes: ω-N^G-monomethylarginines (L-NMMA), asymmetric ω-N^G, N^G-dimethylarginine (ADMA), and ω-N^G, N'^G-dimmethylarginine (SDMA) (figure 1.1) (Vallance et al. 1992).

Free cellular methylargines are released through proteolysis of mono- and di-methylated proteins in the cytosol. Previous studies demonstrated that production and elimination of methylated proteins in the cell is closely correlated to total proteome synthesis and degradation (Miyake and Kakimoto 1976). To date, there is no evidence for the generation of L-NMMA, ADMA and SDMA from free L-arginine through an enzyme-catalyzed reaction. In addition, demethylation of free methylarginines has not been demonstrated yet. Thus, protein turnover and type I PRMT activity in the cell together might control intracellular ADMA levels (Vallance et al. 1992).

1.1.1.1 The PRMT family of enzymes: biological function

Eleven PRMTs have been identified in humans. The PRMTs have been classified into two groups: type I and type II, depending on enzyme activity. All PRMTs can catalyze the formation of L-NMMA, while type I PRMTs form ADMA and type II, SDMA (Bedford and Richard 2005). The members PRMT1, PRMT3, PRMT4, PRMT6 and PRMT8 belong to the type I group, while PRMT5, PRMT7 and PRMT9 belong to the type II group (figure 1.2). Although PRMT2 is structurally very similar to PRMT1, enzymatic activity has not been demonstrated to date (Scott et al. 1998). Furthermore, PRMT10 and PRMT11 enzymatic activity has not been demonstrated.

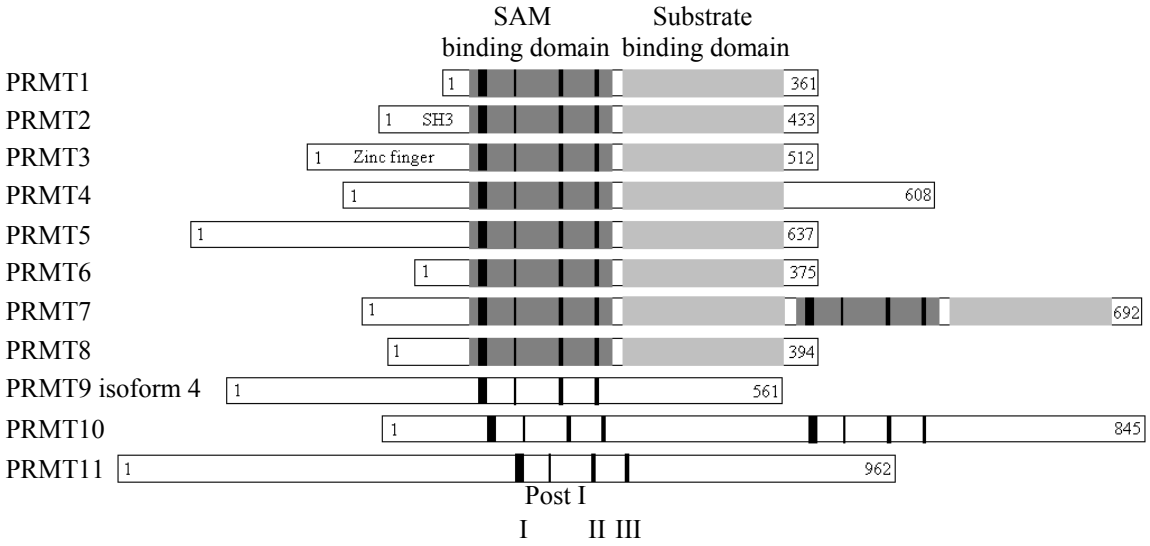

Figure 1.2 The protein arginine methyltransferase family. Currently, 11 PRMTs have been identified. The mammalian methyltransferases contain conserved motifs: post I, II, III (black), SAM (dark grey) and substrate (light grey) binding domain. PRMT2 and PRMT3 have SH3 and zinc finger domain, respectively. Both PRMT7 and PRMT10 harbor a repetition of PRMT conserved motifs. The length of the proteins is indicated by the number of amino acids (modified from Pahlich et al. 2006).

All PRMTs contain an S-adenosyl methionine (SAM) binding domain, with conserved motifs I, II, and III and a substrate binding domain. Additionally, PRMT2 and PRMT3 possess a SH3 and Zn^{2+} finger domain, respectively, which increase the specificity for substrate binding (Bedford and Richard 2005) (figure 1.2).

Proteins which have been demonstrated as PRMT substrates harbor glycine and arginine-rich (GAR) conserved motifs, often RGG, RXR and RG, where R indicates L-arginine, G, glycine and X, any amino acid (Najbauer et al. 1993). The PRMT targets have been identified by different approaches: during the identification of new interaction partners (Abramovich et al. 1997; Chen et al. 1999; Passos et al. 2006; Nicod 2007), through *in vitro* substrate screens (Lee and Bedford 2002; Wada et al. 2002; Kim et al. 2004) or by proteomic-based mass spectrometric approaches (Boisvert et al. 2003; Ong et al. 2004).

Methyltransferases regulate several cellular processes in bacteria, yeast and mammalian cells. These enzymes are implicated in the modulation of nuclear export (McBride et al. 2005) and import (Xu et al. 2001), or protein-protein interaction (Bedford

et al. 2000). They are also involved in RNA processing (Cote et al. 2003), signal transduction (Mowen et al. 2004), transcription (Hassa et al. 2008) and DNA repair (Boisvert et al. 2003; Boisvert et al. 2005) (tabele1.1).

Tabele 1.1 List of Protein arginine methyltransferases and their biological functions. H indicates histone and R methylated arginine residue implicated in protein methylation. ? PRMT type not specified (modified from Pal and Sif 2007).

PRMT	Type	Biological functions
PRMT1	I	Transcriptional activation (H4R3), nuclear localization, DNA repair, signaling
PRMT2	?	Transcriptional coactivator, nuclear retention, apoptosis
PRMT3	I	Ribosome assembly
PRMT4/CARM1	I	Transcriptional activation (H3R2, H3R17, H3R26), muscle differentiation, T cell development, tumorigenesis
PRMT5	II	Transcriptional repression (H3R8 and H4R3),
PRMT6	I	HIV replication, DNA repair
PRMT7	II	Imprinting in male germ cell (H4R3)
PRMT8	I	Unknown
PRMT9	II	Unknown
PRMT10	?	Unknown
PRMT11	?	Unknown

The predominant methyltransferase in mammalian cells is PRMT1 and 85% of cellular protein arginine methylation appears to be performed by this enzyme (Gary and Clarke 1998; Pawlak et al. 2002). The PRMT1 was first identified as an interaction partner of the BTG/TOB protein family (BTG1, BTG2) (Lin et al. 1996) and the cytoplasmic domain IFNAR1 chain of the α, β interferon receptor, using a yeast two hybrid screen (Abramovich et al. 1997). The PRMT1 is localized to the nucleus (Frankel et al. 2002; Passos et al. 2006; Robin-Lespinasse et al. 2007), while cytosolic localization of this enzyme was also observed under native and modified conditions in various cell

types (Herrmann et al. 2005). Homozygous PRMT1 knockout mice die at embryonic day 6.5, indicating that protein methylation is required for early postimplantation development. However, embryonic cells derived from PRMT1$^{-/-}$ mice were viable, although the PRMT1 expression and activity were dramatically reduced in those cells (Pawlak et al. 2000).

The major targets of PRMT1 are nuclear core histones H3 and H4, and members of heterogenous nuclear ribonucleoprotein (hnRNP) family including Src-associated in mitosis of 68 kDa (Sam68), hnRNP A1, A2, R and K, mediating RNA processing and export (Pal and Sif 2007). The PRMT1 interacts directly with p65 and synergistically coactivates transcription complex formation leading to the activation of NF-κB (nuclear factor-κB) gene expression (Hassa et al. 2008). The involvement of PRMT1 was also observed in the regulation of cellular signal transduction. Methylation of NFAT interacting nuclear protein 45 (NIP45) enhanced cytokine production in Th (T helper) cells (Mowen et al. 2004). The PRMT1 is also the predominant methyltransferase implicated in alterations to the subcellular localization of proteins, for instance, posttranslational modification of Sam68 is required for its nuclear localization (Cote et al. 2003) and RNA binding activity (Rho et al. 2007).

Although the methyltransferase activity of PRMT2 has not been demonstrated, PRMT2 regulates transcription *in vivo* by binding a number of hormone receptors (ERα, ERβ, PR, TRβ, RARα) in a ligand-independent manner (Qi et al. 2002). Similar to PRMT1, PRMT2 is involved in the regulation of NF-κB-dependent gene expression, and promotes apoptosis. Studies in mice exposed to normoxic (21% O_2) or hypoxic (4% O_2) conditions confirmed the role of PRMT2 in the development of pulmonary hypertension (Yildirim et al. 2006).

The PRMT3 harbors a characteristic N-terminal zing finger motif, which is required for the binding to RNA-associated proteins in extracts from RAT1 fibrobrast cells (Frankel and Clarke 2000). The PRMT3 knockout mice are viable, but embryos differ in size to wild-type animals, and are characterized by hypomethylation of cellular proteins (Swiercz et al. 2007).

The PRMT4, also known as coactivator-associated arginine methyltransferase 1 (CARM1), together with PRMT1, acts with a number of transcription factors, further regulating gene expression in the cell. The best-known substrates for this

methyltransferase are: CARM1 itself, histone H3, HuD, HuR, ILF3, p300/CBP, PABP1 and TARPP (Pahlich et al. 2006). Heterozogous PRMT4-deficient mice are normal and fertile, whereas homozygous knockout mice die at birth due to abnormalities in lung structure, suggesting an important role of PRMT4 in mouse development (Pawlak et al. 2000).

Both PRMT5 and PRMT7 belong to the type II class of methyltransferases. Symmetric dimethylation by PRMT5 affects many nuclear proteins, including histones H4, H2A, H3, SmD1, SmD3, Sm B/B, coilin, LSm4, SPT and the most-investigated substrate, MBP (myelin basic protein), indicating nuclear localization of this protein in the cell (Bedford and Richard 2005). The PRMT5 participates in RNA processing, as a component of 20S methylosome and interaction partner of fibrillarin (Friesen et al. 2001; Meister et al. 2001; Pahlich et al. 2006).

A second type II methyltransferse, PRMT7, contains duplication of conserved PRMT motifs and deletion of one SAM-binding domain, leading to loss of enzymatic activity. Three substrates for PRMT7 have been identified: histones H2A, H4, and MBP (Lee et al. 2005).

The PRMT6 comprises 375 amino acids, therefore, due to its size, is most similar to PRMT1. Nevertheless, its functions differ from PRMT1, since PRMT6 methylates DNA polymerase β and the HIV TAT protein (Boulanger et al. 2005; Miranda et al. 2005; El-Andaloussi et al. 2006). It has been proposed that PRMT6 might modulate protein-protein and protein-DNA interaction by methylation of high-mobility group proteins (HMGs), HMGA Ia and HMGA Ib (Frankel et al. 2002; Sgarra et al. 2006). Interestingly, it has been demonstrated that PRMT6 undergoes automethylation (Frankel et al. 2002).

The most unique methyltransferase is PRMT8. Due to the fact that it possesses N-terminal myristoylation signal, PRMT8 is localized to the plasma membrane. The comparison of all human methyltransferases demonstrated that PRMT1 and PRMT8 share the highest degree of identity, including substrate specificity and type I activity (Lee et al. 2005).

Specific substrates as well as biological functions of PRMT9, PRMT10 and PRMT11 have not been determined.

1.1.2 Role of cellular ADMA

In vitro and *in vivo* studies have demonstrated that ADMA, but also L-NMMA, inhibits the activity of all three isoforms of nitric oxide synthase (NOS), including neural NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) (Vallance et al. 1992; MacAllister et al. 1994). These enzymes are responsible for the formation of nitric oxide (NO), a major vasodilator in the cardiovascular system. The reaction requires L-arginine as a substrate (figure 1.3). The NO induces vasodilatation, but also acts as an inhibitor of LDL (low density lipoprotein) oxidation, platelet aggregation, monocyte and leukocyte adhesion to the endothelium, superoxide radical generation, and smooth muscle cell proliferation (Garg and Hassid 1989; Kubes et al. 1991; Boger et al. 1995; Hogg et al. 1995; Boger et al. 1998). Since NOS activity can be inhibited by ADMA, this methylarginine may affect all processes in which NO plays significant role.

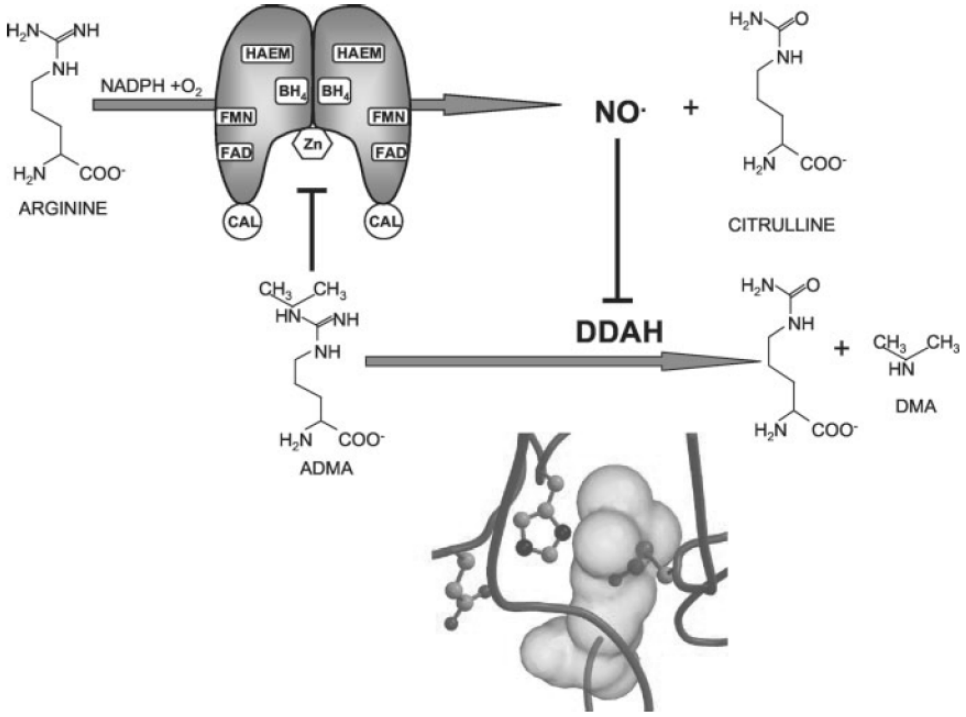

Figure 1.3 ADMA inhibits NOS activity and is metabolized by DDAH. L-arginine converts to nitric oxide (NO) and citrulline. Optimal enzyme activity requires a broad variety of cofactors: flavin adenine dinucleotide (FAD), Haem and tetrahydrobiopterin (BH4) and calmodulin (Cal). The ADMA is degraded by N^G, N^G–dimmethylarginine dimethylaminohydrolase (DDAH), which is controlled by intracellular NO concentrations (modified from Vallance and Leiper 2004). DMA; diamine, FMN; flavin mononucleotide, NADPH; nicotinamide adenine dinucleotide phosphate.

Not only NOS is a target for ADMA. Recently published observations have demonstrated that ADMA, as well as L-NMMA and SDMA, can act as a competitor of L-arginine for transport through the plasma membrane cationic amino acid transporter (y+). This may happen when the intracellular concentration of methylarginine is very high (Bogle et al. 1995). Therefore, SDMA can also affect NO production by lowering L-arginine bioavailability (Beltowski and Kedra 2006).

The ADMA can be transported to the neighboring cells and affects their NO generation. This interaction has been demonstrated between macrophages and endothelial cells (Fickling et al. 1999), suggesting that similar mechanism may occur between other cells as endothelium and smooth muscle cells (Vallance and Leiper 2004).

1.1.3 Elimination of ADMA by DDAH

All methylarginines are released from the body by urinary excretion, but levels of ADMA are additionally controlled by enzyme called $N^G N^G$–dimethylarginine dimethylaminohydrolase (DDAH). Previously published observations revealed that more than 90% of ADMA is degraded by this enzyme, resulting in the generation of citrulline and dimethylamines (McDermott 1976; Ogawa et al. 1987). The human DDAH gene contains an 858-base pair open reading frame encoding a single 285-amino acid protein. The DDAH exists in two isoforms: DDHA1 and DDAH2. Although the enzyme activity of the two isoforms is similar, the tissue distribution varies between DDAH1 and DDAH2. Interestingly, DDAH1 appears to overlap with nNOS expression, whereas DDAH2 is mainly expressed in tissue containing eNOS (Leiper et al. 1999; Tran et al. 2003).

The DDAH activity might be reversibly inhibited by the S-nitrosylation of cysteine residues (figure 1.3). This reaction leads to oxidation of a sulfur atom of the active site cysteine, resulting in deactivation of this protein residue. The S-nitrosylation of DDAH provides a potential mechanism that reduces NO generation by inactivation of DDAH, consequently increasing intracellular ADMA levels, and finally inhibiting NOS activity (Leiper et al. 2002).

1.2 Alterations to methylarginine metabolism in human

1.2.1 Human cancer

Modulation of PRMT expression has already been demonstrated in human cancers such as prostate and breast cancer. These forms of tumor are primarily hormone dependent. Due to the fact that methyltransferases are known coactivators of nuclear receptors, the overexpression of PRMTs in these cancer types should be expected (Bedford and Richard 2005). Indeed, studies on human prostate carcinoma revealed enhanced levels of PRMT4 in primary prostate tissue specimens, and lowering its expression resulted in inhibition of cell proliferation, suggesting that PRMT4 may be essential for tumor progression (Majumder et al. 2006). Other studies have demonstrated that inhibitors of PRMT4, as well as PRMT1, can suppress estrogen and androgen receptor-mediated transcriptional activation (Cheng et al. 2004). In addition, PRMT5 is involved in the pathogenesis of breast cancer by inhibiting the expression of tumor suppressors (Moggs et al. 2005). The role of this enzyme in tumorigenesis was supported by the finding that PRMT5 was overexpressed in gastric carcinoma (Kim et al. 2005).

1.2.2 Viral pathogenesis

It has been suggested that protein arginine methylation may play an important role in viral pathogenesis. The human immunodeficiency virus (HIV) transactivator (TAT) protein was identified as the first HIV protein containing arginine residues methylated by PRMT6, and inhibition of arginine methylation leads to enhanced HIV gene expression (Kwak et al. 2003; Boulanger et al. 2005). While increasing protein methylation may offer some protection against HIV infection, inhibition of this protein modification may prevent hepatitis delta virus replication (Li et al. 2004).

1.2.3 Multiple sclerosis

Myelin basic protein is a substrate for PRMT5 and PRMT7, and exibits enhanced methylation levels in multiple sclerosis. Although the physiological relevance of this

modification is unknown, it has been suggested that the posttranslational modification of arginine residues in MBP plays an important role in multiple sclerosis (Kim et al. 2003). The explanation might be that methylated MBP serves as autoantigen, similar to methylated Sm and coilin in lupus erythomatosus (Carroll 2004).

1.2.4 Abnormal ADMA levels in human disorders

The accumulation of ADMA has been demonstrated in a wide range of disorders. Abnormalities in methylarginine metabolism manifest by altered cellular, as well as tissue and urinary ADMA concentration (Tran et al. 2003). The elevated ADMA levels have been found, for example, in patients with atherosclerosis (Miyazaki et al. 1999), hypercholesterolemia (Boger et al. 1998), muscular dystrophy (Inoue et al. 1979) and stroke (Yoo and Lee 2001).

As ADMA is eliminated from the body by urinary excretion, altered ADMA levels were first demonstrated for patients with chronic kidney failure. In those patients, the plasma ADMA concentration was increased from 1 to 3 μM, compared to between 0.4 to 0.7 μM for healthy humans (Vallance et al. 1992). It has been demonstrated that there is a relationship between plasma levels and the degree of endothelial dysfunction (Cross et al. 2001). Patients who had elevated ADMA levels in plasma also exhibited enhanced risk for cardiovascular disorders and higher mortality (Vallance 2001; Zoccali et al. 2001). Unfortunately, therapy with L-arginine did not clearly demonstrate a beneficial effect on patients with renal failure (Hand et al. 1998; Cross et al. 2001).

Levels of ADMA are elevated in animal models of type I and type II diabetes, and in patients with overt type II diabetes or insulin resistance (Paiva et al. 2003; Xiong et al. 2003). Glucose can suppress DDAH activity, and this may explain the increased methylarginine concentrations observed in this disease (Lin et al. 2002).

Chronic heart failure (CHR) is characterized by the heart's inability to pump sufficient blood to maintain normal circulation. Levels of ADMA are increased in patients with CHR and lower ventricular contraction and heart rate (Vallance and Leiper 2004). There is increasing evidence that systemic administration decreases systemic vascular resistance, mean arterial pressure, increases ventricular stroke volume and cardiac output (Wu and Meininger 2000). Thus, elevated ADMA levels together with the

effect of L-arginine supplementation, suggests a potential role for methylarginine metabolism in the pathophysiology of CHR in humans (Vallance and Leiper 2004).

1.3 Pulmonary arterial hypertension

1.3.1 Characteristics of pulmonary arterial hypertension

Pulmonary arterial hypertension (PAH) is a severe, progressive and fatal disease which is defined as a mean pulmonary arterial pressure higher than 25 mm Hg at rest or 30 mm Hg during exercise (Puri et al. 2007). It is a rare disorder with an estimated prevalence of 1-2 cases per million, and is twice as common in women as in men (Martin et al. 2006).

The common presenting symptom of patients with PAH is dyspnea on exertion. Other symptoms such as chest pain, lightheadedness and palpitations may also be observed, nevertheless the final diagnosis of PAH may be confirmed only by chest X-ray or transthoracic Doppler-echocardiography (Martin et al. 2006).

The World Health Organization (WHO) classified PAH according to the etiology of various forms of this disease. Thus, PAH has been separated into five categories: idiopathic (IPAH), familial (FPAH), associated (APAH) with variety of diseases (collagen vascular disease, HIV infection, portal hypertension), associated with significant venous or capillary involvement, and persistent pulmonary hypertension of the newborn (Martin et al. 2006).

With current available therapies, the progression of PAH can not be cured, but improvement and quality of life of patients with PAH can be achieved (Chan and Loscalzo 2008). Therefore, the understanding of the molecular mechanisms involved in the pathogenesis of PAH has become an important issue for scientists studying this complex disease.

1.3.2 Histopathological abnormalities

Pulmonary arterial hypertension is characterized by changes in the structure and function of smooth muscle cells, endothelial cells and fibroblasts contributing to vascular

remodeling (figure 1.4), altered tone and vasoreactivity. Two histopathological abnormalities contribute greatly to the elevated arterial pressure observed in PAH. Medial thickening occurs through the hypertrophy and hyperplasia of smooth muscle cells; and second, muscularization of distal vessels occurs due to the proliferation and differentiation of fibroblasts and pericytes (Meyrick and Reid 1980; Strange et al. 2002; Nicod 2007).

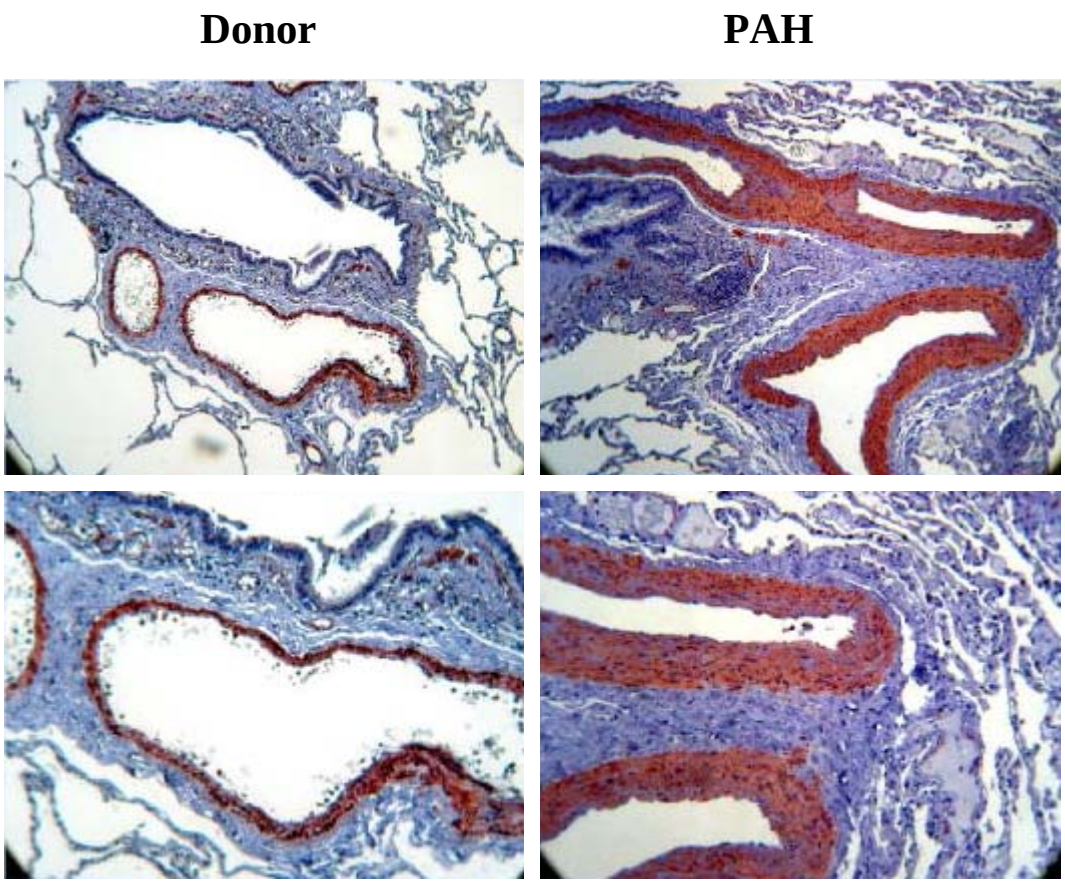

Figure 1.4 Histopathological changes observed in pulmonary arterial hypertension (PAH). Pulmonary arterioles in a normal patients (left) and in patients with PAH (right) with significantly hypertropic tunica medium (modified from Eickelberg and Seeger 2005).

The plexiform lesion is one of the characteristic pathological abnormalities in PAH. It is made up of a monoclonal population of endothelial cells and smooth muscle cells. It has been proposed that differentiated and proliferating endothelial cells might

form plexiform lesions, indicating the clear involvement of these cells in the development of PAH (Eickelberg and Seeger 2005; Nicod 2007). Endothelial cells may also contribute to vascular wall thickening by synthesizing a number of growth factors and vasodilators that directly regulate matrix deposition and smooth muscle cell proliferation (Martin et al. 2006).

1.3.3 Genetic determinants of pulmonary arterial hypertension

Several observations have indicated that in the pathogenesis of PAH, some genetic components are involved that initiate or cause progression of the disease.

1.3.3.1 The bone morphogenetic protein pathway

During recent years, genetic discoveries have drawn the attention to bone morphogenetic proteins (BMPs) and the BMP receptor II (BMPR-II) (Lane et al. 2000).

The BMPs are members of the transforming growth factor (TGF)-β family. Their involvement in the regulation of cellular processes such as growth, differentiation and apoptosis has been previously reported. It has been demonstrated that a number of cell types in humans have ability to synthesize and secrete BMPs, for instance, smooth muscle cells and endothelial cells (Nohe et al. 2002).

The BMP receptors play an important role in cell-cell interaction and intracellular signal transduction. After binding of BMP to the BMPR-II, BMPR-I is recruited into the heteromeric complex, resulting in phosphorylation of cytoplasmic transcription factors, called Smads. Phosphorylated Smads bind to common Smads which are required to translocate receptor-regulated Smads into the nucleus. Binding of complex-containing transcription factors to DNA activates specific gene expression regulating cell differentiation and cell proliferation (Massague and Chen 2000).

Heterozygous mutations of the BMPR-II gene have been found in approximately 60% of patients with a family history of the disease (familial PAH) and in 20% of patients with sporadic IPAH (Newman et al. 2001). Interestingly, genetic disturbances to genes encoding BMPR-II led to decreased protein expression, which has been reported in patients with idiopathic form of PAH. Recently published studies have involved BMPR-II mutations in the modulation of protein-protein interaction. The BMPR-II

variants that contain amino acid substitutions present in PAH patients exhibited a reduced affinity for receptor for activated protein kinase C (RACK1) resulting in a significant increase in PASMC proliferation, which is characteristic of this disorder (Zakrzewicz et al. 2007).

1.3.3.2 Activin receptor-like kinase 1

Defect in genes encoding activin receptor-like kinase (ALK)-1 causes hereditary hemorhagic telangiectasia (HHT), an autosomal dominant inherited disease, characterized by mucocutaneous telangiectasis, causing gastro-intestinal blood loss. Mutations in the ALK-1 gene might cause HHT to develop together with PAH, or PAH alone (Trembath et al. 2001).

1.3.3.3 Serotonin

The last gene known to be implicated in the pathogenesis of PAH is the serotonin gene, which encodes a protein which interacts with a specific transporter to enter PASMC, and induces cell proliferation. Due to the fact that platelet and plasma 5-hydroxytryptamine (5-HT, serotonin) levels are increased in clinical hypertension, it has been proposed that serotonin transporter gene polymorphisms might be genetic determinants of a variety of forms of PAH (Abenhaim et al. 1996; Eddahibi et al. 2002; Nicod 2007).

1.3.4 Humoral regulators in pulmonary arterial hypertension

There is a growing evidence that vasoconstriction and vascular remodeling observed in chronic pulmonary hypertension is caused by an imbalance in vasoconstrictor and vasodilator activity (figure 1.5).

1.3.4.1 Prostacyclin

Prostacyclin (prostaglandin I_2, PGI_2), a potent vasodilator and an inhibitor of platelet aggregation, is produced by endothelial cells. Patients with PAH exhibit an imbalance in the local production of PGI_2 and thromboxane (arachidonic acid

metabolites), and lowered expression of PGI_2 synthase (Christman et al. 1992; Tuder et al. 1999; Strange et al. 2002) (figure 1.5).

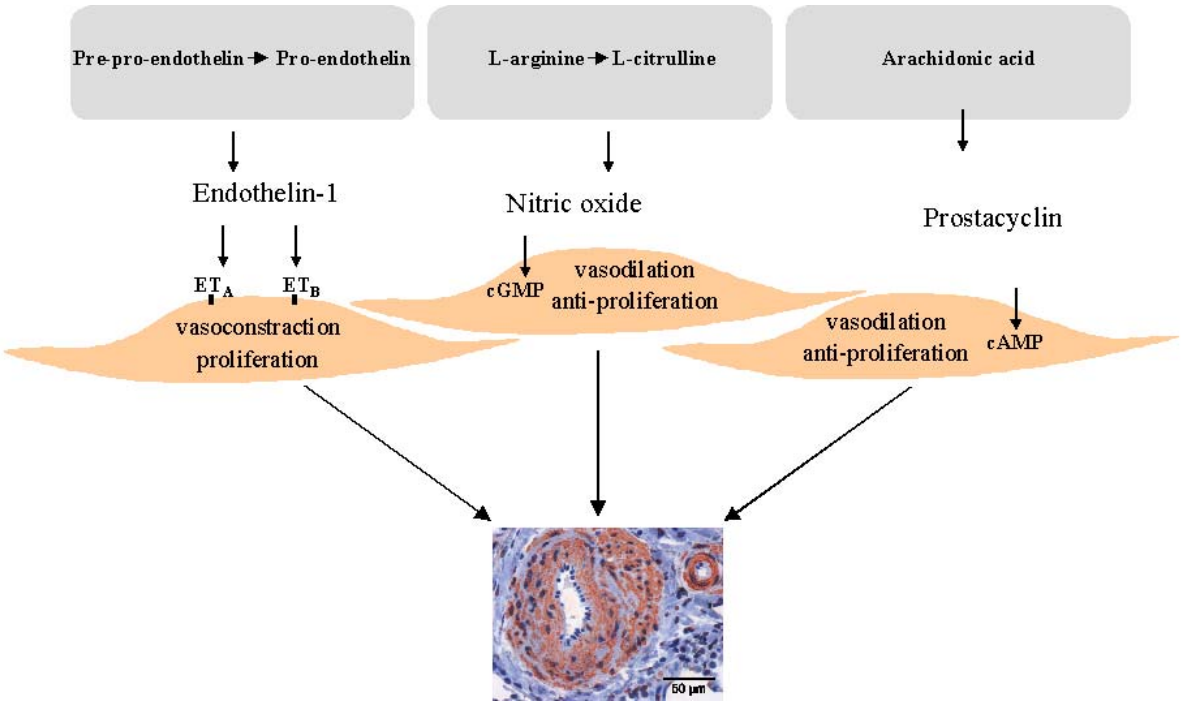

Figure 1.5 Consequences of pulmonary artery endothelial cell dysfunction on pulmonary artery smooth muscle cell tone and proliferation. Dysfunctional endothelial cells (grey) decrease the generation of prostacyclin, nitric oxide (NO) and increase the production of endothelin-1 leading to vasoconstriction and proliferation of pulmonary smooth muscle cells (orange) (modified from Humbert et al. 2004). ET_A; ETA receptor, ET_B; ETB rereceptor, cAMP; cyclic adenosine monophosphate, cGMP; cyclic guanosine monophosphate.

It has previously been demonstrated that PGI_2 has an inhibitory effect on vascular smooth muscle cell proliferation and vascular remodeling *in vivo* (Todaka et al. 1999). Pharmacological analogs of PGI_2 (iloprost, cicaprost) reduce the growth of distal human PASMC. All these findings suggest that a prostacyclin imbalance might markedly influence pulmonary vascular tone and structure in PAH (Wharton et al. 2000).

1.3.4.2 Endothelins

The endothelins (ET-1, -2, -3) are synthesized from precursors by endothelin-converting enzymes (ECEs) (Strange et al. 2002). The cells, which are mainly responsible for production and release of ET-1 in the lung are endothelial and epithelial cells, as well as vascular smooth muscle cells after cytokine stimulation (Woods et al. 1999). Endothelin-1 exhibits high binding affinity to the G-protein-coupled receptors ETA receptor (ET_A) and ETB receptor (ET_B), which differ in their distribution pattern in the lung. The ET_A is predominantly expressed in proximal pulmonary arteries, while ET_B occurs in vascular smooth muscle cells in distal resistance vessels. Both endothelin receptors have a significant impact on vasoconstriction and proliferation (Zamora et al. 1993; McCulloch et al. 1996) (figure 1.5).

1.3.5 Nitric oxide generation in PAH

1.3.5.1 Nitric oxide production

Nitric oxide is one of the major endothelium-derived vasoactive mediators. Nitric oxide is produced in the endothelium by endothelial nitric oxide synthase. The NO generation results in the activation of soluble guanylate cyclase, which increases cyclic GMP (guanosine monophosphate) levels in vascular smooth muscle cells. This activates a protein kinase, which leads to the inhibition of calcium influx and decreased calcium-calmodulin stimulation of myosin light chains. This, in turn, reduces the phosphorylation of myosin light chains, lowering smooth muscle tension, resulting in vasodilation (Boger and Bode-Boger 2000; Galley and Webster 2004) (figure 1.5).

Studies with knockout mice have supported the significant impact of eNOS on pulmonary vascular homeostasis. Indeed, eNOS-deficient mice exhibit mild pulmonary hypertension, under normobaric normoxia, and increased susceptibility to hypoxia-induced pulmonary hypertension (Fagan et al. 1999; Strange et al. 2002). In contrast, overexpression of eNOS in transgenic mice prevents vascular remodeling in the lung (Budhiraja et al. 2004).

Although animal experiments have clearly demonstrated an important role for eNOS in vasodilation, results from human studies are not consistent. Patient data have

suggested increased, decreased and unchanged expression of eNOS in PAH (Giaid and Saleh 1995; Xue and Johns 1995; Tuder et al. 1999).

1.3.5.2 L-arginine content in patients with PAH

Limitation of L-arginine might modulate NO concentrations in humans. Indeed, PAH patients with reported decreased NO levels have lower L-arginine content in their serum due to enhanced activity of arginase, an enzyme that converts L-arginine to ornithine and urea. Oral L-arginine supplementation reduces pulmonary pressures in these patients, but does not completely halt the progression of the disease (Mori and Gotoh 2000; Morris et al. 2003).

1.3.6 ADMA metabolism in PAH

It is well established that ADMA is a naturally occurring inhibitor of eNOS. Lowering NO production by blocking eNOS activity, leads to cardiovascular diseases such as PAH. In addition, DDAH metabolizes 90% of circulating ADMA. Both enzymes, eNOS or DDAH, are known to be involved in human PAH pathogenesis, by regulation of ADMA metabolism (figure 1.6). However, it remains unclear whether ADMA is a pathophysiologically relevant mediator of pulmonary hypertension (Vallance and Leiper 2004).

It has been reported that heterozygous DDAH1-deficient mice accumulate ADMA which account for reduced vascular NO signaling, endothelial dysfunction and elevated systemic and pulmonary artery pressure. In contrast, transgenic mice overexpressing DDAH1 exhibit reduced systolic blood pressure and systemic vascular resistance. These observations implicate DDAH1 in preventing pulmonary vascular remodeling (Vallance and Leiper 2004).

Initial reports on the serum ADMA content in PAH have revealed markedly increased ADMA levels in patients compared to matched controls as well as an important role for ADMA in the prediction of survival in patients with this disorder (Kielstein et al. 2005).

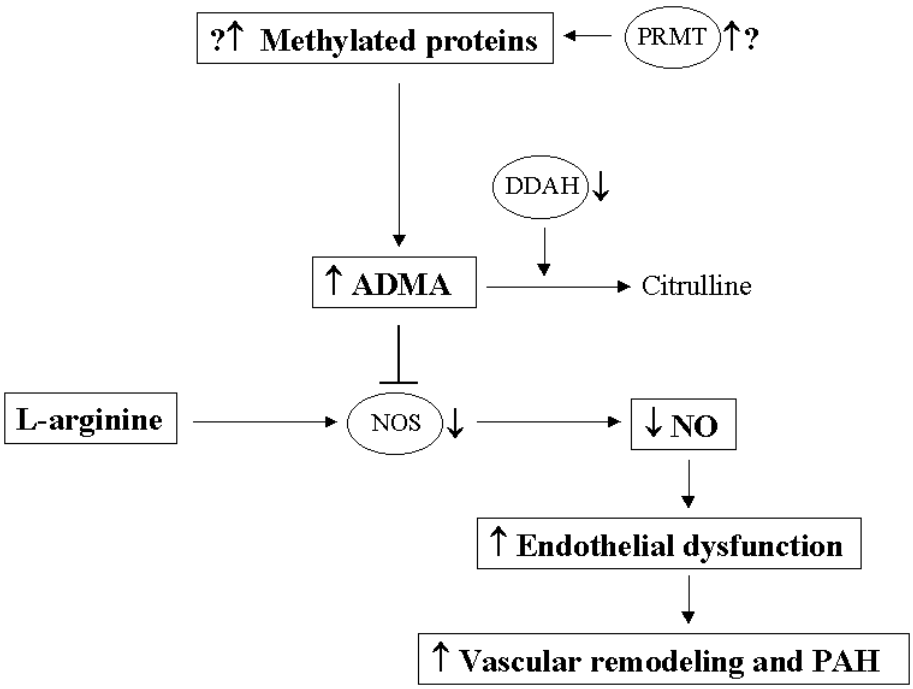

Figure 1.6 Possible roles played by enzymes involved in ADMA metabolism in PAH (modified from Pullamsetti et al. 2005). PRMT; protein arginine methyltransferase, DDAH; N^G, N^G–dimmethylarginine dimethylaminohydrolase, ADMA; asymmetric ω-N^G, N^G-dimethylarginine, NO; nitric oxide, NOS; nitric oxide synthase, PAH; pulmonary arterial hypertension.

Interestingly, elevated levels of methylarginines have also been encountered in monocrotaline-treated rats, a well established animal model of pulmonary hypertension (Pullamsetti et al. 2005). The DDAH expression observed in both animal and human hypertensive lungs supported the theory that modulation of DDAH activity may represent a new strategy for the treatment of PAH.

2 Aim of the study

The implication of protein arginine methylation in human cardiovascular disorders has been suggested by previous studies. However, the available data are fragmentary and the interplay of methylarginine formation and degradation has not been studied in detail so far. Therefore, the expression and activity of methylarginine metabolizing enzymes were analyzed in cell culture, mouse organs, and lung homogenates from PAH patients. In detail, specific aims of this research were:

1) to develop a novel method for quantitative assessment of free versus protein-incorporated methylarginine *in vitro* and *in vivo*,

2) to assess tissue-specific patterns of protein arginine methylation in the mouse cardiovascular system,

3) to analyze the expression and activity of ADMA metabolic enzymes in mouse organs,

4) to analyze the protein arginine methyltransferase expression and activity in patients suffering from pulmonary arterial hypertension,

5) to applicate an siRNA technology to further investigate the role of PRMT1 in pulmonary arterial hypertension.

3 Materials and Methods

3.1 Materials

3.1.1 Equipment

ABI PRISM 7500 Sequence Detection System	Applied Biosystems, USA
Cell Culture Incubator; Cytoperm2	Heraeus, Germany
Chroma SPIN-1000 DEPC-H_2O Columns	Biosciences, Clontech, USA
Developing machine; X Omat 2000	Kodak, USA
Electrophoresis chambers	Bio-Rad, USA
Film cassette	Sigma-Aldrich, Germany
Filter Tip FT: 10, 20, 100, 200, 1000	Greiner Bio-One, Germany
Filter units 0.22 µm syringe-driven	Millipore, USA
Freezer -20 °C	Bosch, Germany
Freezer -40 °C	Kryotec, Germany
Freezer -80 °C	Heraeus, Germany
Fridge +4 °C	Bosch, Germany
Fusion A153601 Reader	Packard Bioscience, Germany
Gel blotting paper 70 × 100 mm	Bioscience, Germany
Glass bottles: 250, 500, 1000 ml	Fischer, Germany
GS-800™ Calibrated Densitometer	Bio-Rad, USA
HPLC system:	
ASI-100 auto sampler	Dionex, USA
P680 gradient pump	Dionex, USA
RF-2000 fluorescence detector	Dionex, USA
Data acquisition system Chromeleon 6.60	Dionex, USA
Light microscope Olympus BX51	Olympus, Germany
µBondapak™ C18 guard column	Waters, USA
Mini spin centrifuge	Eppendorf, Germany

Multifuge centrifuge, 3 s-R	Heraeus, Germany
Nanodrop®	Peqlab, Germany
Oasis MCX solid-phase extraction cartridges	Waters, USA
PCR-thermocycler	MJ Research, USA
Pipetboy	Eppendorf, Germany
Pipetmans: P10, P20, P100, P200, P1000	Gilson, France
Power Supply; Power PAC 300	Bio-Rad, USA
Petri dish with vents	Greiner Bio-One, Germany
Pipette tip: 200, 1000 µl,	Sarstedt, Germany
Pipette tip 10 µl	Gilson, USA
Quantity One software	Bio-Rad, USA
Radiographic film X-Omat LS	Sigma-Aldrich, Germany
Serological pipette: 5, 10, 25, 50 ml	Falcon, USA
SunFire™ C18 column	Waters, USA
Single-use syringe	Braun, Germany
Test tubes: 15, 50 ml	Greiner Bio-One, Germany
Tissue culture chamber slides	BD Falcon, USA
Tissue culture dish 100 mm	Greiner Bio-One, Germany
Tissue culture flask 250 ml	Greiner Bio-One, Germany
Tissue culture plates: 6, 48 well	Greiner Bio-One, Germany
Trans blot transfer medium (0.2 µm)	Bio-Rad, USA
Western blot chambers:	
Mini Trans-Blot	Bio-Rad, USA
Mini-Protean 3 Cell	Bio-Rad, USA
Vortex machine	Eppendorf, Germany
Vacuum-manifold	Millipore, USA
Vacuum centrifuge	Eppendorf, Germany

3.1.2 Reagents

Acetone	Roth, Germany
Acetonitrile	Roth, Germany
Acrylamide solution, Rotiphorese Gel 30	Roth, Germany
Agarose	Invitrogen, UK
Albumine, bovine serum	Sigma-Aldrich, Germany
Ammonium persulphate	Promega, Germany
Ammonium sulphate	Sigma-Aldrich, Germany
β-glycerophosphate	Sigma-Aldrich, Germany
β-mercaptoethanol	Sigma-Aldrich, Germany
Bromophenol blue	Sigma-Aldrich, Germany
Calcium chloride	Sigma-Aldrich, Germany
Complete™ Protease inhibitor	Roche, Germany
DEPC water	Roth, Germany
D-(+)-Glucose	Sigma-Aldrich, Germany
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, Germany
D-MEM medium	Gibco BRL, Germany
DNA Ladder (1 kb)	Promega, USA
Ethylendinitrilo-N, N, *N´, N´,* -tetra-acetic-acid (EDTA)	Promega, USA
Ethylene glycol-bis (2-amino-ethylether)-N,N,*N',N'* -tetraacetic-acid (EGTA)	Sigma-Aldrich, Germany
Dulbecco's phosphate buffered saline 10×	PAA Laboratories, Austria
Dulbecco's phosphate buffered saline 1×	PAA Laboratories, Austria
Ethanol absolute	Riedel-de Haën, Germany
ECL Plus Western Blotting Detection System	Amersham Biosciences, UK
Ethidium bromide	Roth, Germany
Fetal calf serum (FCS)	Gibco BRL, Germany
Gel extraction kit	Qiagen, Germany
Glycine	Roth, Germany

Hydrochloric acid	Sigma-Aldrich, Germany
2-(-4-2-hydroxyethyl)-piperazinyl-1-ethansulfonate (HEPES)	Sigma-Aldrich, Germany
[^{3}H]-thymidine	GE HealthCare, UK
Igepal CA-630	Sigma-Aldrich, Germany
L-arginine	Sigma-Aldrich, Germany
Magnesium chloride	Sigma-Aldrich, Germany
Magnesium sulfate	Sigma-Aldrich, Germany
Metafectene™	Biontex, Germany
Methanol	Fluka, Germany
M-MLV reverse transcriptase	Promega, USA
N,*N*,*N'*,*N'*-tetramethyl-ethane-1,2-diamine (TEMED)	Bio-Rad, USA
Oligo(dT)$_{15}$ Primer	Promega, USA
Opti-MEM medium	Gibco BRL, Germany
ortho-phthaldialdehyde (OPA)	Grom-chromatography, Germany
PCR Nucleotide Mix	Promega, USA
Penicillin-streptomycin	PAA Laboratories, Austria
Potassium acetate	Sigma-Aldrich, Germany
Potassium borate	Grom-chromatography, Germany
Potassium chloride	Merck, Germany
Potassium phosphate	Sigma-Aldrich, Germany
Precision Plus Protein™ Standards	Bio-Rad, USA
2-Propanol	Merck, Germany
QIAprep Spin Miniprep Kit	Qiagen, Germany
Quick Start™ Bradford Dye Reagent	Bio-Rad, USA
RNAsin inhibitor	Promega, Germany
RNeasy Midi Kit	Qiagen, Germany
Smooth muscle cell medium 2	Promocell, Germany
Sodium acetate	Sigma-Aldrich, Germany

Sodium chloride	Merck, Germany
Sodium dodecyl sulfate (SDS)	Promega, USA
Sodium ortho vanadate	Sigma-Aldrich, Germany
Sodium phosphate	Sigma-Aldrich, Germany
Sodium sulfate	Merck, Germany
SuperSignal® West Pico Chemiluminescent Substrate	Pierce, USA
SYBER® Green PCR Kit	Qiagen, Germany
Trichloroacetic acid	Sigma-Aldrich, Germany
Tris	Roth, Germany
Triton X-100	Promega, USA
Trypsin/EDTA	Gibco BRL, Germany
Tween 20	Sigma-Aldrich, Germany
X-treme GENE siRNA transfection reagent	Roche, Germany

3.2 Cells

A549 cell line (human, lung carcinoma), epithelial	DSMZ, Germany
Human primary pulmonary arterial smooth muscle cells (hPASMC)	Promocell, Germany

3.3 Animals

C57BL/6N mice	Charles River, Germany

3.4 Samples from mice

3.4.1 Broncho-alveolar lavage fluid, serum and tissues

All animal studies in the project were performed according to the guidelines of the University of Giessen and approved by the local authorities (Regierungspräsidium Giessen, no. II25.3–19c20–15; GI20/10-Nr.22/2000). Tissue samples were obtained from specific pathogen-free female C57BL/6N mice weighing 18-20 g. From mice under anesthetic, BALF and serum, were collected and all mouse tissues were surgically excised, washed in ice-cold 1 x PBS and frozen in liquid nitrogen for further analyses.

3.5 Samples from human origin

3.5.1 Lung tissue

Lung tissue biopsies were obtained from 10 organ donors, mean age 36.4 ± 13.5 years, six females, four males) and 12 patients with pulmonary arterial hypertension (33.3 ± 12.1 years, nine females, three males). The study protocol was approved by the Ethics Committee of the Justus-Liebig-University School of Medicine (AZ 31/93). Informed consent was obtained from each subject for the study protocol.

3.5.2 Serum and broncho-alveolar lavage fluid

The group examined consisted of seven volunteers (mean age 37.4 ± 2.1 years, three females, four males) who had never smoked and had no history of either cardiac or pulmonary disease, and had normal chest X-rays and pulmonary function. Informed consent was obtained from each subject for the study protocol.

3.6 Methods

3.6.1 RNA isolation

The RNA was isolated from cell cultures according to the manufacture's instructions provided with the RNeasy Midi Kit.

3.6.2 RNA and DNA determination

The concentration of isolated RNA or DNA was measured according to a protocol from Peqlab by applying 1.5 µl of the sample to a Nanodrop® spectrophotometer.

3.6.3 Reverse transcription reaction

Reverse transcriptase polymerase chain reaction (RT-PCR) is an enzymatic reaction during which reverse transcriptase (RT) generates cDNA complementary to RNA.

Each reaction was performed with 500 ng of RNA and 4 µl of oligo$(dT)_{15}$ (100 µg/ml) primers and was diluted with RNAse free water to a final volume of 10 µl. The sample was mixed in a PCR tube and heated (70 °C) for 5 min to allow primers to bind specifically to polyA tail of mRNA. The reaction was cooled on ice for 5 min, and later the rest of required reagents were combined.

RT-Mix:

Components	**Volume**	**Final concentration**
5× RT Buffer	5 µl	1 ×
10 mM dNTP mix	0.5 µl	0.2 mM
RNAsin inhibitor (1 U/µl)	0.5 µl	0.5 U
MMLV Reverse transcriptase (1 U/µl)	0.5 µl	0.5 U
RNAse free water	8.5 µl	not applicable

The reaction was performed at 40 °C for 60 min and completed at 70 °C for 15 min. The resulting complementary DNA (cDNA) was further used for polymerase chain reaction or stored at -20 °C.

3.6.4 Polymerase chain reaction

Polymerase chain reaction (PCR) is an enzymatic reaction, carried out by DNA polymerase and permits the amplification from selected fragments of genomic DNA. Product specificity is determined by the DNA primers used in the reaction.

3.6.4.1 Semi-quantitative PCR

The reaction was performed according to a protocol from the Go Taq® Flexi DNA polymerase kit, and all components were combined as follows:

PCR-mix:

Components	Volume	Final concentration
5× PCR Buffer (free $MgCl_2$ free)	10 µl	1 ×
10 mM dNTP mix	1 µl	0.2 mM
25 mM $MgCl_2$	2 µl	1 mM
10 µM forward primer*	1 µl	0.2 mM
10 µM reverse primer*	1 µl	0.2 mM
DNA (template)	1 µl	not applicable
GoTaq® Flexi DNA polymerase (5U/µl)	0.25 µl	1.25 U

* The primers are listed in Table 6.1

All components were mixed in a 0.5 ml tube and the final volume was adjusted with distilled, autoclaved water up to 50 µl. The sample was transferred to a PCR thermocycler and denatured for 3 min at 94 °C. The reaction was performed for 36 cycles. Each cycle contained three steps: denaturation (separation of double-stranded DNA), annealing (binding of primers to specific region of DNA), and elongation (extending of generated product). The steps were carried out as follows:

PCR program:

Step	Temperature	Time
Denaturation	95 °C	1 min
Annealing	60 °C	1 min 30 s
Elongation	72 °C	2 min

The reaction was completed by a final extension step at 72 °C for 30 min.
The sample was further analyzed by DNA gel electrophoresis or stored at -20 °C.

3.6.4.2 Real-time PCR

Real-time PCR is a variation of the polymerase chain reaction, which is commonly used for amplification and quantification of specific fragments of cDNA. During the reaction, fluorescence dye (SYBER® Green) binds to amplify double-stranded DNA and a signal is detected after each cycle of PCR reaction. The level of fluorescence is proportional to the amount of amplified DNA.

The reaction was performed according to a protocol from the SYBER® Green PCR kit using a Sequence Detection System 7500 Fast from Applied Biosystems. All components were combined as follows:

Real-time PCR mix:

Components	Valume	Final concentration
Platinum® Syber® Green qPCR SuperMix-UDG	13 µl	1 ×
50 mM $MgCl_2$	1 µl	2 mM
10 µM forward primer*	0.5 µl	0.2 mM
10 µM reverse primer*	0.5 µl	0.2 mM
DNA template	1 µl	not applicable

* The list of all primers is described in Table 6.1

The sample was filled up with distilled, autoclaved water to final volume of 25 µl. The real-time PCR was performed for 45 cycles as follows:

Real-time PCR program:

Step	Temperature	Time
Denaturation	95 °C	5 s
Annealing	60 °C	5 s
Extension	72 °C	30 s

Human porphobilinogen deaminase (PBGD), an equally expressed gene, served as a control (reference gene) for all real-time PCR reactions. Relative transcripts abundance of targeted genes was expressed in ΔCt values ($\Delta Ct = Ct^{reference} - Ct^{target}$). The proper size of the amplicons was assessed by agarose gel electrophoresis.

3.6.5 Gel electrophoresis

Gel electrophoresis is a technique which allows the separation of either nucleic acids (DNA, RNA) or proteins according to their physical properties (size, electric charge and other physical properties).

3.6.5.1 DNA gel electrophoresis

For preparation of 1% agarose gels, agarose was dissolved in 1 × Tris-acetate-EDTA (TAE) buffer containing 0.5 µg/ml ethidium bromide, a fluorescent intercalating dye. Before loading onto the gel, DNA samples were mixed 5:1 with 6 × agarose gel-loading buffer. Electrophoresis was performed at 100 V, in 1 × TAE buffer, for 45-60 min. Separated nucleic acids were visualized with short wavelength ultraviolet light (λ 257 nm).

1 × TAE buffer:

40 mM Tris acetate, pH 8.0
1 mM EDTA, pH 8.0

6 × agarose gel-loading buffer:

0.025% (w/v) bromophenol blue
40% (w/v) sucrose

3.6.5.2 Protein gel electrophoresis

To separate proteins, according to their size, SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed. The 10% resolving gel mixture was poured between two glass plates with spacers between, and allowed to polymerize. The stacking gel was poured on the top of resolving gel, and the comb was inserted in to the gel to form the wells. Before sample was loaded on to gel, protein samples were mixed with 10 × SDS gel-loading buffer and heated at 95 °C for 7 min. Electrophoresis was carried out in 1 × SDS-running buffer at 120 V for 1 h.

5% stacking gel:

5% acrylamide/bisacrylamide
125 mM Tris-HCl, pH 6.8
0.1% SDS (w/v)
0.1% APS (w/v)
0.1% TEMED (v/v)

10% resolving gel:

10% acrylamide/bisacrylamide
375 mM Tris-HCl, pH 6.8
0.1% SDS (w/v)
0.1% APS (w/v)
0.1% TEMED (v/v)

10 × SDS-loading buffer:

625 mM Tris-HCl, pH 6.8
50% (v/v) glycerol
20% (w/v) SDS
9% (v/v) β-mercaptoethanol
0.3% (w/v) bromophenol blue

1 x SDS running buffer:

25 mM Tris
250 mM Glycine
0.1% (w/v) SDS

3.6.6 Protein isolation

In order to isolate proteins from cell culture and tissue samples, two alternative methods were applied.

3.6.6.1 Protein isolation from cell culture

Cells were washed twice with ice-cold 1 × phosphate-buffered saline (PBS). The same buffer was applied to cell monolayers (100 µl/cm^2), and cells were detached from culture plates by scraping, and transferred to 1.5 ml tube. After centrifugation for 3 min at 3000 g, the pellet was resuspended in 100 µl of cell-lysis buffer. The cell lysate was incubated for 1 h on ice and centrifuged at 16000 g for 15 min. The resulting supernatant was used as a crude cell extract, and stored at -20 °C.

1 × PBS, pH 7.4:

0.08% (m/v) NaCl
0.02% (m/v) KCl
0.115% (m/v) $Na_2HPO_4 \cdot 2H_2O$
0.02% (m/v) $KH_2PO4 \cdot 2H_2O$

Cell-lysis buffer:

20 mM Tris-HCl, pH 7.5
150 mM NaCl
1 mM EDTA
1 mM EGTA
0.5% Igepal CA-630
2 mM Na_3VO_4 *
Complete™, protease inhibitor mix *

*Added immediately prior to homogenization

3.6.6.2 Protein isolation from tissue

Mouse or human tissue was homogenized in liquid nitrogen followed by addition of ice-cold tissue lysis buffer. Tissue lysate was then passed 3 × through a 0.9 mm needle fitted to a RNAse-free syringe. Homogenized tissue was incubated for 1 h on ice and

centrifuged for 15 min at 16000 g. The resulting supernatant was used as a crude tissue extract and stored at -20 °C.

Tissue lysis buffer:

20 mM Tris-HCl, pH 7.5
150 mM NaCl
1 mM EDTA
1 mM EGTA
1% (v/v) Triton X-100
2 mM Na_3VO_4 *
Complete™, protease inhibitor mix *

*Added immediately prior to homogenization

3.6.6.3 TCA precipitation

Trichloroacetic acid (TCA) precipitation is a method commonly used for protein concentration and purification. It allows the separation of macromolecules, such as proteins, from other organic compounds (fats, amino and nucleic acids), present in the protein crude extract.

Proteins were precipitated by mixing 100 µl of tissue/cell extract with an equal volume of 20% (v/v) trichloroacetic acid for 20 min on ice. After centrifugation at 16000 g for 12 min, the supernatants were removed and the protein pellets were washed with 100 µl ice-cold acetone for 60 min at -20 °C. The suspension was centrifuged at 16000 g for 12 min and resulting protein pellet was dissolved in 100 µl of distilled water.

3.6.6.4 Hydrolysis

Total hydrolysis of precipitated protein fractions (20 µl) was achieved by gas-phase hydrolysis with 100 µl of 6 M HCl at 110 °C for 16 h. Samples were dried by use of a vacuum centrifuge and stored at -20 °C until further analyzed.

3.6.6.5 Protein determination

Protein determination was performed using Quick Start™ Bradford Dye Reagent and measured spectrophotometrically on a Fusion A153601 Reader. The method is based on chemical, colorimetric reaction between Coomassie Brilliant Blue G-250 dye and aromatic amino acids residues (Bradford 1976). The color change is proportional to the amount of protein in the sample, and can be measured as an absorbance by spectrophotometer. The Bradford method requires a calibration curve to be generated each time the assay is run before unknown protein concentrations can be determined.
Sample (10 µl) was mixed in a 96-well plate with 200 µl of Quick Start™ Bradford Dye reagent. Reaction mixtures were incubated for 15 min at room temperature. The absorbance was measured at 570 nm. For preparation of calibration, five dilutions (0.05-0.4 µg/µl) of bovine serum albumin (BSA) were used.

3.6.7 Subcellular fractionation

To obtain nuclear and cytosolic fractions, cells were washed twice with ice-cold 1× PBS, scraped in PBS and collected by centrifugation at 3000 g for 3 min. Cells were resuspended in ice-cold hypotonic lysis buffer and incubated on ice for 10 min. After incubation, nuclear and cytosolic fractions were separated by centrifugation at 5000 g for 5 min. The supernatant served as the cytosolic fraction, and was stored at -20 °C for further investigation. Hypertonic lysis buffer was added to the pellet, mixed and incubated on ice for 45 min. Nuclear soluble compounds were separated by centrifugation at 12000 g for 10 min, 4 °C. The nuclear fraction was stored at -20 °C for further analysis.

Hypotonic-lysis buffer:

20 mM HEPES, pH 7.9
10 mM KCl
1 mM EDTA
1 mM EGTA
0.2% (v/v) NP-40
10% (v/v) glycerol
2 mM Na_3VO_4 *
Complete™, protease inhibitor mix *

Hypertonic-lysis buffer:

20 mM HEPES, pH 7.9
10 mM KCl
420 mM NaCl
1 mM EDTA
1 mM EGTA
20% (v/v) glycerol
2 mM Na_3VO_4 *
Complete™, protease inhibitor mix *

*Added immediately prior to homogenization

3.6.8 Analysis of basic amino acids

3.6.8.1 Isolation of basic amino acids

All procedures were performed as previously described (Bulau et al. 2006). Tissue/cell extracts and amino acid hydrolysates were subjected to crude fractionation on Oasis MCX cation exchange solid-phase extraction (SPE) columns. For tissue/cell extracts, 50 µl of each sample was combined with 10 µl of L-homoarginine (4 pmol/ µl) as internal standard and adjusted to a final volume of 1 ml with PBS. Amino acid hydrolysates were dissolved in 1 ml PBS. All conditioning, washing, and elution steps were performed on a vacuum manifold with a capacity of 10 columns, at a flow rate of 0.5 ml/min. The SPE columns were conditioned with 2 ml of eluent solution followed by 2 ml of PBS prior to sample application. Samples were passed through the SPE cartridges and the contaminating components were rinsed with 2 ml of 0.1 M HCl followed by 2 ml methanol. Basic amino acids were eluted with 1 ml of H_2O/methanol/ammonia (45/50/5; v/v). The samples were dried by use of vacuum centrifuge at 65 °C. Eluates were redissolved in 230 µl of distilled water and centrifuged at 14000 g for 2 min.

3.6.8.2 Derivatization with OPA reagent

Amino acids form highly fluorescence adducts when reacted with *ortho*-phthaldialdehyde (OPA) under basic conditions. The products of this reaction

exhibit optimal excitation at 330 nm and emission at 450 nm and can be quantified by fluorescence detection.

All procedures were performed as previously described (Bulau et al. 2006). The OPA was freshly prepared in potassium borate buffer, according to the manufacturer's instructions. Sample (50 µl) was combined with 62.5 µl of OPA, immediately transferred to the auto sampler, and injected exactly after 2 min.

3.6.8.3 Chromatographic separation by HPLC

Quantification of amino acids was performed on a HPLC system with the data acquisition system Chromeleon 6.60. Separation was performed according to a method previously described (Bulau et al. 2006; Bulau et al. 2007). Fluorescent amino acid derivatives were separated on a SunFire™ C18 column (4.6 × 150 mm; 3.5 µm particle size; 100 Å pore size) with a µBondapak™ C18 guard column at 25 °C and a flow rate of 1.1 ml/min. After injecting 125 µl of the sample, separation was performed under isocratic conditions with HPLC running buffer as solvent. The isocratic conditions were maintained for 25 min. In order to elute strongly-bound compounds, the column was flushed with 50% (v/v) acetonitrile for 15 min and reequilibrated under isocratic conditions for 25 min prior to the next injection. Fluorescent derivatives were detected at excitation and emission wavelengths of 330 and 450 nm, respectively. L-arginine, ADMA and SDMA were quantified by two separated steps. For detection of ADMA and SDMA, the gain of the detector was switched to a hundred-fold higher sensitivity.

HPLC running buffer (pH 6.8):

8.8% (v/v) acetonitrile
25 mM potassium phosphate
25 mM sodium phosphate

3.6.8.4 DDAH activity assay

The activity of DDAH was assayed by directly measuring the amount of ADMA metabolized by the enzyme.

For tissue extracts and serum, 25 µl of each sample was combined with ADMA/SDMA solution (500 pmol/µl each) and adjusted to a final volume of 0.5 ml with 0.1 M sodium phosphate buffer (pH 6.5). After incubation for 2 h at 37 °C, samples were directly subjected to crude fractionation on Oasis MCX cartridges, HPLC separation and fluorescence detection. As assay blank, ADMA and SDMA from the crude tissue extracts were directly quantified.

3.6.9 Western blot analysis

Western blot analysis allows the visualization of levels of candidate proteins using specific antibodies for recognition.

3.6.9.1 Immunoblotting

Proteins separated by SDS-PAGE were transferred from the polyacrylamide gel to a polyvinylidene difluoride (PVDF) membrane. The membrane was first activated in 100% methanol, and transfer was performed in transfer buffer at 100 V for 1 h.

Transfer buffer (pH 7.4):

24 mM Tris
193 mM glycine
10% (v/v) methanol

3.6.9.2 Protein visualization

The membrane was incubated in blocking solution for 1 h followed by incubation with appropriate primary antibodies in blocking buffer at 4 °C overnight.* Then, the membrane was washed 3 × with washing buffer and incubated with horseradish proxidase (HRP) labeled secondary antibodies for 1 h at room temperature. The membrane was washed 5 × for 10 min. Proteins were detected using chemiluminescence by Enhanced Chemiluminescent Immunoblotting System and membrane was exposed to radiographic film.

To apply second antibody, the membrane was incubated in stripping buffer for 10 min, washed in washing buffer for 5 min, and protein visualization was performed as previously described.

*Primary and secondary antibodies are listed in Tables 6.2 and 6.3

Blocking buffer:

5% (w/v) non-fat dry milk
1 × PBS
0.1% (v/v) Tween-20

Washing buffer:

1 × PBS
0.1% (v/v) Tween-20

Stripping buffer:

62.5 mM Tris-HCl, pH 6.8
2% (w/v) SDS
100 mM β-mercaptoethanol

3.6.9.3 Densitometric analysis

Densitometric analysis of autoradiographies was performed using a GS-800 Calibrated Densitometer and 1-D analysis software Quantity One (Bio-Rad, USA). Protein expression was normalized to the appropriate house-keeping protein used in the experiment.

3.6.10 Immunohistochemistry

To elucidate the localization of proteins of interest in lung tissue, immunohistochemical analysis was performed using a Histostain-SP Kit. Whole lung sections (3µm) were deparaffinized in xylene 3 × for 10 min followed by dehydration in a graded alcohol series. Antigen retrieval was performed in citrate buffer (pH 6.0) for 20 min at 100 °C. Slides were washed 2 × for 5 min in 1 × PBS. To block activity of endogenous peroxidase, sections were immersed in 3% (v/v) H_2O_2 for 20 min. Sections

were incubated with blocking reagent (provided with the kit) for 10 min at room temperature to prevent non-specific binding. Primary antibodies* were applied to sections and incubated at 4 °C overnight. Sections were washed with 1 × PBS for 5 min and incubated with secondary biotinylated antibodies followed by streptavidin-conjugated enzyme and chromogenic reagent, separately for 10 min. Slides were developed for 5 min with diaminobenzidine (DAB) and counterstained with Mayers hematoxylin. Finally, sections were mounted using and examined using Olympus BX51 microscope.

*Primary antibodies are listed in Table 6.2

3.6.11 Cell culture

3.6.11.1 Culture of mammalian cells

3.6.11.1.1 A549 cells

Cells were grown in tissue culture flasks in D-MEM medium containing 10% (v/v) heat-inactivated fetal calf serum (FCS) at 37 °C, 5% CO_2, 95-100% humidity. The cell line was passaged when attaining 80-90% confluence. During passaging, cells were washed with 1 × PBS and incubated with 3 ml of Trypsin/EDTA solution for 3 min at 37 °C, after which 7 ml of D-MEM medium was added. Cells were transferred to new tissue culture flasks after 1:5 dilution with medium.

3.6.11.1.2 Pulmonary artery smooth muscle cells

Pulmonary arterial smooth cells (PASMC) were grown in tissue culture flasks in smooth muscle cell medium 2 at 37 °C, 5% CO_2, 95-100% humidity. The cell line was passaged when attaining 80-90% confluence. During passaging, cells were washed with 1 × PBS and incubated with 3 ml of trypsin solution for 3 min at 37 °C after which 7 ml of medium was added. Cells were transferred to new tissue culture flasks after 1:5 dilution with medium.

Trypsin solution:

0.25% (m/v) Trypsin
1.23 g/ml EDTA

3.6.11.2 Transient transfection

3.6.11.2.1 siRNA technique

The PASMC were plated onto 6-well tissue culture dishes in smooth muscle cell medium 2 to obtain 60-70% confluence. After 16 h, medium was exchanged and cells were incubated in 1:50 diluted medium (starvation conditions) for 4-5 h. In order to downregulate PRMT1, siRNA sequence* against this gene was used. To exclude possible non-specific effects of PRMT1 siRNA, the experiment was controlled by the use of a universal siRNA negative control commercially available from Ambion. Transfection reagent (X-treme GENE silencing or Metafectane) was mixed with Opti-MEM medium and incubated at room temperature for 5 min. Afterwards, siRNA was added, gently mixed and left for 20 min. Cells were transfected using transfection complexes, incubated 4-5 h followed by addition of fresh smooth muscle cell medium 2. Cells were harvested for 16-48 h, washed with 1 × PBS and stored at -20 °C for further analysis.

*List of siRNAs is shown in Table 6.4

3.6.11.3 Proliferation assay

A proliferation assay quantifies cell proliferation. When assessed by tritium incorporation it is based on the measurement of radioactivity incorporated into the DNA during cell growth.

The PASMC were grown on 48-well tissue culture plates in smooth muscle cell medium 2. After transfection, using siRNA, and incubation for 16-48 h, cells were pulsed with 0.6 μCi of [^{3}H]-thymidine for 4-5 h. Cells were washed 5 × with 1 × PBS and solubilized in 300 μl of 0.5 M NaOH. After overnight incubation at 4 °C, the contents of each well were combined with 8 ml of scintillation fluid and radioactive counts were measured in a liquid scintillation counter.

4 Results

4.1 Development of a method for quantification of methylarginine content in biological samples

To study protein-incorporated versus free-cellular methylarginine levels either in tissue or in cell culture extracts, a novel HPLC-based technique was developed.

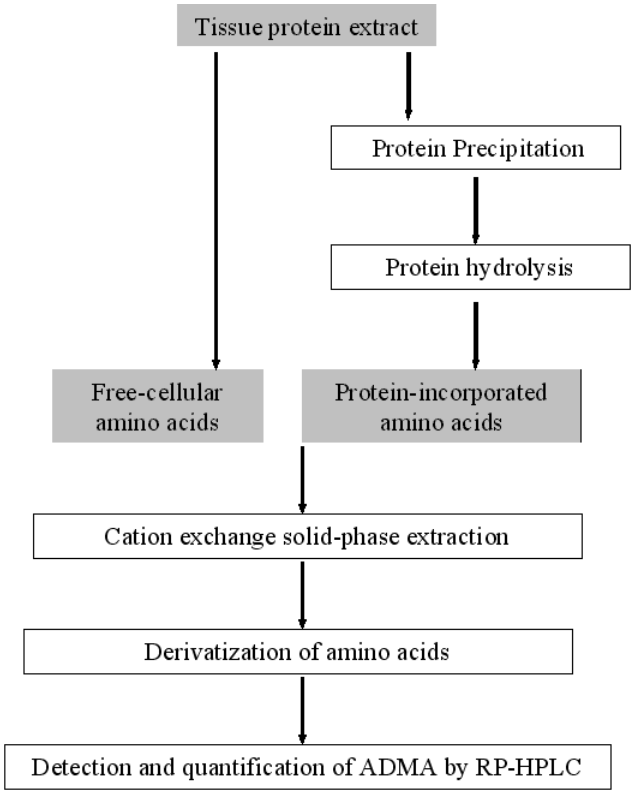

Figure 4.1 Strategy for the quantification of protein-incorporated and free-cellular L-Arg, ADMA and SDMA in human and mouse tissue homogenates. ADMA; asymmetric ω-N^G, N^G-dimethylarginine, RP-HPLC; reverse phase-high performance liquid chromatography.

Crude tissue homogenates were subjected to SPE followed by derivatization of basic amino acids with OPA (*ortho*-phthaldialdehyde) reagent and further analyzed by RP-HPLC (figure 4.1).

The quantification of protein-incorporated methylarginines additionally required protein precipitation and protein hydrolysis of prior to cation exchange SPE, reaction with OPA and chromatographic separation, as outlined in figure 4.1.

4.1.1 Standard curves and calibration

For method validation, calibration was performed using six standards spanning the varied concentration range for L-Arg, L-NMMA, ADMA and SDMA.

Standard solutions were combined with 15 pmol of internal standard L-homoarginine (L-H Arg) and subjected to SPE, derivatization and chromatography as described in the Materials and Methods. Calibration curves were analyzed by plotting the peak area ratios of analyte (L-Arg, L-NMMA, ADMA, SDMA) to the area of the internal standard versus analyte quantity and resulted in linear coefficients (R^2) of 0.9981, 0.9992, 0.9989, 0.9998 and 0.9989 for L-Arg, L-NMMA, L-H Arg, ADMA and SDMA, respectively (figure 4.2).

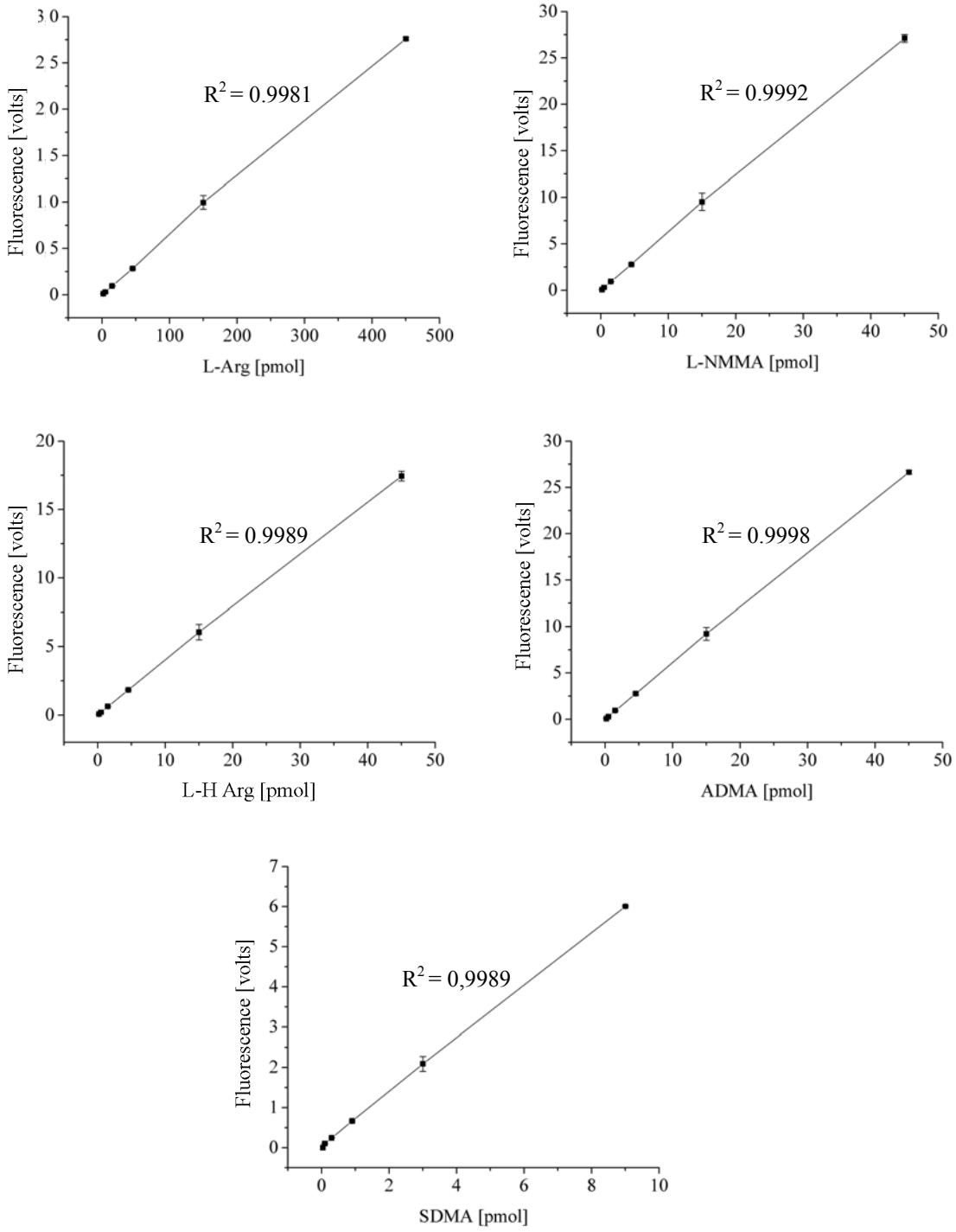

Figure 4.2 Linear regression of standard curves. Calibration was performed using the concentration range of 1.5 to 450 pmol (60 nM to 18 µM) for L-Arg; 0.15 to 45 pmol (6 nM to 1.8 µM) for L-NMMA and ADMA and 0.09 to 9 pmol (3.6 nM to 0.36 µM) for SDMA. L-NMMA; ω-N^G-monomethylarginine, L-H Arg; ω-N^G-homoarginine, ADMA; asymmetric ω-N^G, N^G-dimethylarginine, SDMA; ω-N^G, N'^G-dimethylarginine.

4.1.2 Recovery

In order to validate the recovery of methylated L-Arg derivatives after protein precipitation and protein hydrolysis standard solution containing L-Arg, L-NMMA, L-H Arg, ADMA and SDMA were subjected to acid hydrolysis followed by SPE and chromatographic separation. As illustrated in table 4.1, all compound recoveries were ≥84 %. To analyze the application of L-H Arg as internal standard, relative recoveries were calculated and defined as the absolute recovery divided by the recovery of internal standard. The relative recoveries for all amino acids were almost 100% (table 4.1).

Tabele 4.1 Absolute and relative recovery of analytes subjected to protein hydrolysis followed by SPE and chromatography.
Absolute recovery was calculated as percentage of peak area obtained after RP-HPLC. Relative recovery was determined as percentage of the recovery of L-H Arg. Data are presented as means ± SD, n = 6. L-NMMA; ω-N^G-monomethylarginine, L-H Arg; ω-N^G-homoarginine, ADMA; asymmetric ω-N^G, N^G-dimethylarginine, SDMA; ω-N^G, N'^G-dimethylarginine.

	Absolute recovery	Relative recovery
L-Arg	84.56 ± 2.50%	96.78 ± 2.67%
L-NMMA	84.45 ± 3.83%	96.61 ± 2.68%
L-H Arg	87.46 ± 4.45%	Not applicable
ADMA	88.08 ± 4.08%	100.74 ± 1.87%
SDMA	87.83 ± 5.06%	100.42 ± 2.69%

To further confirm that protein precipitation does not alter the levels of methylated protein, MBP from bovine brain was analyzed. The MBP is a well-characterized protein, which contains monomethylargin and symmetrically dimethylarginines. In order to measure extent of protein methylation, MBP was subjected to protein precipitation followed by acid hydrolysis.

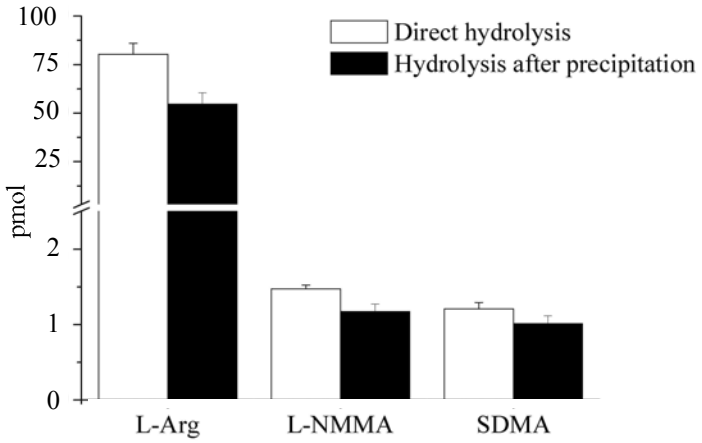

Figure 4.3 Protein precipitation does not alter protein methylation. L-Arg, L-NMMA and SDMA content in amino acid hydrolysates of purified MBP is similar before and after precipitation. Data are presented as mean ± SD in pmol, n = 6. MBP; myelin basic protein, SD; standard deviation**,** L-NMMA; ω-N^G-monomethylarginine, L-H Arg; ω-N^G-homoarginine, SDMA; ω-N^G, N'^G-dimethylarginine.

Quantification of L-Arg, L-NMMA and SDMA in MBP after direct hydrolysis with or without protein precipitation suggested no significant differences in relative methylarginine content (figure 4.3). Asymmetric dimethylation of MBP arginine residues was not detected.

4.1.3 Dynamic metabolism of L-Arg, ADMA and SDMA under proteosome inhibition

To prove that the HPLC method was suitable for the observation of dynamic changes in protein arginine methylation in biological samples, A549 cells were cultured with or without the proteosome inhibitor MG-132 for 2 h. Inhibition of protein degradation led to a significant increase in L-Arg levels in protein hydrolysates (58.2 ± 3.7 versus 47.3 ± 5.6 nmol/mg protein), as illustrated in figure 4.4 A, with a

concomitant decrease of free L-Arg levels (3.01 ± 0.3 versus 4.14 ± 0.4 nmol/mg protein), as illustrated in figure 4.4 B.

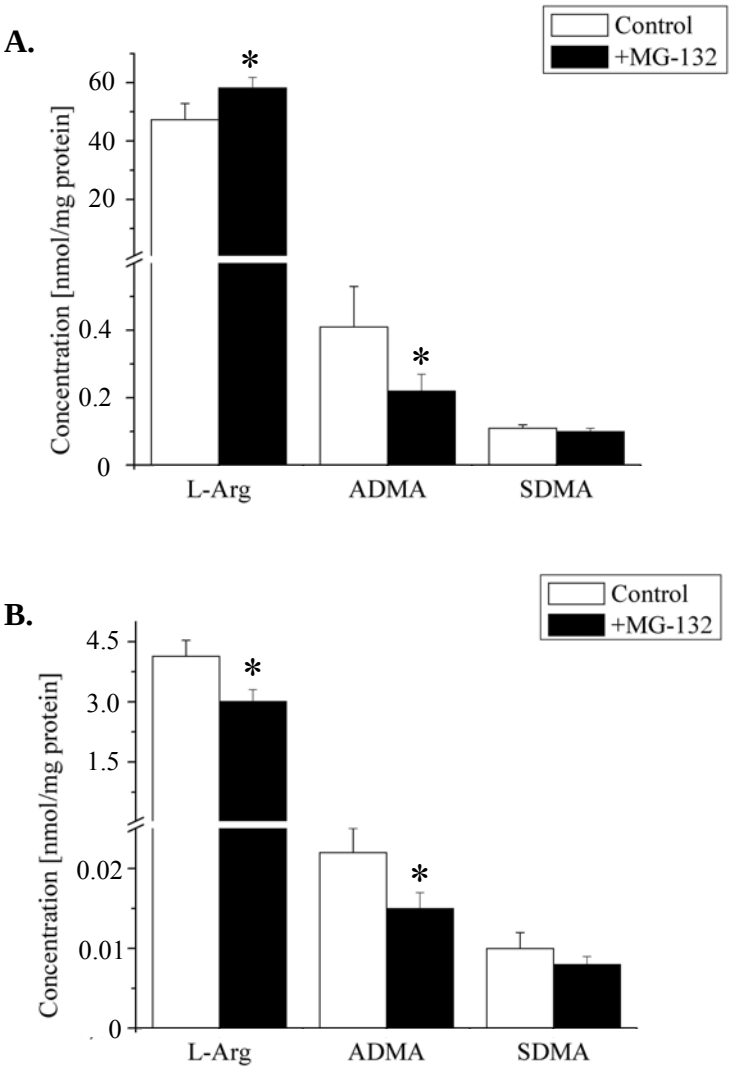

Figure 4.4 Degree of protein methylation in protein hydrolysates (A) and crude extracts (B) in A549 cells after treatment with a proteosome inhibitor. The A549 cells were cultured in absence or presence of MG-132 (5 μM) for 2 h. The concentration of L-Arg, ADMA and SDMA was assesed by HPLC. Data are presented as mean ± SD, n = 4,* $P < 0.05$, two-tailed *t*-test. SD; standard deviation, HPLC; high performance liquid chromatography, ADMA; asymmetric ω-N^G, N^G-dimethylarginine, SDMA; ω-N^G, N'^G-dimethylarginine.

Treatment with MG-132 significantly reduced free-cellular ADMA levels (0.015 ± 0.003 versus 0.022 ± 0.003 nmol/mg protein; figure 4.4 B), but also significantly decreased ADMA levels in protein hydrolysates (0.22 ± 0.05 versus 0.41 ± 0.12 nmol/mg protein), as illustrated in figure 4.4 A. Significant differences in SDMA concentrations were not observed.

4.2 Analysis of ADMA metabolism

4.2.1 Methylarginine content in mouse organs of the cardiovascular system

In order to assess tissue-specific protein arginine methylation, HPLC-based quantification of protein-incorporated and free methylarginines was employed. The L-Arg, ADMA and SDMA levels were assessed in mouse homogenates from lung, heart, kidney and liver.

4.2.1.1 Protein-incorporated L-arginine, ADMA and SDMA

To analyze whether different organs exhibited distinct methylation characteristics, L-Arg and its methylated forms were measured in protein hydrolysates and crude extracts derived from mouse organs of cardiovascular system. As figure 4.5 illustrates, lung proteins exhibited a four-fold higher degree of asymmetrical and a two-fold higher degree of symmetrical arginine methylation (ADMA: 4.23 ± 2.19, SDMA 0.37 ± 0.17 nmol/mg protein) compared with heart (ADMA:1.11 ± 0.53, SDMA 0.22 ± 0.007 nmol/mg protein), kidney (ADMA: 1.41 ± 0.26, SDMA 0.27 ± 0.003 nmol/mg protein) or liver (ADMA: 1.04 ± 0.42, SDMA: 0.21 ± 0.007 nmol/mg protein) hydrolysates, which exhibited almost identical levels of protein-incorporated ADMA and SDMA. The L-arginine concentrations did not exhibit any significant differences between samples.

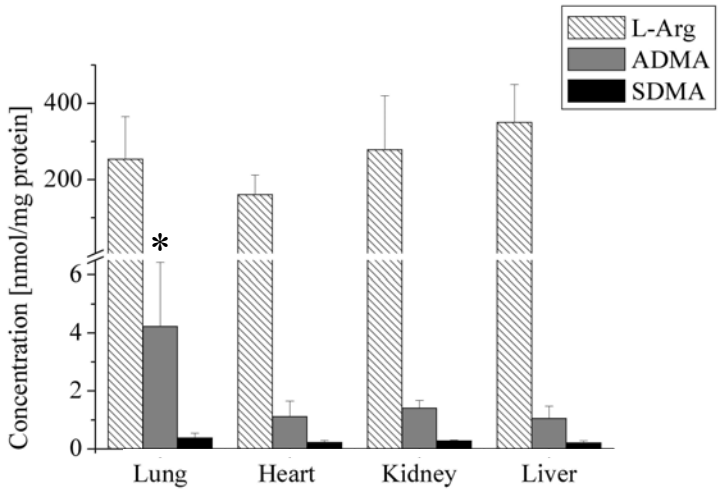

Figure 4.5 Concentration of protein-incorporated L-Arg, ADMA and SDMA in mouse lung, heart, kidney and liver. Data are presented as mean ± SD, n = 6, * $P < 0.05$, one-way ANOVA, lung versus all tissues. SD; standard deviation, ANOVA; analysis of variance, ADMA; asymmetric ω-N^G, N^G-dimethylarginine, SDMA; ω-N^G, N'^G-dimethylarginine.

4.2.1.2 Free-cellular methylarginines

Levels of free cellular ADMA and SDMA were similar in the lung (ADMA: 0.010 ± 0.004, SDMA 0.0017 ± 0.0005 nmol/mg protein) and heart (ADMA: 0.014 ± 0.004, SDMA 0.0026 ± 0.005 nmol/mg protein); while the kidney (ADMA: 0.118 ± 0.04, SDMA 0.033 ± 0.012 nmol/mg protein) and the liver (ADMA: 0.088 ± 0.02, SDMA 0.011 ± 0.003 nmol/mg protein) exhibited significantly higher concentrations of both methylarginines, as illustrated in figure 4.6.

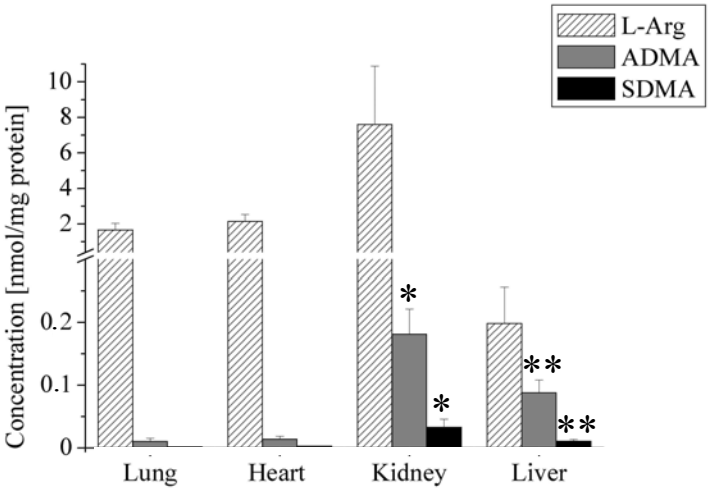

Figure 4.6 Concentration of free-cellular L-Arg, ADMA and SDMA in mouse lung, heart, kidney and liver. Data are presented as mean ± SD, n = 6, * $P < 0.05$; kidney versus lung and heart, ** $P < 0.05$, liver versus lung and heart, one-way ANOVA. ANOVA;analysis of variance, SD; standard deviation, ADMA; asymmetric ω-N^G, N^G-dimethylarginine, SDMA; ω-N^G, N'^G-dimethylarginine.

4.2.2 L-Arg, ADMA and SDMA in serum and BALF

Enhanced levels of protein-incorporated ADMA and SDMA observed in the mouse lung might reflect the occurrence of the methylarginines, either in mouse BALF or serum. To examine levels of methylarginines in biological fluids and to analyze species-specific content of protein arginine methylation, levels of L-Arg, ADMA and SDMA in serum and BALF from mouse as well as human were assessed.

4.2.2.1 Concentration of L-arginine derivatives in mouse and human serum

The ADMA levels in mouse serum were significantly higher when compared to human serum (0.91± 0.18 versus 0.42 ± 0.06 μM). SDMA (0.3 ± 0.15 versus 0.42 ± 0.7 μM) exhibited lower concentrations in mouse serum compared to human serum. No significant differences were observed for of L-Arg (112.39 ± 17.78 versus 69.51 ± 11.92 μM), as illustrated in figure 4.7 A. Moreover, ratios of ADMA to SDMA as well as ratio of L-Arg to SDMA in mouse serum were higher than in human serum

(ADMA/SDMA: 3.5 ± 1.2 versus 1.04 ± 0.24, L-Arg/SDMA: 428.37 ± 146.79 versus 168.01 ± 23.62; figure 4.7 B).

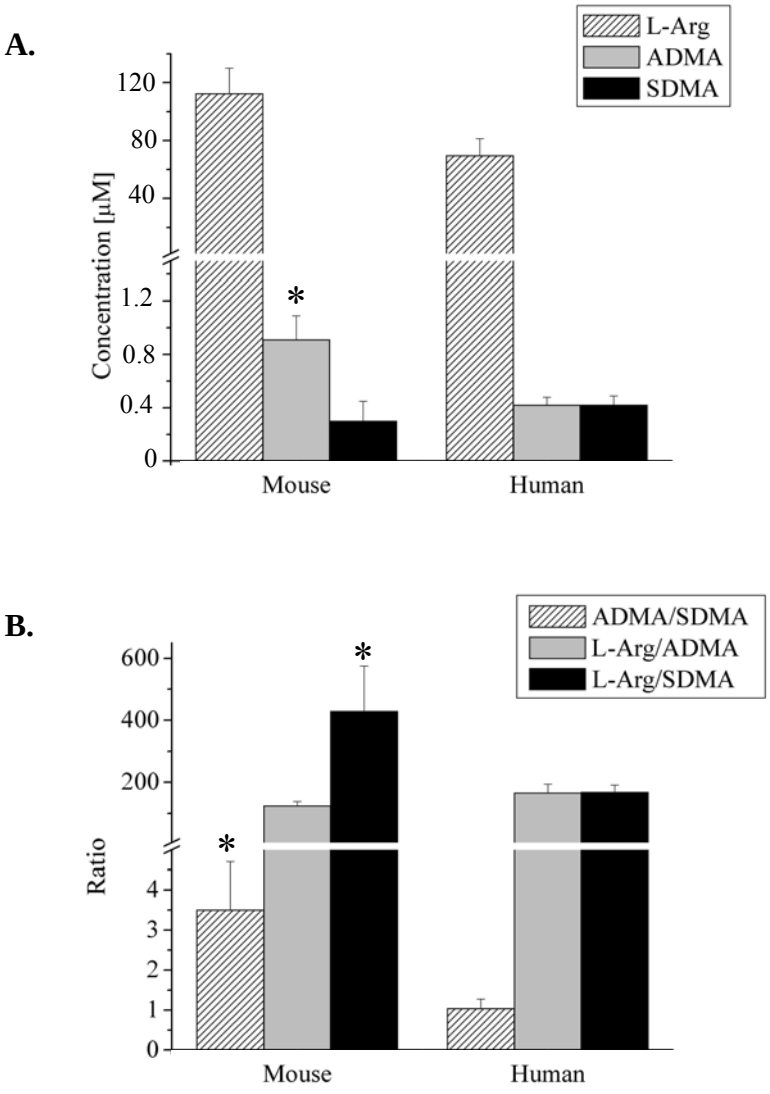

Figure 4.7 Concentration (A) and ratios (B) of methylarginines in mouse and human serum. Data are presented as mean ± SD, mouse: n = 10, human: n = 7, * P < 0.001, two-tailed *t*-test. SD; standard deviation, L-NMMA; ω-N^G-monomethylarginine, ADMA; asymmetric ω-N^G, N^G-dimethylarginine, SDMA; ω-N^G, N'^G-dimethylarginine.

4.2.2.2 Methylargine content in human and mouse BALF

Both ADMA and SDMA were detected in mouse and human BALF. As illustrated in figure 4.8 A, the ADMA concentration was significantly higher in the mouse compared to the human (0.053 ± 0.02 versus 0.03 ± 0.013 pmol/mg protein), whereas the opposite situation was observed for SDMA (0.0102 ± 0.004 versus 0.031 ± 0.006 pmol/mg protein).

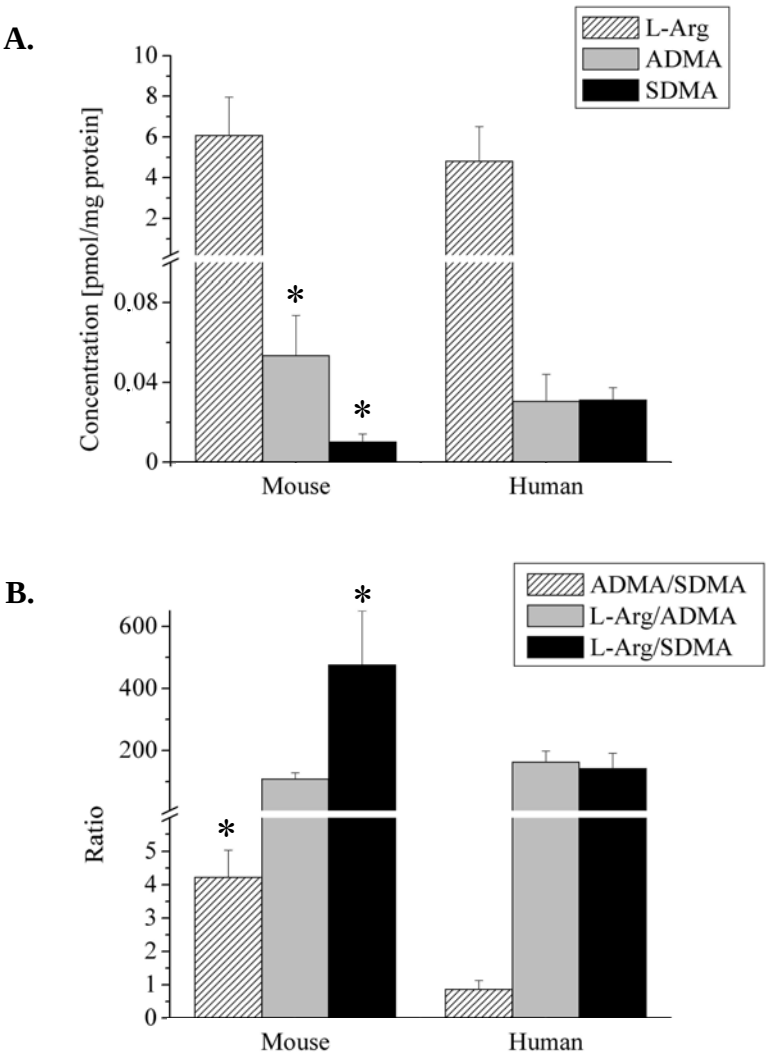

Figure 4.8 Concentration (A) and ratios (B) of methylarginines in mouse and human BALF. Data are presented as mean ± SD, mouse: n = 10, human: n = 7, * P < 0.001, two-tailed *t*-test. L-NMMA; ω-N^G-monomethylarginine, ADMA; asymmetric ω-N^G, N^G-dimethylarginine, SDMA; ω-N^G, N'^G-dimethylarginine, BALF; broncho-alveolar lavage fluid, SD; standard deviation.

The L-Arg levels did not differ between mouse and human (6.07 ± 1.88 versus 4.81 ± 1.71 pmol/mg protein). Interestingly, the ratios of ADMA to SDMA (4.23 ± 0.81 and 0.8684 ± 0.2659), L-Arg to ADMA (108.34 ± 20.05 and 162.9 ± 35.7) and L-Arg to SDMA (475.83 ± 174 and 142.14 ± 49.47) in BALF were similar to those observed in serum. Taken together, mouse and human tissues displayed different methylarginine concentrations and ratios, both in the serum and the BALF. In contrast, both species demonstrated a similar methylarginine distribution between serum and BALF.

4.2.3 Expression of PRMTs in mouse lung, heart, liver and kidney

The ADMA is produced through methylation of protein arginine residues by PRMT type I enzymes (Bedford and Richard 2005). To further investigate whether enhanced levels of protein-incorporated ADMA observed in the mouse lung correlated with increased PRMT expression, western blot followed by densitometric analysis were performed on crude mouse extracts. As illustrated in figure 4.9 A, expression levels of both type I and type II PRMTs varied in the mouse lung, heart, kidney and liver.

Densitometric analysis (figure 4.9 B) revealed that mouse lungs expressed significantly higher levels of PRMT1, PRMT2 and PRMT6 compared to other organs.

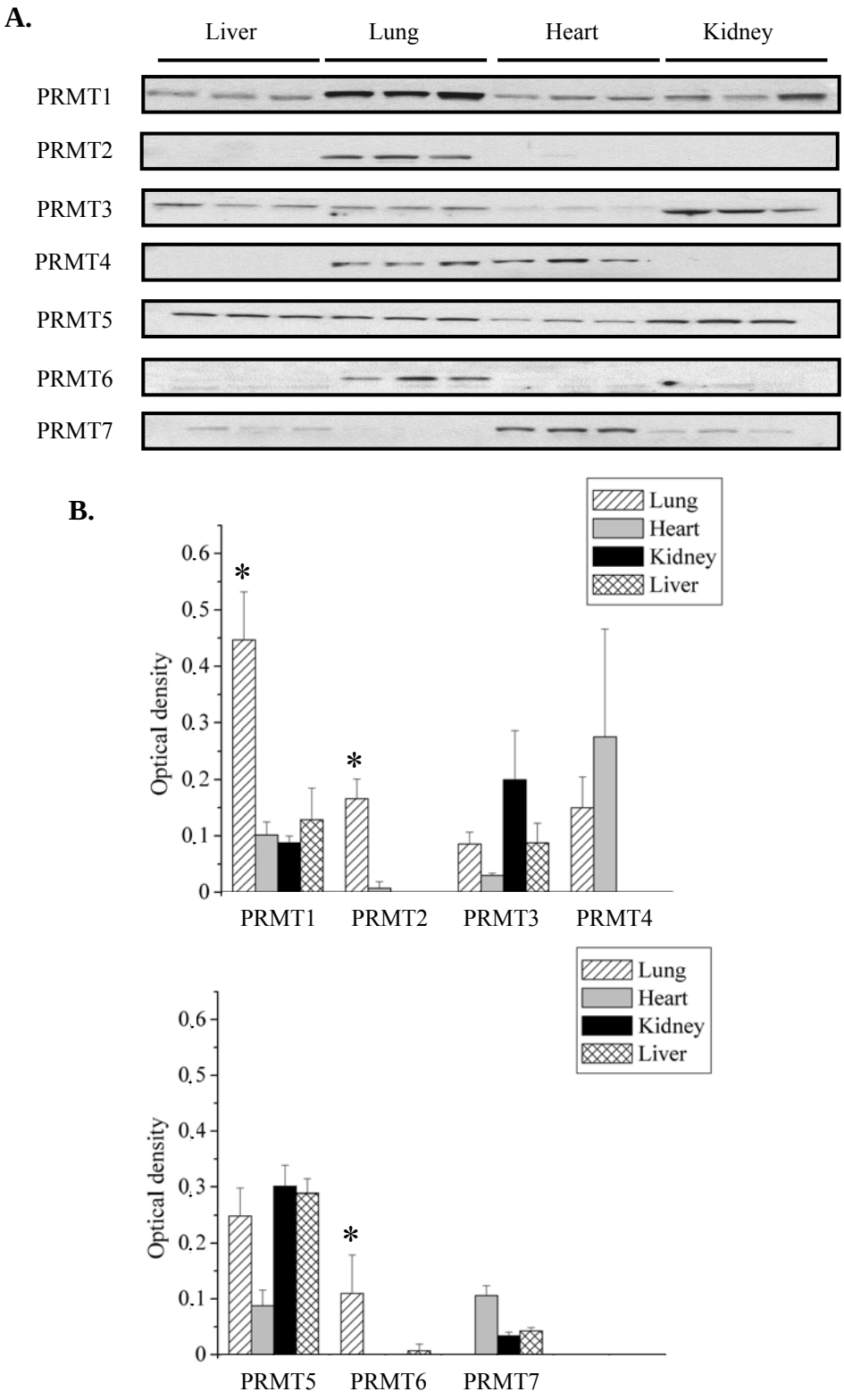

Figure 4.9 Expression pattern of PRMT enzymes in mouse tissues. Western blot (A) was performed with 20 µg protein from crude organ extracts from mice followed by densitometric analysis (B). In panel B, data are presented as mean ± SD, * $P < 0.05$, lung versus all tissues, one-way ANOVA. SD; standard deviation, ANOVA; analysis of variance

4.2.4 Analysis of ADMA degradation

Cellular ADMA occuring in mammals can be degraded and converted to citrulline and mono- or di-methylamines by DDAH (Bedford and Richard 2005). To study tissue-specific ADMA degradation, western blot analysis of DDAH expression and HPLC measurement of DDAH activity were performed.

4.2.4.1 Expression of mouse DDAH isoforms

To examine the expression levels of DDAH isoforms, western blot analysis was performed on crude extracts derived from mouse lung, heart, kidney and liver (figure 4.10 A). Densitometric analysis revealed that DDAH1 was significantly more expressed in the kidney and liver, compared to the lung, while in the heart, DDAH1 was not detected (figure 4.10 B, left panel). In contrast, DDAH2 was equally expressed in the liver, lung and heart, whereas significantly lower expression of this isoform was observed in kidney lysates.

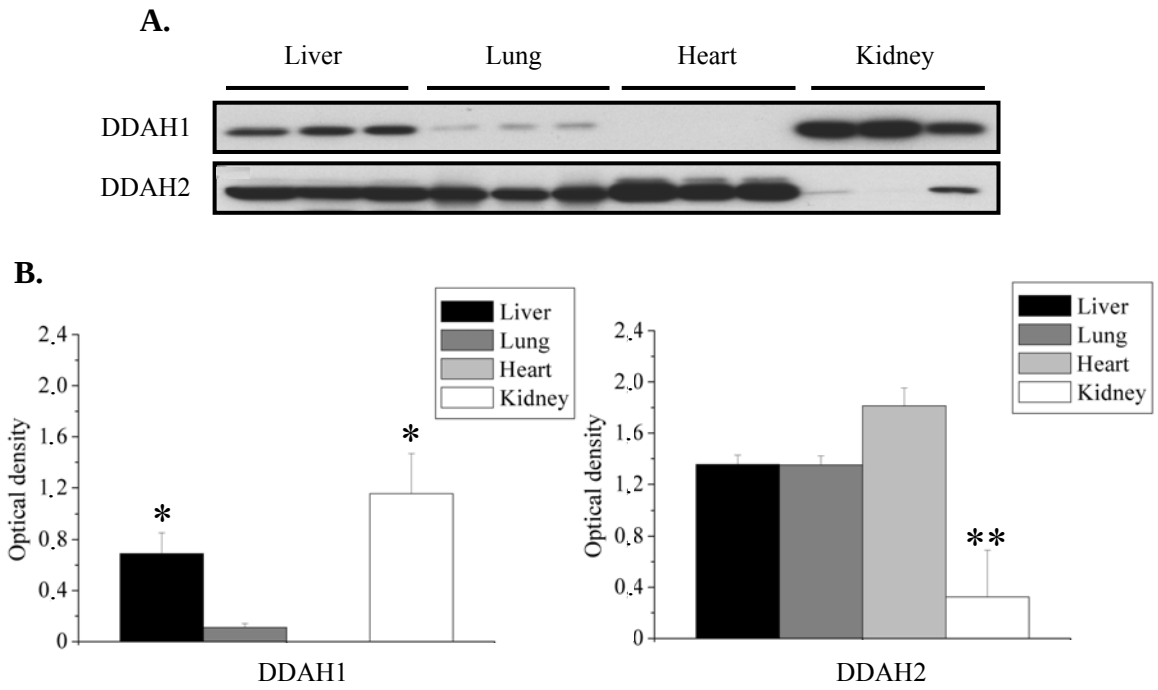

Figure 4.10 Expression of DDAH isoforms in mouse tissues. Western blot analysis (A) was performed with 20 µg protein from crude organ extracts from mice followed by densitometric analysis (B). Data are presented as mean ± SD;* $P < 0.05$, kidney and liver versus heart and lung, ** $P < 0.05$, kidney versus all tissues, one-way ANOVA. ANOVA; analysis of variance, DDAH; N^G,N^G–dimmethylarginine dimethylaminohydrolase, SD; standard deviation HPLC; high performance liquid chromatography, ADMA;. asymmetric ω-N^G, N^G-dimethylarginine.

4.2.4.2 Tissue-specific activity of DDAH

To investigate whether expression of DDAH isoforms was correlated with tissue-specific degradation of ADMA, studies of DDAH activity were performed. To study enzyme activity *in vitro*, mouse protein extracts were incubated with a standard solution containing ADMA and SDMA and subjected to HPLC-based method for quantification.

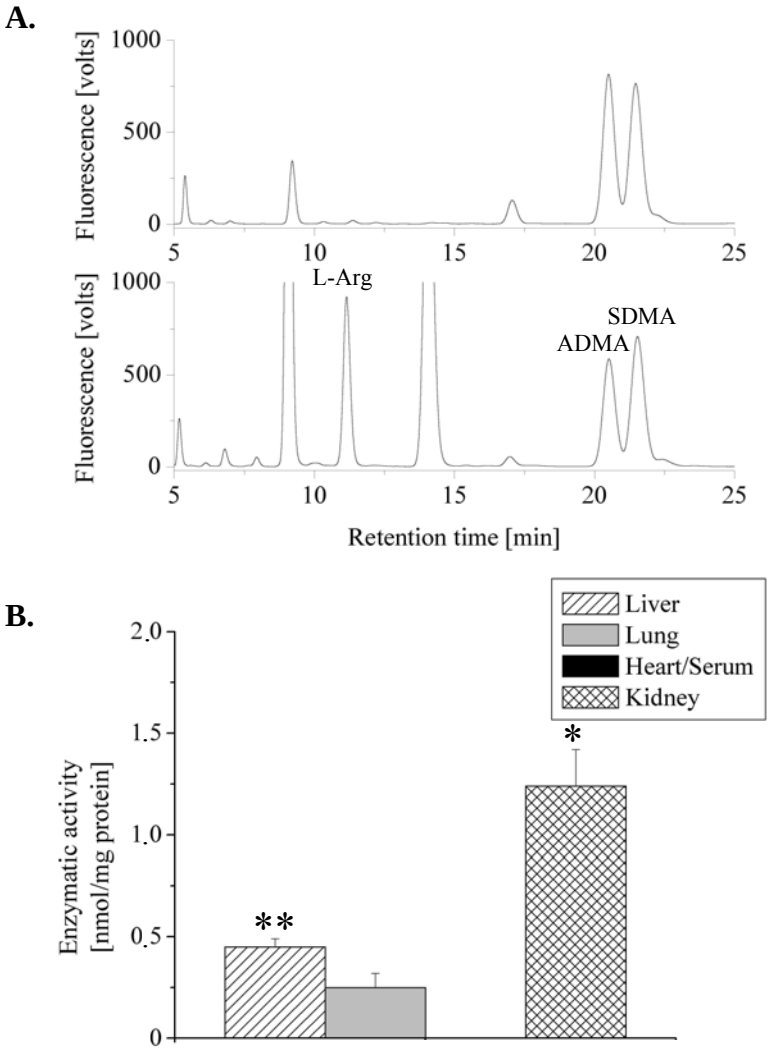

Figure 4.11 DDAH activity in mouse tissues. HPLC chromatograms of combined standard containing 15 pmol ADMA and SDMA (A upper panel) and from crude lung extract incubated with an identical amount of combined standard (A, lower panel). Enzymatic degradation of 0.75 pmol of ADMA in 25 µl of lung, heart, kidney and liver extracts. Data are presented as mean ± SD, n = 5, * $P < 0.05$, kidney versus all tissues, one-way ANOVA; ** $P < 0.05$, liver versus lung, two-tailed *t*-test. ANOVA; analysis of variance, SD; standard deviation HPLC; high performance liquid chromatography, ADMA; asymmetric ω-N^G, N^G-dimethylarginine, DDAH; N^G,N^G–dimmethylarginine dimethylaminohydrolase, SDMA; ω-N^G, N'^G-dimethylarginine.

Measurement of DDAH activity was based on the determination of tissue-specific ADMA degradation (nmol ADMA/mg tissue protein) as illustrated in figure 4.11 A. As figure 4.11 B indicates, kidney lysates exhibited the highest DDAH activity compared with those from the liver and lung. Moreover, degradation was found to be significantly higher in the liver versus the lung. The ADMA degradation was not detected in heart lysates and serum. Significant SDMA degradation was not observed in any tissue investigated.

4.3 Protein arginine methylation in pulmonary arterial hypertension

4.3.1 Methylarginine content in tissue lung homogenates from PAH patients

To analyze whether protein arginine methylation and cellular ADMA metabolism is altered in patients with pulmonary arterial hypertension, protein-incorporated versus free cellular methylarginine levels were assessed in lung homogenates from PAH patients and healthy donors.

4.3.1.1 Protein-incorporated L-Arg, ADMA and SDMA

Proteins were isolated from human lung tissue and crude extracts were subjected to protein precipitation followed by acid hydrolysis (as described in the Materials and Methods 1.1.1.2). Chromatographic analysis revealed that L-Arg (PAH: 131.48 ± 48.15 nmol/mg protein; Donor: 162.370 ± 52.45 nmol/mg protein) and SDMA levels (PAH: 0.099 ± 0.039 versus Donor: 0.131 ± 0.1 nmol/mg protein) did not differ significantly, whereas significant reduction in protein-incorporated ADMA concentration (PAH: 0.545 ± 0.226 versus Donor: 0.846 ± 0.358 nmol/mg protein) was observed in the lung homogenates from PAH patients compared to healthy donors, as illustrated in figure 4.12.

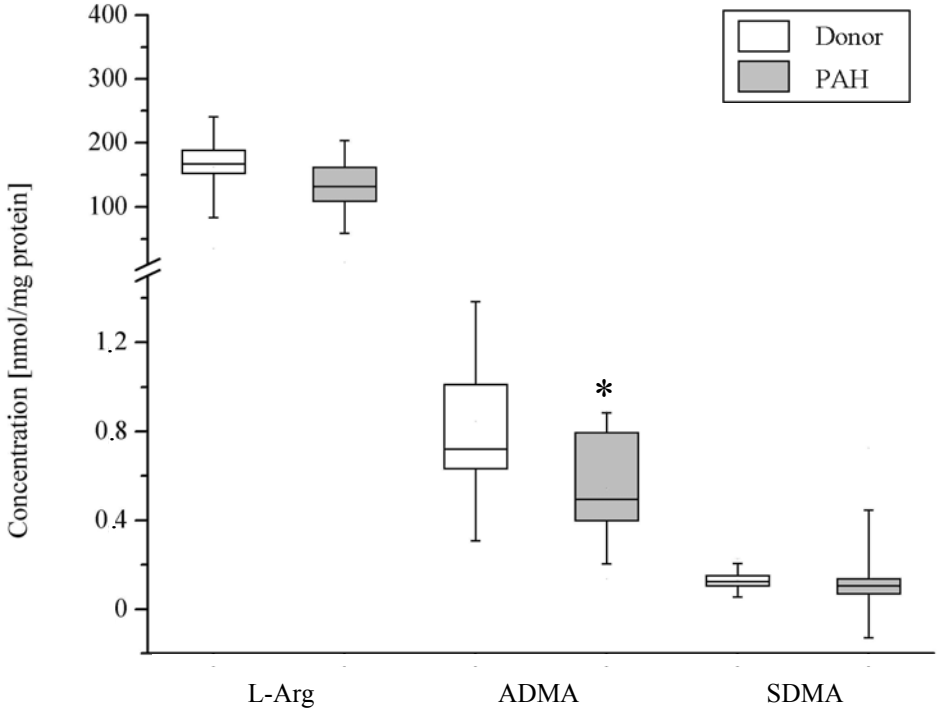

Figure 4.12 Box plots of protein-incorporated L-Arg, ADMA and SDMA levels in the lung tissue homogenates from PAH patients and healthy donors. The box represents the 25th and 75th percentile range of scores and the bar within the box represents the median. The verticale represents SD; PAH: n = 11, Donor: n = 10, * P < 0.05; two-tailed *t*-test. SD; standard deviation, asymmetric ω-N^G, N^G-dimethylarginin, SDMA; ω-N^G, N'^G-dimethylarginine.

4.3.1.2 Free-cellular methylarginines

In contrast to protein-incorporated methylarginine, significantly higher levels of free-cellular SDMA (PAH: 0.018 ± 0.006 versus Donor: 0.009 ± 0.002 nmol/mg protein) were observed in PAH patients compared to donors. The L-Arg (PAH: 4.97 ± 3.55 versus Donor: 4.31 ± 1.32 nmol/mg protein) and ADMA levels (PAH: 0.027 ± 0.021 versus Donor: 0.028 ± 0.015 nmol/mg protein) did not differ significantly (figure 4.13).

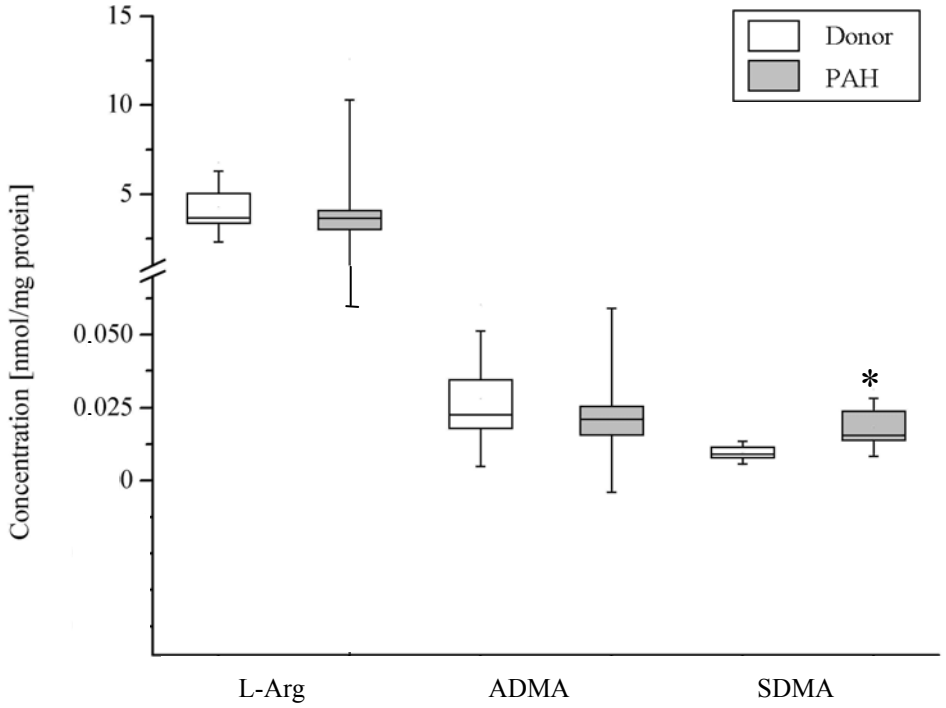

Figure 4.13 Box plots of free-cellular L-Arg, ADMA and SDMA levels in the lung tissue homogenates from PAH patients and healthy controls. The box represents the 25th and 75th percentile range of scores and the bar within the box represents the median. The vertical represents SD; PAH: n = 11, Donor: n = 10, * $P < 0.05$, two-tailed *t*-test**.** PAH; pulmonary arterial hypertension, SD; standard deviation, asymmetric ω-N^G, N^G-dimethylarginin, SDMA; ω-N^G, N'^G-dimethylarginine.

4.3.2 Expression of PRMTs in the lungs from PAH patients and healthy donors

In order to elucidate whether the decreased concentration of protein-incorporated ADMA observed in the lung of PAH patients correlats with reduced expression of type I PRMTs, western blot analysis was performed (figure 4.14 A). Analysis of PRMT1-6 by

densitometry revealed reduced expression of type I PRMTs (PRMT1, PRMT3, PRMT6) in the lung homogenates from PAH patients versus healthy donors. Importantly, PRMT1 was significantly less expressed in PAH lung tissue compared with donors (figure 4.14 B).

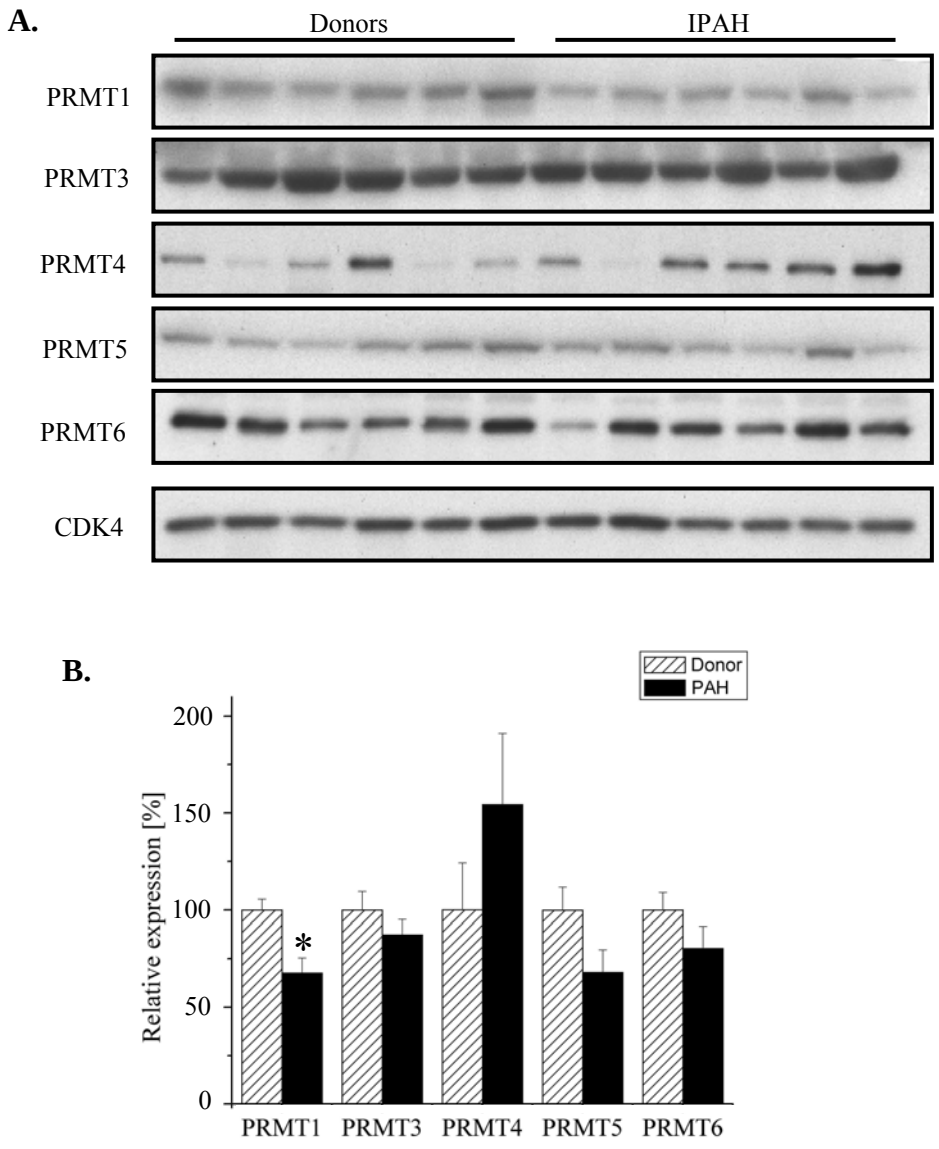

Figure 4.14 Expression of PRMTs in the lung homogenates derived from PAH patients and healthy donors. Western blot analysis (A) was performed with 20 µg protein from human lung extracts followed by densitometric analysis (B). CDK4 served as loading control. Data are presented as mean ± SEM, * $P < 0.05$; two-tailed *t*-test. SEM; standard error of the mean, PRMT; protein arginine methyltransferase.

4.3.3 Western blot analysis of asymmetric dimethylated proteins in lung homogenates

To further analyze differences in protein-incorporated methylarginine levels and to examine whether down-regulation of type I PRMTs observed in PAH patients has a strong impact on the methylation of specific proteins, western blot analysis was performed. For this purpose, the ADMA specific antibody ASYM24 was used. An alteration in the methylation pattern of asymmetric dimethylated proteins was observed in the PAH patients compared to healthy subjects (figure 4.15). Importantly, significant differences in the degree of methylation were observed among proteins at the size between 50 and 75 kDa and at around 25 kDa.

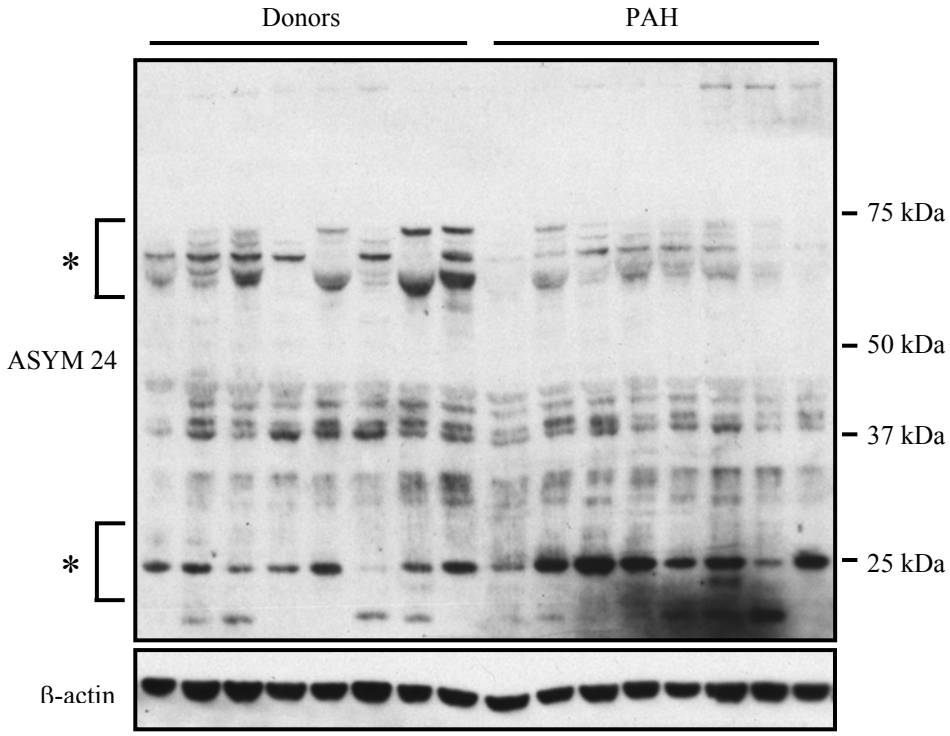

Figure 4.15 Analysis of asymmetric dimethylated proteins in PAH (n=8) and donor (n=8) lung homogenates. Protein extracts (20µg) were subjected to western blot analysis with an ASYM24 antibody. An asteriks indicates proteins with altered degree of asymmetric dimethylation. β-actin served as a loading control.

4.3.4 Immunohistochemical analysis of lung sections

In order to analyze whether PRMT1 was localized to particular human cell types, immunohistochemistry was performed on donor and PAH patient lung sections. The PRMT1 staining was detected mainly in PASMC of human lungs, as evident in figure 4.16. The expression pattern and localization of PRMT1 did not differ between PAH patients and healthy subjects. The α-SMA staining served as a marker for PASMC localization in the lung sections.

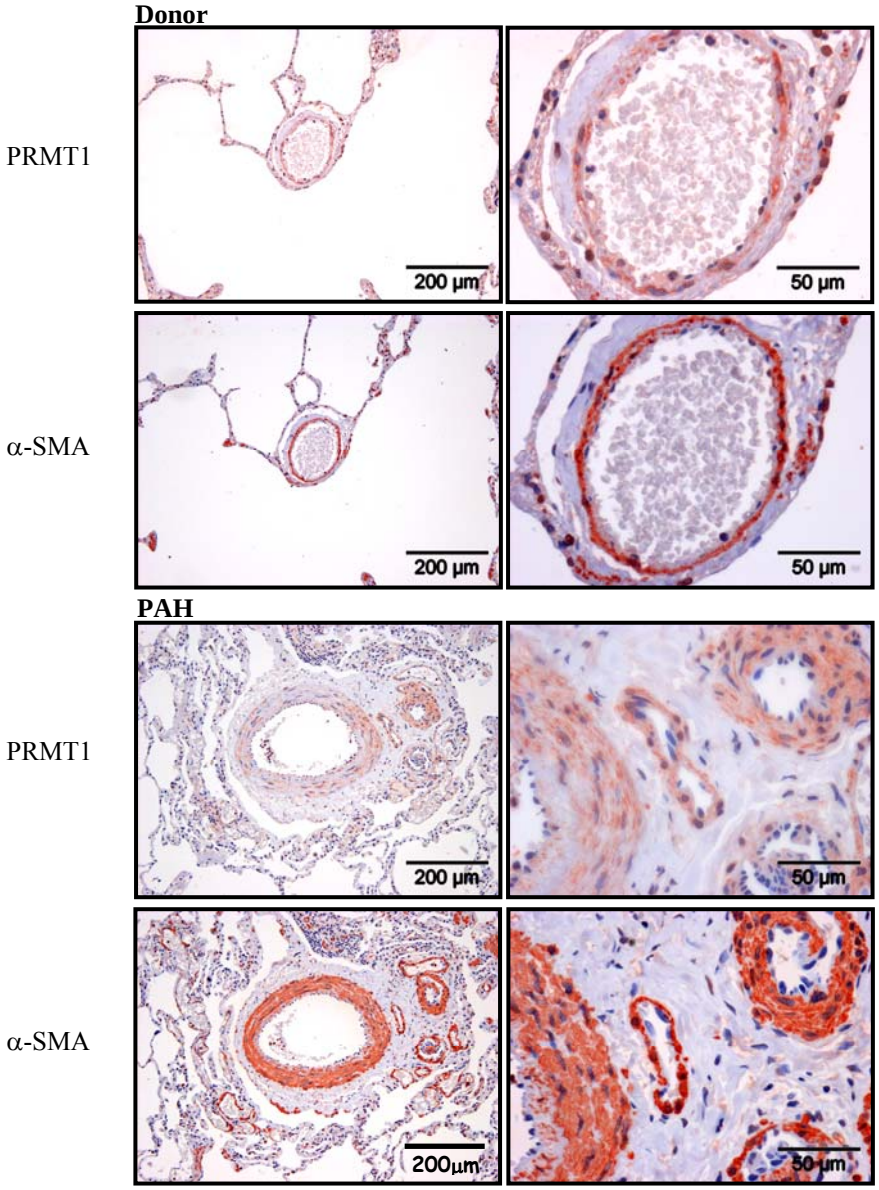

Figure 4.16 Localization of PRMT1 in the human lung sections. Lung sections were obtained from PAH patients and transplanted healthy donors. Data are representative for at least three independent samples from at least three different patients or donors.

4.3.5 Subcellular fractionation of proteins from human PASMC

To support our previous observations from immunohistochemical analysis and also to elucidate the cellular compartmentalization of PRMT1, subcellular fractionation of proteins from PASMC isolated from healthy donors was performed. Western blot analysis indicated that PRMT1 was localized in the cytosol and the nucleus, whereas higher levels were detected in the nucleus with concomitantly increased asymmetric dimethylation of proteins revealed by the ASYM24 antibodies. To examine the purity and enrichment of cellular fractions, western blot analysis was performed. Lamin A/C served as a specific marker for nuclear compartment, while purity of cytosolic fraction was assessed by determination of tubulin (figue 4.17).

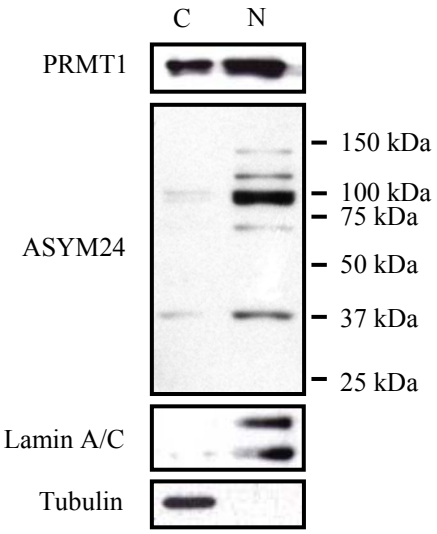

Figure 4.17 Subcellular fractionation of human PASMC. 10 μg of nuclear (N) and cytosolic (C) fractions were subjected to western blot analysis with antibodies against PRMT1, ASYM24, Lamin A/C and tubulin.

4.3.6 PRMT1 silencing in PASMC

To study whether decreased protein-incorporated ADMA levels, as observed in PAH patients, might alter PASMC proliferation, a hallmark of PAH pathogenesis, knockdown of PRMT1 in PASMC, followed by the assessment of PASMC proliferation, was performed.

4.3.6.1 Optimalization of siRNA transfection

To reduce expression of PRMT1 in PASMC, an siRNA sequence directed against PRMT1 mRNA was used. The PASMC are characterized by low transfection efficiency.

To optimize the transfection protocol, two transfection reagents were examined. The PASMC were transfected with siRNA either using Metafectene or X-treme GENE siRNA transfection reagent, and the efficiency of down-regulation was assessed by analyzing mRNA levels of PRMT1 by real-time PCR. Surprisingly, after transfection with siRNA and Metafectene, a significant reduction of PRMT1 expression was not observed, either at a siRNA concentration of 50 nM or 100 nM (figure 4.18 A) compared to transfection with negative control siRNA. Altered levels of the gene of interest in PASMC were already observed after transfection using 50 nM siRNA and X-treme GENE siRNA transfection reagent (figure 4.18 B left panel), whereas a higher dose of PRMT1 siRNA increased the effect of silencing. After 12 h, 24 h and 36 h, PRMT1 expression was significantly reduced using 100 nM siRNA and the X-treme GENE siRNA transfection reagent (figure 4.18 B, right panel).

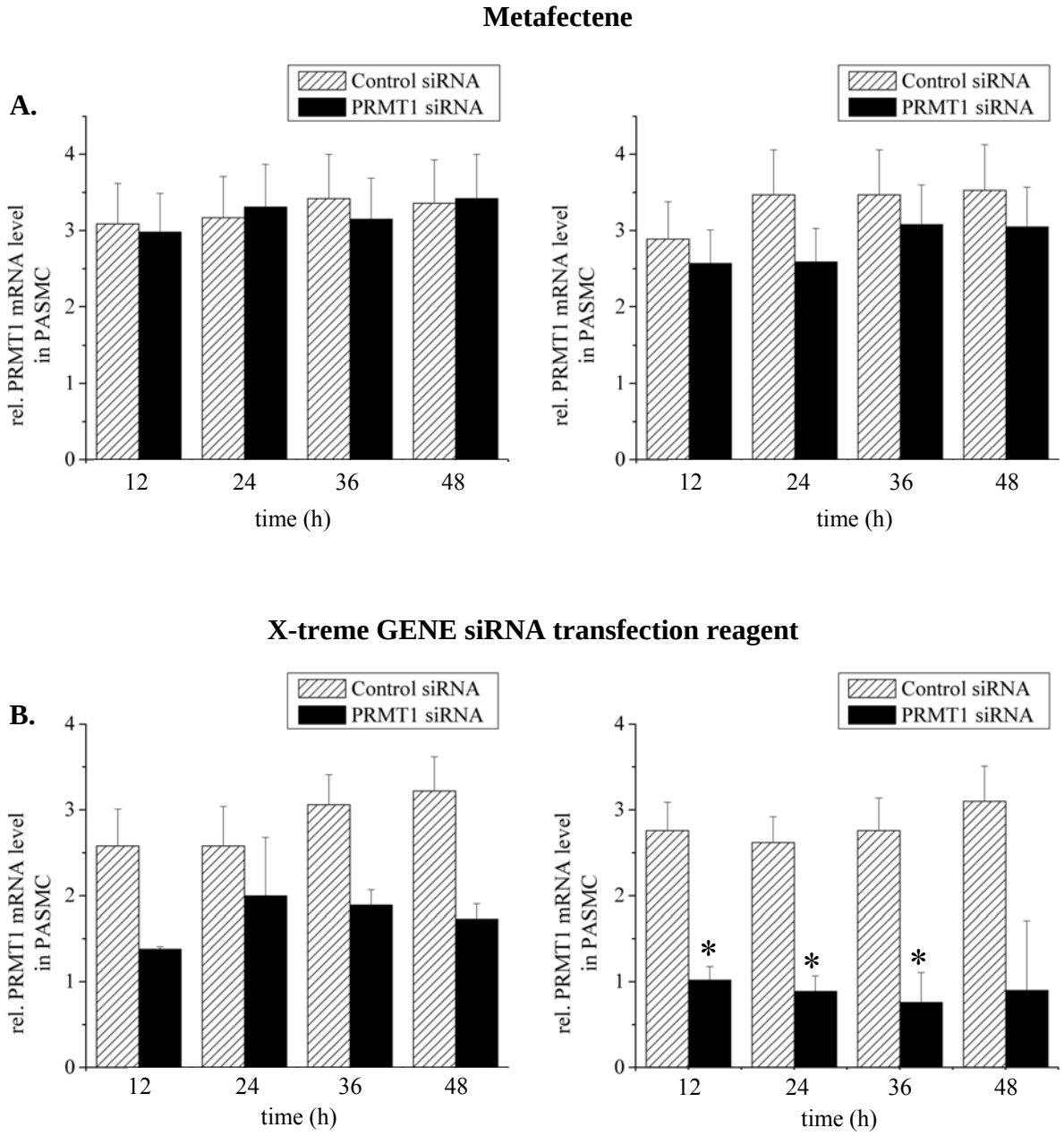

Figure 4.18 Optimization of PRMT1 knockdown in PASMC. The PRMT1 mRNA levels were assesed by real-time PCR. For optimization, two transfection reagents were used: Metafectene at a final concentration of 50 nM (A, left panel) and 100 nM (A, right panel) and the X-treme GENE siRNA transfection reagent, at a final concentration of 50 nM (B, left panel) and 100 nM (B, right panel). Data are presented as mean ± SEM, n = 3,* $P < 0.05$, two-tailed *t*-test.

4.3.6.2 Specificity of PRMT1 knockdown in PASMC

To analyze the specificity of PRMT1 silencing, and to examine whether PRMT1 siRNA might alter the expression of other members of the PRMT family, the mRNA levels of PRMT4, a PRMT1 homolog, were assessed by real-time PCR and western blot analysis. The mRNA as well as protein levels of PRMT4 were not affected in PASMC by treatment with 100 nM siRNA against PRMT1 (figure 4.19 A, B). In addition, the highest reduction in PRMT1 expression at the protein level was observed after 36 and 48 h after transfection, as illustrated in figure 4.19 B.

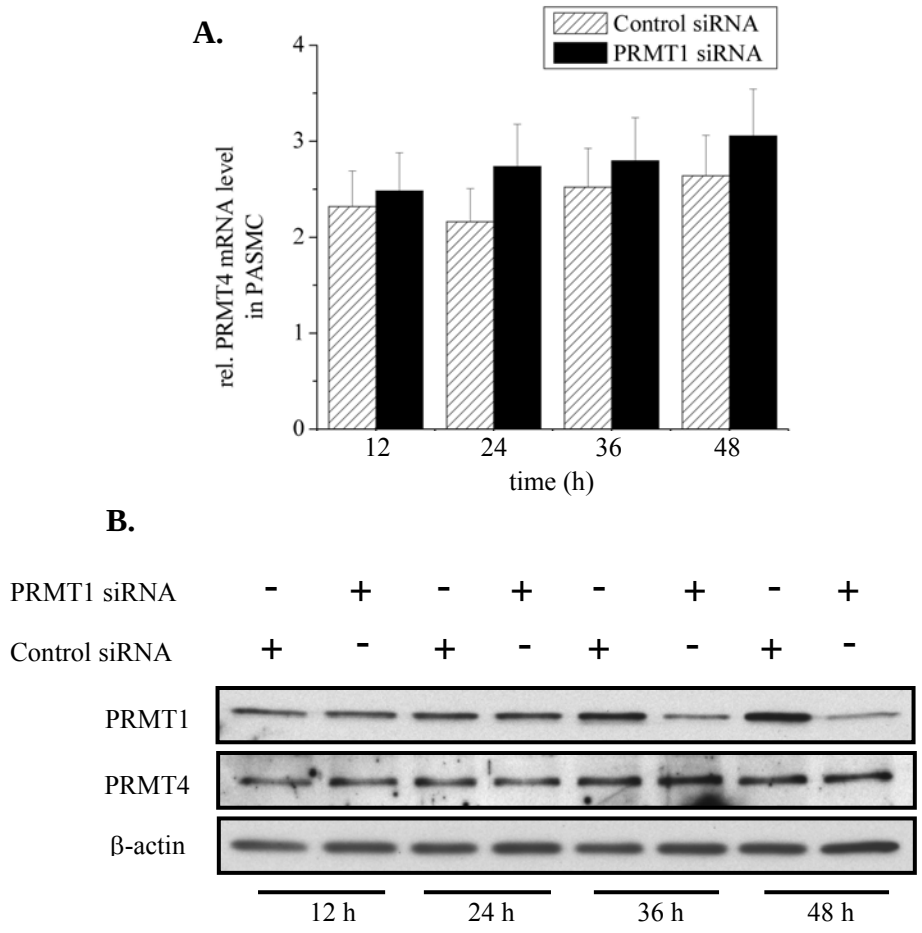

Figure 4.19 PRMT1 siRNA did not affect mRNA and protein levels of PRMT4. The PRMT4 mRNA and protein levels in PASMC were assesed by real-time PCR (A) and western blot analysis (B).

4.3.6.3 Effect of reduced PRMT1 expression on cell proliferation

To assess whether alterations to PRMT1 expression would affect PASMC proliferation, [H^3]-thymidine incorporation, using PASMC transfected with siRNA directed against PRMT1, was performed. As figure 4.20 illustrates, knockdown of PRMT1 in PASMC resulted in significant enhanced PASMC proliferation compared to negative control siRNA-treated cells.

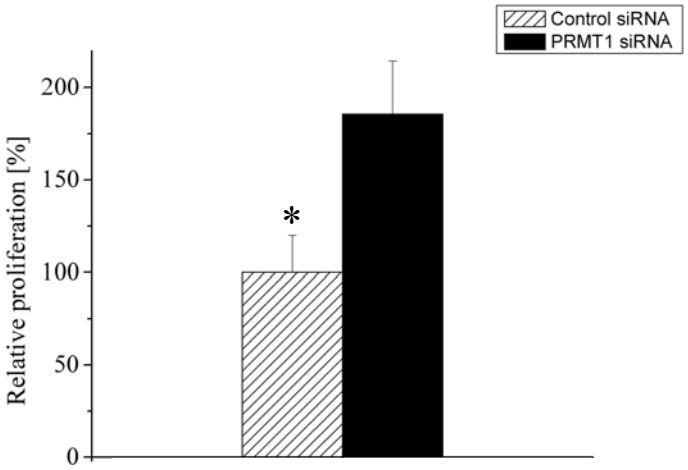

Figure 4.20 Increased PASMC proliferation after transfection with siRNA directed against PRMT1. The proliferation of PASMC was analyzed by [H^3]-thymidine incorporation assay. Data are presented as mean ± SEM; n = 4,* $P < 0.05$, two-tailed *t*-test.

5 Discussion

5.1 Protein arginine methylation in the cardiovascular system

5.1.1 Quantitative assessment of free versus protein-incorporated methylarginine *in vitro* and *in vivo*

To date, L-Arg, L-NMMA, ADMA and SDMA have been determined by enzyme-linked immunosorbent assay (ELISA) (Valtonen et al. 2005), high-performance liquid chromatography (HPLC) (Heresztyn et al. 2004), capillary electrophoresis (CE) (Causse et al. 2000), and liquid chromatography/mass spectrometry (LC/MS) (Pi et al. 2000; Vishwanathan et al. 2000; Teerlink et al. 2002; Huang et al. 2004). However, while these approaches are suited to the assessment of methylarginine in biological fluids, such as plasma, urine or cerebrospinal fluid, they do not allow the quantification of protein arginine methylation in the proteome of biological samples. Therefore, an approach for the simultaneous quantification of free and protein-incorporated L-Arg, L-NMMA, ADMA, and SDMA in cellular and tissue protein extracts was developed. This method entailed the isolation of cellular proteins by precipitation and hydrolysis, isolation/purification of methylarginines by SPE, derivatization with OPA, and finally RP-HPLC separation with fluorescence detection (figure 4.1).

Since this detection method relies on the quantification of methylated arginines after protein precipitation and acid hydrolysis, the approach was validated by assessing the amount of methylated arginines before and after protein hydrolysis using a standard solution of L-Arg, L-NMMA, L-H Arg, ADMA, and SDMA. For all components, the recovery rates were greater than 80%. Using the calculated recovery of the internal standard L-H Arg the relative recovery for each component was close to 100%, demonstrating that the use of L-H Arg as an internal standard ensures precision equal to that obtained with L-NMMA (Teerlink et al. 2002; Heresztyn et al. 2004).

Using this assay, the detection limit for all arginine forms was approximately 100 fmol (0.003 μM in crude extracts, based on a signal-to-noise ratio of 3:1), which is comparable to other HPLC methods with fluorescence detection (Teerlink et al. 2002; Heresztyn et al. 2004) and with LC/MS-based approaches (Huang et al. 2004).

To confirm that the protein precipitation procedure used did not selectively enrich or deplete specific (methylated) arginines, the extent of arginine methylation was measured in a well-characterized purified protein, MBP. The recovery of L-Arg, L-NMMA, and SDMA in amino acid hydrolysates of MBP from bovine brain did not change after protein precipitation. Approximately 2% of arginine residues in MBP were monomethylated, while symmetric dimethylation occurred in approximately 1.5%. Asymmetric dimethylation of arginine residues was not detected, which is in agreement with a previous study on the characterization of MBP methylation (Deibler and Martenson 1973). This indicates that the precipitation procedure did not selectively enrich or deplete specific mono- and/or symmetric dimethylated proteins, thus allowing accurate quantification of methylarginines in protein precipitates.

In order to show that the described method is suitable for monitoring dynamic changes in arginine methylation patterns in biologically relevant samples, A549 epithelial cells, cultured under different conditions, were assayed. Specifically, the impact of protein degradation on the amount of methylated arginines was analyzed in the absence or presence of the proteosome inhibitor MG-132. Inhibition of the proteosome led to an expected increase of L-Arg levels in protein hydrolysates, with a concomitant decrease in free L-Arg levels, suggesting decreased protein breakdown. Interestingly, a strong reduction in free ADMA, as well as in protein-incorporated ADMA levels was observed after MG-132 treatment, while SDMA levels in the same samples did not change. This led to selective changes in the arginine/ADMA, arginine/SDMA, and ADMA/SDMA ratios of protein-incorporated methylarginine. Therefore, these data suggest that the application of the proteosome inhibitor results in the specific inhibition of type I PRMT activity without affecting type II PRMT activity. Current and future work will therefore strive to understand the mechanisms that regulate PRMT activity via the proteosome pathway.

In conclusion, a novel method for the accurate quantification of arginine methylation in complex biological samples was developed.

5.1.2 Analysis of methylarginine metabolism in the cardiovascular system

To date, the interplay between methylarginine synthesis and degradation *in vivo* has not been described. Thus, PRMT and DDAH activity in mouse lung, heart, liver, and kidney homogenates was determined. To this end, HPLC-based quantification of protein-incorporated and free methylarginine was employed, combined with immunoblotting and ADMA degradation assays, for the assessment of tissue-specific patterns of arginine methylation.

Arginine methylation of proteins is catalyzed by the action of protein arginine methylatransferases. Type I enzymes exhibit variable tissue distribution in humans, which correlates with the localization of specific target proteins (Wada et al. 2002; Boisvert et al. 2003). In the cardiovascular system, PRMT expression has been only reported in the human heart, endothelial cells and smooth muscle cells (Vallance and Leiper 2004).

Initially, the present study clearly demonstrated the presence of protein arginine methyltransferases in the mouse lung, heart, liver and kidney. The mouse lung exhibited significantly higher expression of type I PRMTs (PRMT1, PRMT2, PRMT6) compared to the heart, kidney and liver.

To elucidate whether the increased pulmonary expression of type I PRMTs correlated with increased asymmetrical dimethylation of lung proteins, protein hydrolysis of tissue proteins and HPLC analysis were performed. In lung protein hydrolysates, arginine residues exhibited an almost four-fold higher degree of asymmetrical and two-fold higher degree of symmetrical dimethylation, compared with arginine residues from heart, kidney, and liver tissue. Free cellular methylarginine levels are dependent upon PRMT activity, the rate of protein degradation, the rates of ADMA metabolism by DDAHs, degradation of ADMA and SDMA by pyruvate aminotransferase, and the rates

of active methylarginine uptake and release. Similar free cellular ADMA and SDMA levels were found in the lung and heart, whereas the kidney and liver exhibited significantly higher concentrations for of dimethylarginines. Kidney lysates exhibited the highest levels of both dimethylarginines. Because kidney proteins did not display a higher degree of protein-incorporated methylarginine, the increased ADMA and SDMA levels may be a result of active renal methylarginine uptake, as suggested in previously published studies (Vallance et al. 1992; MacAllister et al. 1996; Al Banchaabouchi et al. 2000). Kidney homogenates exhibited significantly higher DDAH1 expression and ADMA degradation activity compared with liver, lung, or heart homogenates, suggesting that the kidney provides the main route for the clearance and metabolic conversion of circulating methylarginines.

Moreover, kidney lysates displayed the highest capacity for ADMA degradation, although DDAH2 expression was significantly lower than that observed in the liver, lung, or heart. Furthermore, no significant degradation of SDMA (as a consequence of pyruvate aminotransferase activity) was observed. These findings suggest that renal metabolism of ADMA is due to the metabolic action of DDAH1 and not DDAH2 and pyruvate aminotransferase. This is the first report on the direct characterization of methylarginine metabolism in the liver. Liver lysates displayed four- to eight-fold higher levels of free cellular ADMA and SDMA compared with the lung and heart. Because liver proteins did not exhibit a higher degree of protein-incorporated methylarginine, the increased ADMA and SDMA levels may be a result of active hepatic methylarginine uptake by the y+ transporter, as suggested in previously published studies (Siroen et al. 2004; Siroen et al. 2005). Liver lysates also exhibited significantly higher DDAH1 expression and ADMA degradation activity than did lysates from lung and heart tissue. A previously published study on the characterization of ADMA clearance in rat plasma after nephrectomy has suggested that ADMA does not require the kidney for its elimination from the plasma (Carello et al. 2006). Moreover, a transplanted liver graft is capable of clearing circulating ADMA in human patients (Siroen et al. 2004). Thus, these data suggest that the liver provides an alternative route for the clearance and metabolic conversion of circulating ADMA. Together, kidney and liver tissues exhibited a high

capacity for ADMA degradation, supporting the idea that both organs provide complementary routes for clearance and metabolic conversion of circulating ADMA.

Compared with the heart, pulmonary expression of PRMTs was significantly increased and correlated with enhanced asymmetrical and symmetrical dimethylation of proteins in the lung. In contrast, similar levels of free cellular ADMA and SDMA were observed in the lung and heart. Lung homogenates exhibited DDAH1 expression and ADMA degradation activity, which was not detected at all in heart lysates, suggesting that the lung is capable of metabolic conversion of free cellular methylarginine. Heart lysates did not display a capacity for ADMA degradation, although their DDAH2 expression was slightly higher than those of lung lysates. Therefore, relevant pulmonary degradation of ADMA is a result of DDAH1 activity, whereas no contribution of DDAH2 is evident. Furthermore, ADMA and also SDMA were detected in mouse and human BAL fluid. The BAL fluid of mouse and human exhibited an ADMA/SDMA ratio is similar to the ratio observed in the respective sera, suggesting that the similar levels are a result of diffusion through paracellular spaces. Circulating ADMA levels are raised in patients with pulmonary hypertension and in experimental models of pulmonary hypertension (Arrigoni et al. 2003; Millatt et al. 2003; Kielstein et al. 2005; Pullamsetti et al. 2005). In the rat model of chronic hypoxia-induced pulmonary hypertension, the effect was caused by a decreased expression and activity of DDAH1 (Millatt et al. 2003). Thus, the overall pulmonary ADMA output reflects a balance of PRMT activity, rates of protein turnover, intracellular DDAH1 activity, and active extrusion from the cell. The relative contribution of each component remains to be determined, but the salient findings of the present investigation can be summarized as: 1) pulmonary expression of type I PRMTs was correlated with enhanced protein arginine methylation of the lung proteome; 2) pulmonary ADMA degradation was undertaken by DDAH1; and 3) BAL fluid and serum exhibited almost identical ADMA/SDMA ratios. Together, these observations suggest that methylarginine metabolism by the lung significantly contributes to circulating ADMA and SDMA levels.

5.2 Analysis of protein arginine methylation in pulmonary arterial hypertension

5.2.1 Lung methylarginine content of patients with pulmonary arterial hypertension

Studies have reported elevated plasma levels of ADMA in patients with pulmonary arterial hypertension (Kielstein et al. 2005; Pullamsetti et al. 2005). However, other investigators have not detected any changes in plasma methylargine content, suggesting the involvement of alternative mechanisms, as such increased arginase activity, to the pathogenesis of PAH (Xu et al. 2004). Nevertheless, there is growing evidence that pulmonary vascular remodeling and endothelial dysfunction observed in chronic hypertension are influenced by ADMA metabolism (Strange et al. 2002; Valkonen et al. 2005). Initially, the methylarginine content of lungs from PAH patients was analyzed. Tissue homogenates from PAH lungs exhibited significantly lower protein-incorporated ADMA levels compared to lungs from healthy donors, whereas SDMA levels were not changed. The observed reduction of ADMA concentration in lung protein hydrolysates was probably caused by impaired type I PRMT expression as PRMT1, PRMT3, and PRMT6 expression was significantly reduced in PAH patients.

In contrast, free ADMA content was similar in lung crude extracts from PAH patients compared to healthy donors, demonstrating that free cellular ADMA levels are not lowered as a consequence of reduced type I PRMT expression. A previously published study has demonstrated markedly reduced lung DDAH expression in PAH patients (Pullamsetti et al. 2005). Therefore, the free cellular concentration of asymmetric dimethylarginine might remain unchanged due to less degradation by DDAH.

Symmetric dimethylarginine is implicated in endothelial dysfunction, which characterizes pulmonary hypertension. This L-Arg analog inhibits the y+ transporter that mediates the intracellular uptake of L-Arg causing lowered NO production (Closs et al. 1997; Kielstein et al. 2006). In addition, SDMA is not metabolized by DDAH enzyme, therefore, intracellular SDMA levels, generated by proteolysis of methylated proteins,

might have a direct impact on plasma levels (Palm et al. 2007). This study has demonstrated that the free cellular SDMA concentration was two-fold higher in PAH patients compared to healthy donors. These data are consistent with previously published reports, which demonstrated elevated plasma levels of SDMA in patients with PAH, as well as in monocrotaline-treated rats, suggesting that increased SDMA may contribute to prolonged pulmonary vasoconstriction and lung vascular remodeling (Pullamsetti et al. 2005).

5.2.2 Analysis of asymmetric dimethylated proteins in PAH compared to healthy lungs

Studies using the ASYM24 antibody, which recognizes asymmetric dimethylated proteins, confirmed that PAH patients exhibit a reduction in protein arginine methylation (Boisvert et al. 2003; Cote et al. 2003; Robin-Lespinasse et al. 2007). The greatest spectrum of methylation has been observed in 50 to 75 kDa proteins. A well-characterized PRMT target is Sam68, a nuclear protein with an apparent molecular mass of 68 kDa (Babic et al. 2004). Posttranslational protein modifications of Sam68, such as acetylation (Babic et al. 2004), phosphorylation (Najib et al. 2005) and sumoylation (Babic et al. 2006) have been described. Interestingly, phosphorylation of this protein by Src kinase promotes cell proliferation (Lukong et al. 2005). However, the involvement of Sam68 methylation in the regulation of cell proliferation has not been demonstrated to date. Lung homogenates from PAH patients exhibited reduced levels of asymmetric dimethylation of some type I PRMT targets, suggesting that methylation of Sam68 might be impaired. Thus, it would be of interest to elucidate whether methylation of Sam68 regulates cellular processes, which are connected to development of PAH, for instance smooth muscle cell proliferation.

5.2.3 Analysis of PRMT1 localization in the human lung

5.2.3.1 Immunohistochemical analysis of PRMT1 tissue localization

In the cardiovascular system, type I PRMTs are expressed in the heart, smooth muscle cells and endothelial cells (Vallance and Leiper 2004), whereas studies on chronic hypoxia-induced pulmonary hypertension have demonstrated localization of PRMT1 primarily in the lung airway and type II epithelial cells (Yildirim et al. 2006). In the present study, PRMT1 localization was characterized in the lungs of patients with PAH and healthy donors. Staining of lung specimens localized PRMT1 to PASMC, which is consistent with previous published observations, suggesting the expression of all type I PRMTs in vascular cells (Vallance and Leiper 2004). The expression pattern and localization of PRMT1 did not differ between lungs of patients suffering from PAH and donor lungs.

5.2.3.2 Cellular compartmentalization of PRMT1 in PASMC

Subcellular fractionation of human PASMC derived from healthy subjects revealed predominant nuclear localization of PRMT1, but PRMT1 was also observed in the cytosol. The well-characterized PRMT1 targets are nuclear core histones and members of hnRNP family, which mediate RNA processing and import from the nucleus (Bedford and Richard 2005; Passos et al. 2006). However, previous studies have demonstrated that PRMT1 localization in human embronic kidney 293 cells was six-fold more abundant in the cytoplasm than in the nucleus (Herrmann et al. 2005). In addition, PRMT1 was identified as a interaction partner of cytoplasmic domain IFNAR1 chain of the α, β interferon receptor, suggesting its localization in the cytosolic compartment (Abramovich et al. 1997). Thus, most known substrate proteins for PRMT1 are nuclear, but the enzyme is not exclusivly located in the nucleus (Herrmann et al. 2005). As suggested in previously published studies, PRMT1 is a dynamic protein and its cellular localization depends on cell type and experimental conditions. For instance, after inhibition of methylation by periodate-oxidized adenosine (Adox), PRMT1 accumulates in the nucleus of human embronic kidney 293 cells (Herrmann et al. 2005). Taken

together, these data confirm previously published observations, and demonstrate both nuclear and cytosolic PRMT1 localization in human PASMC.

5.2.4 Functional effects of transient PRMT1 knockout on smooth muscle cell proliferation

Pulmonary arterial hypertension is characterized by increased PASMC proliferation, which causes thickening of the pulmonary vasculature, which subsequently reduces the inner diameter of the lumen of pulmonary arteries, increases pulmonary vascular resistance, and raises pulmonary arterial pressure (PAP) (Smith et al. 2005). Many experimental therapeutic strategies for PAH target molecular mechanism that regulate smooth muscle cell growth.

The PRMT1 accounts for the generation of approximately 85% of asymmetric arginine dimethylation in mammals (Tang et al. 2000), suggesting that alterations to PRMT1 expression and activity might significantly affect total cellular protein arginine methylation and processes regulated by this posttranslational modification. As reduced PRMT1 expression and protein arginine methylation were observed in lung homogenates of PAH patients, the questions of whether down-regulation of PRMT1 expression might affect human PASMC growth was addressed. Interestingly, abrogation of PRMT1 expression by siRNA treatment resulted in significantly increased proliferation of PASMC.

Although previous studies have suggested that altered ADMA levels may regulate the expression of genes involved in cell proliferation (Smith et al. 2005), this is the first report indicating a direct involvement of PRMT1 in the progression of pulmonary vascular remodeling controlled by PASMC.

Protein arginine methylation is implicated in the alteration of protein-protein interactions (Pahlich et al. 2006). A well-known PRMT1 substrate is hnRNP K, a nuclear protein, which regulates RNA processing in the cell. Methylation of arginine residues by PRMT1 reduces hnRNP K interaction with the tyrosine kinase c-Src, resulting in reduced phosphorylation of hnRNP K (Ostareck-Lederer et al. 2006). Since phosphorylation of

other RNA-binding proteins, like Sam68, is implicated in the regulation of cell proliferation (Lukong et al. 2005), the methylation of regulatory proteins by PRMT1 might modulate PASMC proliferation.

In recent years, apoptosis has been linked to the pathogenesis of PAH. It has been suggested that both increased PASMC proliferation and decreased PASMC apoptosis have a relevant impact on pulmonary vasculature (Gurbanov and Shiliang 2006).

Apoptosis, or programmed cell death, is an important biological process that has been connected to atherosclerosis, cancer, neurodegenerative disorders and pulmonary vascular diseases including PAH (Haunstetter and Izumo 1998; Lin et al. 2002). This cellular mechanism may significantly contribute to regression of pulmonary vascular remodeling by the loss of PASMC and reverse pulmonary pressure. One of the stimuli which activates apoptotic cell death is tumor necrosis factor (TNF)-α. Treatment of cells with extracellular TNF-α results in NF-κB-dependent gene expression leading, finally, to cell death (Gurbanov and Shiliang 2006). It has been demonstrated in human embronic kidney 293 cells that upon TNF-α stimulation, PRMT1, CARM1, p65, PARP1 and p300 form a complex, which coactivates NF-κB-mediated transcription. Further experiments on modulation of PRMT1 expression have suggested that NF-Bκ-dependent gene activation might be positively regulated by PRMT1 expression (Hassa et al. 2008). These findings suggest a direct link between PRMT1 expression and regulation of PASMC growth. In sum, decreased PRMT1 levels might simultaneously result in increased cell proliferation and reduced apoptosis of PASMC.

5.3 Conclusions and further perspectives

Methylation of arginine residues plays a fundamental role in cellular processes, such as RNA maturation, DNA repair, signal transduction, protein-protein interaction, protein localization, translation and transcription. One product of this posttranslational modification, ADMA, has been implicated in the pathogenesis of several cardiovascular disorders, such as hypercholesterolemia, atherosclerosis, and PAH. To date, the interplay

between methylarginine synthesis and degradation in cardiovascular disorders has not been described.

To conclude, findings reported in this study describe a novel method for the accurate quantification of arginine methylation in complex biological samples. *In vivo* studies on mice can be summarized as: 1) pulmonary expression of type I PRMTs was correlated with enhanced protein arginine methylation of the lung proteome; 2) pulmonary ADMA degradation was undertaken by DDAH1; and 3) BAL fluid and serum exhibited almost identical ADMA/SDMA ratios. Together, these observations suggest that methylarginine metabolism by the lung significantly contributes to circulating ADMA and SDMA levels.

Studies on PAH patients revealed significantly lower levels of protein-incorporated ADMA in the lungs of PAH patients compared with controls. Western blot analyses demonstrated significantly decreased expression of PRMT1 and asymmetrically dimethylated proteins in lungs from PAH patients. Immunohistochemistry localized PRMT1 expression to PASMC, where it was expressed in the nucleus and the cytosol. The knockdown of PRMT1 in primary PASMC by siRNA technology decreased the level of asymmetrically dimethylated proteins, and led to increased PASMC proliferation. These findings indicated the direct involvement of PRMT1 in the pathogenesis of PAH, and the progression of pulmonary vascular remodeling controlled by PASMC. It would be of interest to elucidate whether methylation of Sam68 regulates cellular processes, which are connected to the development of pulmonary arterial hypertension, for instance smooth muscle cell proliferation. Moreover, PRMT1 might play an important role in the development of PAH by controling NF-κB-dependent gene expression, a well known mechanism of cell proliferation. Therefore, future studies will be focused on the involvement of PRMT1 in modulation of NF-κB signaling in PASMC.

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6 Appendix

Table 6.1 List of primers used for Quantitative RT-PCR amplification.

Gene Bank™ Accession Number	Forward primer (5′-3′)	Reverse primer (5′-3′)	Amplicon size (bp)
Human PRMT1 NM_198318.1	GCTGAGGACATGAC ATCCAA	GAAGAGGTGCCGGT TATGAA	132
Human PRMT4/CARM1 NM_199141.1	ACAGCGTCCTCATCC AGTTC	GCTGGGACAGGTAG CCATAA	162
Human PBGD NM_000190.3	CCCACGCGAATCACT CTCAT	TGTCTGGTAACGGCA ATGCG	69

Table 6.2 List of primary antiobodies used for immunoblotting (IB) and immunohistochemistry (IHCH).

Primary	Host	Dilution		Company	Catalog number
		IB	IHCH		
anti - CDK4	rabbit	1:1000		Santa Cruz	601
DDAH1	goat	1:1000		Everest Biotech	05278
DDAH2	goat	1:1000		Everest Biotech	05277
anti - dimethyl-arginine (ASYM24)	rabbit	1:500		Santa Cruz	07-414
anti - lamin A/C	mouse	1:2000		Santa Cruz	20681
anti - PRMT1	rabbit	1:2000	1:250	Abcam	7027-200
anti - PRMT2	rabbit	1:1000		Abcam	3763-100
anti - PRMT3	rabbit	1:2000		Upstate	07-256
anti - PRMT4	rabbit	1:1000		Upstate	07-080
anti - PRMT5	rabbit	1:2000		Upstate	07-405
anti - PRMT6	rabbit	1:500		Imgenex	12192
anti - PRMT7	rabbit	1:500		Upstate	07-639
anti - α–SMA	mouse		1:500	Sigma-Aldrich	A5228
anti - β–SMA	rabbit	1:2000		Cell signaling	4970
anti - tubulin	mouse	1:2000		Santa Cruz	5286

Table 6.3 List of secondary antiobodies used for immunoblotting (IB).

Secondary	Host	Dilution	Company	Catalog number
HRP-conjugated anti-mouse IgG	rabbit	1:3000	Pierce	31450
HRP-conjugated anti-rabbit IgG	goat	1:3000	Pierce	31460
HRP-conjugated anti-goat IgG	rabbit	1:3000	Pierce	31402

Table 6.4 List of siRNA sequences.

siRNA	Company	Catalog number
PRMT1 (human)	Santa Cruz	41069
Negative control siRNA#1	Ambion	4611

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8 Declaration

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

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PUBLICATIONS:
1) Kamila Kitowska, **Dariusz Zakrzewicz**, Melanie Königshoff, Izabela Chrobak, Friedrich Grimminger, Werner Seeger, Patrick Bulau, and Oliver Eickelberg. "Functional role and species-specific contribution of arginases in pulmonary fibrosis". *Am J Physiol Lung Cell Mol Physiol.* 2008 Jan; 294(1):L34-45.

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1) **Dariusz Zakrzewicz**, Kamila Kitowska, Walter Klepetko, Werner Seeger, Patrick Bulau and Oliver Eickelberg. "Decreased protein arginine methylation in pulmonary arterial hypertension. A novel regulatory mechanism of smooth muscle cell proliferation". American Thoracic Society, 16-21 May 2008 Toronto, Ontario, Canada
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3) Kamila Kitowska, Patrick Bulau, **Dariusz Zakrzewicz**, Melanie Königshoff, Andreas Guenther, Werner Seeger and Oliver Eickelberg. "Bleomycin-induced alterations of Pulonary L-arginine metabolism". The 3rd International Meeting on ADAM, ADMA 2006, 1 December 2006 London, UK

4) **Dariusz Zakrzewicz**, Patrick Bulau, Kamila Kitowska, Werner Seeger and Oliver Eickelberg. "Quantitative assessment of protein arginine methylation in the lung: Determination of free versus incorporated methylarginines". The 3rd International Meeting on ADMA, ADMA 2006, 1 December 2006 London, UK
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6. Patrick Bulau, **Dariusz Zakrzewicz**, Kamila Kitowska, Werner Seeger and Oliver Eickelberg. "Quantitative assessment of protein arginine methylation in the lung: Determination of free versus incorporated methylarginines". American Thoracic Society, 19-24 May 2006 San Diego, California, USA
7) **Dariusz Zakrzewicz**, Patrick Bulau, Kamila Kitowska, Walter Klepetko, Werner Seeger and Oliver Eickelberg. "Decreased Protein Arginine Methyltransferase Activity in Idiopathic Pulmonary Arterial Hypertension". American Thoracic Society, 19-24 May 2006 San Diego, California, USA
8) Patrick Bulau, **Dariusz Zakrzewicz**, Kamila Kitowska, Werner Seeger and Oliver Eickelberg. "Quantitative Bestimmung von asymmetrichem Dimethylarginin (ADMA) in Organen des kardiovaskulären Systems". 47th Annual Meeting of the German Society of Pneumology, 29 March-1 April 2006 Nürnberg, Germany
9) **Dariusz Zakrzewicz**, Patrick Bulau, Kamila Kitowska, Walter Klepetko, Werner Seeger and Oliver Eickelberg. "Decreased Protein Arginine Methyltransferase Activity in Idiopathic Pulmonary Arterial Hypertension". 112th Annual Meeting of the German Society of Internal Medicine, 2006, Wiesbaden, Germany

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